TAILORING ALLOSTERIC MODULATORS OF NMDA RECEPTORS AND GABA-A RECEPTORS FOR NEUROLOGICAL DISORDERS

by

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Abstract

Both N-methyl-D-aspartate (NMDA) receptor, one of the subtypes of ionotropic glutamatergic receptors at the vast majority of excitatory synapses, and the type A γ-aminobutyric acid receptor (GABA\textsubscript{A}R), the principal ionotropic GABARs at the inhibitory GABAergic synapses play important roles in regulating neuronal activities in the mammalian central nervous system (CNS). Their dysfunctions contribute to the pathogenesis of many neurological disorders.

Overactivation of NMDAR-mediated excitotoxicity is a common cause of many neurodegenerative diseases including stroke. There are two prevalent theories, the ‘NMDAR subtype hypothesis’ that proposes that activating GluN2A-containing NMDAR promotes neuronal survival, whereas activating GluN2B-containing NMDARs leads to neuronal death, and the ‘NMDAR location hypothesis’ that suggests that activating synaptic, primarily GluN2A-containing NMDARs favors neuronal survival while activating extrasynaptic, predominantly GluN2B-containing NMDARs induces neuronal death. Since both hypotheses support allosteric modulators that potentiate GluN2A function and inhibit GluN2B function may have promising potentials as a new and more effective class of neuroprotective therapy for stroke, we performed computer-aided virtual screening and in-silico drug design to discover a lead compound 813 that functions as an NMDAR dual allosteric modulator (Ndam) that potentiates GluN1/GluN2A and at the same time inhibits GluN1/GluN2B. We further optimized Ndam813 and thereby developed two more efficacious compounds, Ndam830 and Ndam844.

Ndam830 protected cortical neurons from NMDA-induced excitotoxicity and H\textsubscript{2}O\textsubscript{2}-induced oxidative stress, and also reduced ischemia-induced infarct volume and promoted
behavioral recoveries in rat MCAo models. Thus, our results strongly suggest that Ndam830 by promoting synaptic/GluN2A-containing NMDAR mediated cell survival signaling and inhibiting extrasynaptic/GluN2B-containing receptor mediated cell death signaling, is a novel neuroprotective stroke therapeutic.

Ndam844 is a potent pan-NMDAR potentiator that can potentially treat NMDAR hypofunction related disorders.

Apart from NMDARs, dysfunction of GABA\(_A\)Rs is also implicated in various neurological conditions. Using electrophysiological and biochemical methods, we characterized the functional alterations of two de novo GABA\(_A\)R variants T292S and T292I identified in patients with epileptic encephalopathy (EE) and developmental delay. Moreover, we found clinically approved allosteric modulators of GABA\(_A\)R that may treat the patients carrying the variants. In summary, allosteric modulators of both NMDARs and GABA\(_A\)R showed great therapeutic potential for neurological disorders.
Lay Summary

N-methyl-D-aspartate (NMDA) receptor, one of the excitatory synaptic receptor, and type A γ-aminobutyric acid receptor (GABA_AR), the major inhibitory synaptic receptor, involve a vast range of neuronal activities in the mammalian central nervous system (CNS), thus making them attractive targets for developing therapeutics for neurological disorders. In my thesis, we have developed two novel allosteric modulators for NMDARs, Ndam830 and Ndam844 with distinct subunit-specific activities. Ndam830 is able to protect neurons from ischemic insults both in vitro and in vivo, whereas Ndam844 may treat NMDAR hypofunction related brain disorders such as schizophrenia, major depressive disorders (MDDs), Alzheimer’s disease (AD) and autism spectrum disorders (ASD). We also revealed a new therapeutic potential of approved allosteric modulators of GABA_ARs treating child patients carrying some rare GABA_AR mutations. Further detailed characterization of these allosteric modulators for ionotropic neurotransmitter receptors may facilitate the development of precision medicine strategies for treating various brain disorders.
Preface

Chapter 1: Contains excerpts and illustrations from a previously published co-first author review paper. Ge Yang, [Chen Wenlin], Axerio-Cilies Peter, Wang Yu Tian. (2020) NMDARs in Cell Survival and Death: Implications in Stroke Pathogenesis and Treatment. Trends Mol Med. 26(6):533-551. Yang Ge, Wenlin Chen, Dr. Peter Axerio-Cilies and Dr. Yu Tian Wang wrote and revised the manuscript. Wenlin Chen prepared the original illustrations. All figures were drawn by Wenlin Chen with proper citations in the figure legends.

Chapter 2: Material and methods.

Chapters 3 and 4: A version of this material will be assembled into a manuscript for submission. Specifically, Wenlin Chen proposed the study, screened the compounds, performed the bioactivity assessments with electrophysiological recordings and, biochemical experiments, and designed and performed the study concerning the drug development process, and pharmacological in-vivo profile of drugs. Ndam830 was synthesized by Dr. Paul Foth from UBC chemistry department and Primedicine from Qingdao, China. High performance liquid chromatography (HPLC) measurements of micro-dialysis samples were conducted by Maya Nesbit. The collection of rat cerebrospinal fluid (CSF) was conducted by Wenlin Chen. The groups of Dr. Juan Chen group and Dr. Woei-Cherng Shyu tested the compound in the MCAo stroke model in-vivo. Dr. Lidong Liu provided technical and knowledge support in regards to patch clamp recordings. Dr. Jie Lu offered technical and knowledge support in regards to biochemical experiments. Dr. Yuping Li provided technical support for the intravenous injection of the compound into rats. Dr. Yu Tian Wang designed the study, supervised the overall project and provided insightful advice and timely instructions for the conducting the study. Wenlin performed all data analysis and figure construction for the in vitro experiments. Data analysis
and figure plotting for the HPLC measurements of CSF were completed by Maya Nesbit. Data analysis and figure plotting for the in vivo experiments were conducted by Wenlin Chen, Dr. Juan Chen and Dr. Woei-Cherng Shyu.

Chapter 5: A version of chapter 5 has been published. [Wenlin Chen], Yang Ge, Jie Lu, Joshua Melo, Yee Wah So, Romi Juneja, Lidong Liu and Yu Tian Wang (2022) Distinct Functional Alterations and Therapeutic Options of Two Pathological De Novo Variants of the T292 Residue of GABRA1 Identified in Children with Epileptic Encephalopathy and Neurodevelopmental Disorders. Int. J. Mol. Sci. 23(5), 2723. Wenlin Chen, Yang Ge, Dr. Jie Lu, Yee Wah So, Dr. Romi Juneja, Dr. Lidong Liu and my supervisor Dr. Yu Tian Wang were involved in the conceptualization and design of the study. Dr. Romi Juneja and Dr. Joshua Melo from the Neurology Center of Toronto collected clinical data. Wenlin Chen and Yang Ge performed the electrophysiological experiments, and Dr. Jie Lu performed molecular and biochemical experiments. Wenlin Chen analyzed and modified the structural model of the GABA\textsubscript{A}Rs. Wenlin Chen, Yang Ge, Dr. Jie Lu, Dr. Lidong Liu and Dr. Yu Tian Wang analyzed the data and prepared figures. Wenlin Chen, Yang Ge, Dr. Jie Lu, Yee Wah So, Dr. Lidong Liu and Dr. Yu Tian Wang, wrote and revised the manuscript.

Chapter 6: Conclusions.

Experiments were performed in compliance with regulations at the University of British Columbia. Animal experiments were performed in compliance with institutional requirements at the University of British Columbia and in accordance with guidelines set forth by the Canadian Council on Animal Care. Animals were obtained and handled according to University of British Columbia ethics protocols A20-0133.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABD</td>
<td>Agonist binding domain</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>AMPAR</td>
<td>$\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor</td>
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<tr>
<td>ASD</td>
<td>Autism spectrum disorders</td>
</tr>
<tr>
<td>A(N)TD</td>
<td>Amino-terminal domain</td>
</tr>
<tr>
<td>APV</td>
<td>(2R)-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>BZD</td>
<td>Benzodiazepine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTD</td>
<td>Carboxyl-terminal domain</td>
</tr>
<tr>
<td>DAM</td>
<td>Dual allosteric modulator</td>
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<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
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<tr>
<td>ECD</td>
<td>Electrochemical detection</td>
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<tr>
<td>FLZ</td>
<td>Flumazenil</td>
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<tr>
<td>GluN2A</td>
<td>Glutamate receptor ionotropic, N-methyl-D-aspartic acid receptor 2A</td>
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<td>GluN1</td>
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</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-Aminobutyric acid</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GABA&lt;sub&gt;AR&lt;/sub&gt;</td>
<td>A type γ-Aminobutyric acid receptor</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance-liquid-chromatography</td>
</tr>
<tr>
<td>&lt;sup&gt;1&lt;/sup&gt;H-NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>MDDs</td>
<td>Major depressive disorders</td>
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<tr>
<td>MCAo</td>
<td>Middle cerebral arterial occlusion</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NAM</td>
<td>Negative allosteric modulator</td>
</tr>
<tr>
<td>Ndam</td>
<td>NMDAR dual allosteric modulator</td>
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<td>NDCs</td>
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<tr>
<td>NVP-AAM077</td>
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<tr>
<td>OGD</td>
<td>Oxygen glucose deprivation</td>
</tr>
<tr>
<td>PAM</td>
<td>Positive allosteric modulator</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
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<td>TTX</td>
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</tr>
<tr>
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<td>Thiocochicoside</td>
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Dedication

To my beloved father, Huanhui Chen, and to my beloved mother, Baolian Wang.
Chapter 1: Introduction

1.1 Overall introduction

The proper physiological functioning of the mammalian central nervous system (CNS) relies on the processes of electrochemical excitation and inhibition of neural circuits (Foster and Kemp, 2006). At synapses of the brain, glutamate and gamma-aminobutyric acid (GABA) are the most common excitatory and inhibitory neurotransmitters, activating the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate (NMDA) receptors, and GABA receptors, respectively. Both NMDARs and GABARs play critical roles not only in maintaining the excitation and inhibition (E/I) balance, cognitive activities, but also in signal transduction and neuronal development as well as in a series of neurological disorders such as stroke and epilepsy (Grant and Lovinger, 1995; Foster and Kemp, 2006; Ge et al., 2020).

Through virtual screening and in silico drug design, a lead compound Ndam813 was found to function as an NMDAR dual allosteric modulator (Ndam) that potentiates GluN2A-containing NMDARs and at the same time inhibits GluN2B-containing NMDARs, albeit with very low potency (Axerio-Cilies, 2016). In Chapter 3 of the thesis we explored the possibility that further improving the efficacy of such a dual functioning compound could treat excitotoxic neuronal injuries in vitro and in vivo. Briefly, by employing structure-based in-silico drug design and in-vitro functional screening, we successfully optimized the candidate compound Ndam813 into a new compound Ndam830 with better potency and efficacy. As we expected, Ndam830 protected cortical neurons from NMDA-induced excitotoxicity and H₂O₂-induced oxidative stress in vitro. Ndam830 also reduced the infarct volume in in vivo MCAo models when given pre- and post-operatively. Moreover, post-stroke administration of Ndam830 could accelerate the neurological function recovery in rats. During our drug optimization process, apart from Ndam830, we found
a very potent compound Ndam844 that harbored a distinct modulating pattern. Instead of potentiating GluN1/GluN2A and inhibiting GluN1/GluN2B, Ndam844 strongly potentiated both diheteromeric GluN1/GluN2A and GluN1/GluN2B in the initial electrophysiological screening tests. We then systematically characterize this positive allosteric modulator (PAM) in vitro. The characterization of Ndam844 presented in Chapter 4 would assist in preparing it to be a potential therapeutic for hypofunction of NMDARs related schizophrenia, major depressive disorders (MDDs), Alzheimer’s disease (AD), autism spectrum disorders (ASD) and anti-NMDAR encephalitis. In Chapter 5 of this thesis, we also characterized the functional alterations of two rare de novo GABA_A R variants, α1_T292S and α1_T292I via in vitro electrophysiology and biochemical methods. We found that the two mutations have drastically different impacts on the receptor function: the α1_T292S variant significantly increased, whereas the α1_T292I variant significantly reduced GABA_A R function. The opposite functional impacts of these mutations suggest that this residue is critically important in determining the gating properties of the channel, and that we would need different therapeutic strategies for treating the patients carrying these mutations. We screened and found several clinically approved allosteric modulators of GABA_A R as potential therapeutic candidates for precision medicine for treating patients with the variants. We found that the GABA_A R allosteric inhibitor thiocolchicoside (TCC) (Fisher, 2004) could reduce the gain of function caused by the T292S mutation, thereby normalizing the receptor function. Conversely, a combination of the GABA_A R positive modulators, diazepam and verapamil, largely rescued the loss of function caused by the T292I mutation. The allosteric modulators Ndam830 and Ndam844 tailored for NMDAR-related disorders and TCC, diazepam and verapamil tailored for rare variants of GABA_A Rs may have great potentials for future therapeutic applications.
1.2 Structure, subtypes and distribution of N-methyl-d-aspartate glutamate receptors (NMDARs)

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors in the brain that play critical roles in mediating physiological brain functions such as cognitive activities and also in the pathogenesis of neurodegenerative diseases including stroke (Dirnagl et al., 1999), Alzheimer’s disease (Hardingham, 2019), Huntington’s disease (Fernandes and Raymond, 2009), and Parkinson’s disease (Olivares et al., 2012). NMDAR is a tetrameric protein complex composed of two essential GluN1 subunits and two interchangeable GluN2/3 subunits. The non-GluN1 subunits may be identical for diheteromeric NMDARs or non-identical for triheteromeric NMDARs. They may include any of the four GluN2 (GluN2A-D) and two GluN3 (GluN3A-B) subunits, leading to sixty-four different combinations potentially (Paoletti et al., 2013) (Figure 1.1 A). To date, more than a dozen NMDAR subtypes have been characterized, and each displays distinct pharmacological and signaling attributes (Traynelis et al., 2010). Characterization of the temporospatial expressions of each NMDAR subtype is critical for understanding their functions in brain physiology and dysfunctions in neurological disorders including stroke.

GluN2A- and GluN2B-containing NMDARs are the predominant subtypes expressed in many CNS regions (Figure 1.1 B). The GluN2B subunit expression is high during early development and gradually decreases to a moderate level in adulthood. In contrast, the GluN2A subunit expression is delayed compared with that of the GluN2B subunit. During neuronal maturation, an increased amount of the GluN2A subunits would functionally replace the GluN2B subunits at the synapse during neuronal maturation (Sheng et al., 1994).
Most functional studies are conducted under the assumption that these receptors exist in the diheteromeric forms that can be distinguished by subtype specific allosteric inhibitors. However, triheteromeric GluN1/GluN2A/GluN2B NMDARs may be the most abundant NMDAR composition in adult hippocampal and cortical synapses (Rauner and Köhr, 2011; Hansen et al., 2014; Lu and Du, 2017). However, little is known about their physiological functions because of the difficulty of manipulating this specific NMDAR population experimentally.

Despite their limited expression in the adult brain, atypical NMDAR subtypes also have critical physiological functions (Figure 1.1 A). GluN2C subunit expression is initiated relatively late during development, while GluN2D and GluN3A subunit expressions are transiently activated during development and decline to a low level when reaching adulthood. In the adult rodent brain, GluN2C and GluN2D subunits are expressed mainly in the cerebellum and striatum, respectively. Moreover, GluN2C, GluN2D, and GluN3A subunits are highly expressed in the oligodendrocyte processes and may regulate the signaling of both neuronal and non-neuronal cells (Micu et al., 2006; Doyle et al., 2018). To date, conclusive evidence delineating the exact composition of the atypical NMDAR subtypes in native system is still lacking. One research group has used the diheteromeric GluN2C-containing NMDAR specific allosteric modulator PYD-106 and pan-GluN2C-containing NMDAR allosteric modulator CIQ to show that the triheteromeric GluN2C-containing NMDAR is the main receptor subtype in cerebellar granule cells (Strong et al., 2021). Future studies using allosteric modulators that can distinguish between the diheteromeric and triheteromeric NMDARs, or experiments using single channel recordings of native NMDARs measuring channel kinetics may provide the most conclusive evidence for the relative expression of the diheteromeric and triheteromeric NMDARs.
1.2.1 *N*-methyl-d-aspartate glutamate receptors (NMDARs) and stroke

Stroke is a cerebrovascular disorder characterized by the sudden onset of symptoms and clinical signs, such as unilateral numbness or weakness in the face, arm, or leg that are caused by...
disruption of blood supply (Yew and Cheng, 2015). Stroke is a leading cause of mortality and disability and a significant economic burden in both developing and developed countries worldwide (Mozaffarian et al., 2016; 2019). There are mainly two types of strokes, ischemic and hemorrhagic, with ischemic form accounting for around 80% of cases (Grysiewicz et al., 2008). For ischemic stroke, the most commonly adopted interventions are intravenous thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) and interventional treatment with endovascular thrombectomy (Prabhakaran et al., 2015; Chamorro et al., 2016). Unfortunately, these two methods are only applicable to a small portion of stroke patients due to their limited therapeutic windows (4.5 hours and 6 hours, respectively) (Herpich and Rincon, 2020; Suzuki et al., 2021). To date, the majority of stroke patients do not have good pharmacological treatments so far and therefore novel stroke therapies are urgently needed.

The mechanisms mediating stroke damage are multifactorial (Lee et al., 1999), but NMDAR-mediated excitotoxicity plays a major role (Simon et al., 1984; Rothman and Olney, 1995; Lee et al., 1999). During a cerebral ischemic event, the sudden discontinued supply of blood and oxygen leads to impaired mitochondrial energy metabolism and depolarization of neuronal cells. This results in excessive release and accumulation of extracellular glutamate, which overactivates NMDARs and triggers a massive influx of calcium into the intracellular space (Kristián and Siesjö, 1998), elevating lactate levels (Rossi et al., 2007), and causes acidosis and oxidative stress with the formation of reactive oxidative species (ROS) (Su and Wang, 2020). The extent to which NMDARs are overactivated by extracellular glutamate released into the either synaptic or extrasynaptic space varies depending on the ischemic location and timing. High concentrations of glutamate remain elevated in the core ischemic region a few hours after an ischemic insult. However, in the penumbra region where the insult is less serious,
the glutamate concentration decreases down to normal levels about 1 hour after the ischemic insult (Ceulemans et al., 2010; Kiewert et al., 2010; Baron et al., 2014). The physiological basal extracellular glutamate concentration is around 0.5-5 μM but would rise to toxic levels of 30-80 μM that can last for up to 3 hours and lead to the occurrence of neuronal excitotoxic events (Kanthan et al., 1995; Kiewert et al., 2010). The EC50 of glutamate for NMDARs ranges from 2-5 μM, which is quite low in comparison with the concentrations during an ischemic event (Erreger et al., 2007). As a consequence, NMDARs are overactivated during an ischemic insult, leading to subsequent cell apoptosis mediated by a series of signaling pathways downstream of NMDARs.

In stroke patients and in animal stroke models, territorial infarction involves the hippocampus, thalamus, hypothalamus, striatum and the overlying frontoparietal and temporal cortices (Garcia et al., 1995; Fluri et al., 2015). Damage to these brain areas produces complex motor, sensory, autonomic, and cognitive deficits (Garcia et al., 1995; DeGracia et al., 1996; Bramlett and Dietrich, 2004; Suri et al., 2018).

1.2.1.1 NMDAR subtypes mediating neuronal survival and death

The ‘NMDAR subtype hypothesis’ states that activating GluN2A-containing NMDARs promotes neuronal survival, whereas activating GluN2B-containing NMDARs induces neuronal death (Lai et al., 2011) (Figure 1.2). Precedent development of subtype specific drugs or drugs that can modulate their activities (modulators) to treat stroke in-vitro and in-vivo models of stroke fuels the establishment of the hypothesis (Sattler et al., 1999b; Aarts et al., 2002a). A large body of literature shows that antagonists specific for the GluN2B-containing NMDARs (ifenprodil or Ro 25-6981) attenuate NMDA-induced toxicity in-vitro and a transient medial cerebral artery occlusion (tMCAO) stroke model in-vivo. In contrast, antagonists preferential for
GluN2A-containing NMDARs (NVP-AAM077 or Zn\textsuperscript{2+}) either have no effect or exacerbate neuronal death (Manzerra et al., 2001; DeRidder et al., 2006; Zhou and Baudry, 2006; Liu et al., 2007; Chen et al., 2008; Terasaki et al., 2010; Zhang et al., 2015; Eyo et al., 2018). These studies use pharmacological tools to isolate the functions of each receptor subtype and provide the first evidence for the NMDAR subtype hypothesis. Subsequently, it is postulated that the large intracellular C-terminal domain (CTD) of GluN2A and GluN2B subunits are coupled to different downstream signaling complexes that mediate opposing physiological functions (Lai et al., 2011; Wu and Tymianski, 2018a). For example, neurons expressing mutant GluN2B subunits with different parts of their CTD sequences replaced by the analogous sequences from the GluN2A CTD have increased tolerance to excitotoxic neuronal death compared with neurons expressing wild-type GluN2B (Martel et al., 2012; Vieira et al., 2016; Tang et al., 2018). Remarkably, GluN2B knock-in mice with a point mutation abolishing the tyrosine phosphorylation site at Y1472 experience reduced toxicity following neonatal hypoxic-ischemic injury (Knox et al., 2014). These studies identified key sites in the GluN2B CTD that mediate neuronal death signaling. On the other hand, expressing mutant GluN2A-containing NMDAR with CTD replaced with the GluN2B CTD exacerbates NMDA-induced neurotoxicity, suggesting a neuroprotective function of the GluN2A CTD (Martel et al., 2012). However, there is also controversial evidence suggesting that knocking down GluN2A or abolishing the phosphorylation site Ser1232 on the GluN2A is neuroprotective against NMDA-induced neuronal death (Wang et al., 2003; Zhou et al., 2013b). Further studies using mutant GluN2A constructs will be needed to identify the key domains of the GluN2A CTD and its interacting proteins that contribute to neuronal survival and death signaling during ischemic brain damage. Collectively, experiments using both genetic and pharmacological manipulations demonstrate
that activating GluN2B-containing NMDAR activates the neuronal death signaling pathways associated with the GluN2B CTD. In contrast, the evidence regarding the roles of GluN2A-containing NMDAR in cerebral ischemia using genetic manipulations is less consistent.

Triheteromeric GluN2A/GluN2B-containing NMDARs may constitute a large proportion of NMDARs expressed in the adult hippocampus and cortex. This receptor subpopulation contains the CTD of both GluN2A and GluN2B subunits, therefore may have both pro-survival and pro-death functions. Based on the pharmacology of triheteromeric NMDARs determined in a recombinant system, NVP-AAM077 (GluN1/GluN2A specific antagonist) blocks the majority (>70%) of triheteromeric NMDARs current whereas Ro 25-6981 (GluN1/GluN2B-specific antagonist) blocks a small percentage (<30%) of the NMDAR current (Stroebel et al., 2018). Despite the strong inhibition of triheteromeric NMDAR current by NVP-AAM077, this GluN2A-containing NMDAR antagonist has no effect or exacerbates excitotoxic neuronal death in-vitro and in-vivo, suggesting that triheteromeric NMDARs activities may not contribute to excitotoxicity overall (DeRidder et al., 2006; Liu et al., 2007; Chen et al., 2008). Recently, Zheng et al showed that activating acid-sensing ion channel 1a (ASIC1a) modulates the function of the NMDA receptor and is dependent on the expressed recombinant triheteromeric GluN2A/GluN2B-containing NMDAR but not the diheteromeric GluN2A- or GluN2B-containing NMDARs (Ma et al., 2019). Importantly, blocking ASIC1a using flurbiprofen reduces the infarct volume following MCAo in-vivo, and this effect is stronger in older animals during which the expression of the triheteromeric GluN2A/GluN2B-NMDAR is increased (Ma et al., 2019). This study suggested that the triheteromeric NMDARs may be coupled to pro-death signaling pathways distinct from the diheteromeric NMDARs containing the same subunits. However, further evidence is needed to verify the successful expression of their triheteromeric
NMDARs and to demonstrate the mechanisms through which triheteromeric NMDARs can have unique functions from diheteromeric NMDARs. One major challenge in studying the functions of the triheteromeric NMDARs is the lack of pharmacological tools to separate them from diheteromeric NMDARs. As such, we reason that developing effective subtype specific modulators, especially those are specific for triheteromeric GluN2A- and GluN2B-containing NMDARs, will be particularly important to verify the relative expression as well as the functions of triheteromeric NMDARs.

![Diagram](Image)

**Figure 1.2 Illustration of ‘subtype hypothesis’ of NMDARs in cerebral ischemia.**

In cortical neurons, activation of GluN2A-containing NMDARs promotes neuronal survival, whereas overactivation of GluN2B-containing NMDARs induces neuronal death. The role of triheteromeric GluN1/GluN2A/GluN2B on cerebral ischemia is still unclear (indicated by ?).

After more than a decade since its commencement, the ‘NMDAR subtype hypothesis’ remains the most influential hypothesis explaining the dual roles of NMDARs in brain ischemia (Liu et al., 2007; Wu and Tymianski, 2018a). Several questions still need to be addressed in future studies. First, the existence of triheteromeric NMDARs remains a confounding issue in
nearly all studies dissecting the roles of GluN2A- and GluN2B-containing NMDARs in neuronal survival and death (Liu et al., 2007; Zhang et al., 2007; Liu et al., 2010b; Lai et al., 2011). Future studies that differentially manipulate the activities of each of the three receptor populations are needed to address this issue. Second, both GluN2A- and GluN2B-containing receptors can mediate downstream signaling through ion influx through the channel or the metabotropic change of their CTD conformation following agonist binding (Kessels et al., 2013; Hu et al., 2016; Weilinger et al., 2016b). However, the relative contributions of each subunit’s function toward the magnitude and direction of their downstream neuronal survival and death signaling remain to be examined. Third, NMDAR subtypes may play different roles at different time points following neuronal damage (Sun et al., 2018). For example, overactivation of NMDARs is considered an early event during an ischemic stroke, but not 2-hours after stroke onset (Lai et al., 2011). Moreover, the expression of NMDAR subtypes can both be upregulated and downregulated depending on the time frames post ischemia (Liu et al., 2010b). Therefore, inhibiting NMDAR subtype activities during different time frames may have different results and will need to be clarified in future investigations. Fourth, most of the studies examining the roles of GluN2A and GluN2B-containing NMDAR subtypes in cell death are limited to hippocampal or cortical neurons, but the results might not be generalized to cell types such as the cerebellar Purkinje cells, of which some atypical NMDAR subunits are predominantly expressed (Quillinan et al., 2015). Parallel to the increased understanding of physiological functions of NMDAR subtypes, recent improvement in the structural biology understanding of NMDAR subtypes and their allosteric modulation is particularly encouraging (Tajima et al., 2016; Lu and Du, 2017; Jalali-Yazdi et al., 2018). Based on the novel high-resolution crystal structures, modulators with
high subtype specificity can be more efficiently developed and used to better dissect the roles of which NMDAR subtypes are involved in stroke pathogenesis.

### 1.2.1.2 Interventions based on the NMDAR subtype hypothesis

NMDAR antagonists were once attractive candidates for treating acute ischemic stroke (Lees, 1997; Muir, 2006). With mounting support for the subtype hypothesis, GluN2B-containing NMDAR specific antagonists including Ro 25-6981 and CP-101,606 were developed and were shown to be effective against stroke damage in preclinical studies (Chenard et al., 1995; Fischer et al., 1997). However, clinical trials with these or similar drugs were discouraging due to strong side effects, limited therapeutic time windows, and/or lack of clinical efficacies (Lees, 1997; Merchant et al., 1999). Recently, pH-sensitive antagonists that specifically acted at low pH were developed and could specifically target the GluN2B-containing NMDARs in ischemic regions (Yuan et al., 2015). One lead candidate of this antagonist family achieved similar neuroprotective efficacy and fewer side effects in an animal model of stroke compared with traditional NMDAR inhibitors such as ketamine and Ro 25-6981 (Yuan et al., 2015). This new generation of GluN2B-NMDAR antagonists may be more tolerated by stroke patients, but it is unclear how issues regarding the narrow therapeutic time window will be addressed. On the other hand, enhancing the channel function of GluN2A-containing NMDARs may enhance cell survival signaling and achieve a longer therapeutic time window for ischemic stroke than inhibiting GluN2B-containing NMDAR (Liu et al., 2007). A newly developed GluN2A-containing NMDAR specific positive allosteric modular (PAM) Npam43 was shown to reduce the infarct volume in a middle cerebral artery occlusion (MCAo) stroke model even when administrated 4.5hrs after stroke (Axerio-Cilies, 2016). Another group of GluN2A-specific PAMs were the lead compound, GNE-6901, bound to GluN2A-containing NMDAR with over
100-fold higher affinity than other GluN2-containing subtypes (Volgraf et al., 2016). Indeed, an improved version of its kind, GNE-0723, has recently been proven to improve brain oscillations, synchrony, and cognitive functions in Dravet syndrome and Alzheimer’s disease models (Hanson et al., 2020). Given that activation of GluN2A-containing NMDARs protects neurons against excitotoxicity via activating neuronal survival signaling, rather than blocking cell death signaling pathway, further pre-clinical and clinical studies using these novel GluN2A-containing NMDAR enhancers may provide stronger evidence suggesting a paradigm-shifting hypothesis in NMDAR-based neuroprotective stroke therapy. A selective enhancement of GluN2A-containing NMDARs, rather than the previously proposed NMDAR blockade, may constitute a more promising therapy for stroke.

### 1.2.1.3 Location of NMDARs and ischemic neuronal survival and death

Synaptic NMDAR and extrasynaptic NMDARs are two functionally distinct pools of receptors. Most early studies support the opposing roles of synaptic and extrasynaptic NMDAR, whereby synaptic NMDARs mediate long term potentiation (LTP) and extrasynaptic NMDARs mediate long term depression (LTD) (Lu et al., 2001). It soon become clear that these opposing roles of synaptic and extrasynaptic NMDARs can be extended into neuronal survival and death, where synaptic NMDARs mediate neuronal survival signaling and extrasynaptic receptors activate neuronal death signaling pathways (Figure 1.3, left) (Hardingham et al., 2002; Zhang et al., 2007; Léveillé et al., 2008). These experimental studies give rise to the ‘NMDAR location hypothesis’ (Liu et al., 2007; Hardingham and Bading, 2010), implying that activating synaptic NMDARs is pro-survival while activating extrasynaptic NMDARs is pro-death. Although this hypothesis has been highly influential, it has been challenged by several other studies. Some groups reported that both synaptic NMDARs mediate neuronal death and extrasynaptic
NMDARs mediate neuronal survival. Additionally, activating synaptic and extrasynaptic NMDARs alone is reported to be pro-survival while activating both induces cell death (Stark and Bazan, 2011; Zhou et al., 2013b; Zhou et al., 2013a; Chen et al., 2014) (Figure 1.3, right).

Nevertheless, therapies that modulate the subpopulations of NMDARs by location are shown to be effective in treating ischemic injury in preclinical studies (Lai et al., 2011; Yuan et al., 2015).

Figure 1.3 Two versions of the ‘NMDAR location’ hypotheses in cerebral ischemia.

The traditional ‘NMDAR location’ hypothesis proposes that activating synaptic NMDARs is prosurvival and activating extrasynaptic NMDARs leads to neuronal death (Left). The alternative version of the ‘NMDAR location’ hypothesis argues that activating synaptic or extrasynaptic NMDARs alone promotes neuronal survival, whereas co-activation of the synaptic and extrasynaptic NMDARs is required to induce stroke damage (Right).
Hardingham et al. first demonstrated that co-applying bicuculine and 4-AP enhances neuronal excitability and is anti-apoptotic in cultured neurons mediated by the strong activation of synaptic NMDARs (Hardingham et al., 2002). On the other hand, pre-blocking synaptic NMDAR with transient MK801 incubation followed by glutamate application activates the extrasynaptic NMDARs and cell death signaling pathways (Hardingham et al., 2002; Lu and Du, 2017). These findings suggest that selectively targeting synaptic NMDARs may be beneficial in triggering cell survival pathways and could counteract the cell death cascades that occur after ischemic insult. Subsequently, many studies have confirmed the opposing roles of synaptic and extrasynaptic NMDARs and explored the diverse mechanisms downstream of these two receptor pools (Kaufman et al., 2012; Karpova et al., 2013; Wang et al., 2013; Lau et al., 2015; Wang et al., 2016b). For example, extracellular signal-regulated kinase 1 and 2 (ERK1/2) are differentially regulated by synaptic and extrasynaptic NMDAR activities (Lai et al., 2011). Only synaptic NMDAR activation enhanced phosphorylated ERK expression and protects cultured neurons from sevoflurane induced toxicity (Ivanov et al., 2006). Similarly, synaptic but not extrasynaptic NMDAR activity induces phosphorylation and nuclear transport of Jacob and protects cultured neurons from NMDA-induced neurotoxicity (Karpova et al., 2013). Moreover, the calcium dependent protease m-calpain is induced by extrasynaptic NMDAR activities, while u-calpain is induced by synaptic activity. Blocking m-calpain and knocking out u-calpain decreases and increases NMDA-induced lactate dehydrogenase (LDH) release, respectively (Wang et al., 2013). Taken together, distinct downstream proteins or protein subtypes are preferentially located at synaptic and extrasynaptic sites. These proteins regulate the balance of neuronal survival and death signaling mediated by the functionally opposing pools of NMDARs.
Contrary to the location hypothesis, several scaffold proteins are primarily located in the post-synaptic density mediated signals related to neuronal death rather than neuronal survival (Sattler et al., 1999b; Zhang et al., 2014). For example, suppressing PSD 95 expression by antisense oligodeoxynucleotide reduces the vulnerability of neurons to NMDA-induced insult, and knocking out PSD 93 results in profound neuroprotection after in-vitro oxygen glucose deprivation (OGD) and in-vivo MCAo (Sattler et al., 1999b; Zhang et al., 2014). Moreover, synaptic and extrasynaptic NMDARs are also found to be preferentially regulated by endogenous co-agonists d-serine and glycine, respectively. Exogenously applied RgDAAO that degrades d-serine is neuroprotective, whereas BsGO degrades glycine exacerbated NMDA-induced neuronal toxicity (Papouin et al., 2012). These experiments raise questions against the ‘location hypothesis’ that synaptic NMDARs mediate neuronal survival and extrasynaptic NMDARs are coupled to neuronal death.

Using a similar experimental design as before, recent studies suggest that synergistic activation of synaptic and extrasynaptic NMDARs are required to induce excitotoxic neuronal death. Several independent research groups show that activating synaptic or extrasynaptic NMDARs alone is neuroprotective against OGD and NMDA-induced cell death, and co-activation of synaptic and extrasynaptic receptors exacerbates neuronal toxicity in neuronal culture and brain slices (Zhou et al., 2013b; Zhou et al., 2013a; Chen et al., 2014) (Figure 1.3, right). They argued that some previous studies supporting the location hypothesis fail to confirm the complete blockade of synaptic NMDAR by MK801, and extrasynaptic NMDAR blocker memantine also blocks the tonic activity of synaptic NMDARs (Wroge et al., 2012). Interestingly, Stark et al mechanistically demonstrated the involvement of both synaptic and extrasynaptic NMDARs in neuroinflammation. Activating synaptic NMDARs enhanced the
expression of a key neuroinflammation modulator, cyclooxygenase-2 (COX-2), while activating extrasynaptic NMDAR currents elevated the production of COX-2 substrate arachidonic acid (AA). Based on their unique roles in neuroinflammation, sequential activations of synaptic and extrasynaptic NMDARs are required for maximized COX-2 mediated neurotoxicity (Stark and Bazan, 2011). These findings challenged the long-standing views supporting the opposing roles of synaptic and extrasynaptic NMDARs.

1.2.1.4 Therapies based on the location hypothesis

Even though the validity of the “NMDAR location hypothesis” is still under debate, many interventions based on this idea, including strategies to block extrasynaptic NMDAR activations and to enhance the synaptic NMDAR activation, are proven to be successful in pre-clinical studies (Liu et al., 2007; Xia et al., 2010). Memantine, a drug approved for treating Alzheimer’s disease and the only NMDAR-targeted drug approved by the FDA, is found to preferentially target extrasynaptic NMDARs (Xia et al., 2010). Pre-clinical proof-of-concept studies show that memantine effectively reduces infarct volume and improves the post-stroke behavioral outcome in rabbits following multiple infarct embolic stroke (Lapchak, 2006; Xia et al., 2010), but large randomized clinical trials are still missing, likely due to the high failure rate of NMDAR antagonists in trials against acute ischemic stroke. One group has exploited the size difference of synaptic cleft and designed a hybrid nanodrug by attaching memantine to a gold nanoparticle; this nanodrug can reach extrasynaptic but not synaptic targets, therefore, may reduce the clinical side effects of memantine. Nano-structured memantine shows neuroprotection in cultured neurons and brain slices of mice, and is more effective than free memantine at protecting neurons against NMDA-induced excitotoxicity (Savchenko et al., 2016). On the other hand, activating synaptic NMDAR activities may have neuroprotective effects with a longer therapeutic time.
window than blocking extrasynaptic NMDAR. The NMDAR co-agonist glycine preferentially enhance synaptic but not extrasynaptic NMDAR activities when exogenously applied (Hardingham et al., 2002). Glycine is neuroprotective against NMDA-induced neuronal death applied even up to 4.5 hours after the insult (Liu et al., 2007). A more recent study has shown that extrasynaptic NMDARs are physically coupled intracellularly with transient receptor potential cation channel subfamily M member 4 (TRPM4) and mediate excitotoxicity. These compounds identified via structure-based design to uncouple NMDAR from TRPM4 effectively eliminated NMDAR-mediated toxicity without altering the intracellular calcium signaling (Yan and Bengtson, 2020).

One important thing that is worth mentioning, the ‘NMDAR subtype’ and ‘NMDAR location’ hypotheses are actually highly complementary. In the adult forebrain, GluN2A-containing NMDARs and GluN2B-containing NMDARs are preferentially localized at the synaptic and extrasynaptic sites, respectively (Tovar and Westbrook, 1999; Harris and Pettit, 2007; Liu et al., 2007; Traynelis et al., 2010). Therefore, activating synaptic NMDARs would stimulate prosurvival pathways downstream GluN2A-containing NMDARs while activating extrasynaptic NMDARs would stimulate prodeath pathways downstream GluN2B-containing NMDARs. Furthermore, normal physiological synaptic transmission activates mainly GluN2A-containing NMDARs and results in the maintenance of neuronal survival. During stroke, on the other hand, excessive glutamate would spill over to the extrasynaptic sites where predominant GluN2B-containing NMDARs are located. Stimulation of these GluN2B-containing NMDARs then leads to the activation of downstream pathways that mediate neuronal death (Lai et al., 2011).
1.2.1.5 Neuronal survival signaling complexes (NSCs) and neuronal death signaling complexes (NDCs) downstream of NMDARs

Despite its critical role in neuronal survival and death, NMDAR activation is considered the initial step during stroke-related neuronal damage and is followed by diverse signaling cascades. The neuronal survival signaling complexes (NSCs) are protein assemblies coupled to the pro-survival NMDARs, whereas the neuronal death signaling complexes (NDCs) are coupled to pro-death NMDARs. The complexity of the NMDAR signaling cascades allows for the continuous discovery of novel NSCs and NDCs. More importantly, characterizing these signaling cascades creates the foundation for discovering novel therapeutics that may have superior efficacy and therapeutic time window compared to many other investigational drugs for stroke.

Neuronal activities induced by low concentrations of NMDA are found to be neuroprotective and are mediated by the increased activation of the NSCs (Lai et al., 2014). The most well-characterized neuronal survival pathway downstream of NMDARs is the phosphoinositide-3-kinase (PI3K)/Akt kinase pathway (Zhao et al., 2006). Triggered by Ca^{2+} entry through NMDARs, PI3K and Akt are activated sequentially and phosphorylate a number of targets that inhibit neuronal death or promote neuronal survival (Alessi et al., 1997; Joyal et al., 1997; Wu and Tymianski, 2018a). Moreover, a synaptically localized adaptor protein APPL1 is critical for coupling synaptic NMDARs with the PI3K-Akt signaling cascade (Wang et al., 2012). Interfering peptide dissociating APPL1 with PSD95 or lentiviral knockdown of APPL1 reduced the coupling of NMDARs and PI3K-Akt cascade, thereby reducing neuroprotection provided by synaptic activity in cultured neurons undergoing trophic deprivation (Wang et al., 2012; Wang et al., 2016a). Another important neuronal survival pathway involves the activation
of the Ras/Extracellular signal regulated kinase (ERK) pathway followed by phosphorylation and activation of the pro-survival transcription factor cyclic AMP response element binding protein (CREB) (Wu et al., 2001). Lentiviral transduction of vector expressing CREB in mice motor neurons enhanced motor function recovery following photothrombotic stroke, whereas silencing the virally transduced neurons using hM4Di-DREADD blocked motor recovery in mice (Caracciolo et al., 2018). Therefore, PI3K-Akt and ERK pathways are consistently activated following the pro-survival NMDARs and mediate NMDAR-related neuroprotection.

Several pro-survival signaling proteins and transcription factors downstream of the NSCs can promote neuroprotective effects (Zheng et al., 2010; Andrabi et al., 2011; Lau et al., 2015). For example, brain derived neurotropic factor (BDNF), is a pro-survival neurotrophin and a target gene of CREB (Park and Poo, 2013). Exogenously applied BDNF is neuroprotective against NMDA-induced cell death, and this effect is mediated by increased transcription of activin A and decreased NMDAR-induced calcium overload (Lau et al., 2015). Similarly, Nuclear factor I-A (NFI-A) is a late phase neuroprotective gene activated by synaptic NMDAR activity. NFI-A deficient mice are more sensitive to NMDA-induced cell death in-vitro and intrastriatal NMDA-induced lesion in-vivo (Zheng et al., 2010). Iduna is another pro-survival gene downstream of NMDA activation and inhibits the neurotoxicity induced by poly (ADP-ribose) polymerase (PARP). Transgenic mice overexpressing iduna have smaller lesion in-vivo following intrastriatal injection of NMDA (Andrabi et al., 2011). These downstream signaling proteins are related to NMDAR activation and mediate enduring neuroprotective effects following ischemic insult. Future investigation should focus on further understanding the intracellular pathways that lead to their activation and whether these pro-survival genes and their
associated neuroprotective signaling pathways interact with one another to promote neuronal survival.

In contrast, NMDAR activation also induces neuronal toxicity through activating the NDCs, including the well-characterized GluN2B-PSD95-nNOS complex (Sattler et al., 1999a; Aarts et al., 2002b), GluN2B-DAPK-P53 complex (Tu et al., 2010; Pei et al., 2014), and GluN1-PTEN complex (Ning et al., 2004). The GluN2B-PSD95-nNOS complex is rapidly assembled following NMDA-induced neurotoxicity and dissociating either the GluN2B-PSD95 or PSD95-nNOS interaction is neuroprotective (Sattler et al., 1999a; Aarts et al., 2002b). Upon formation of this death complex, there are several ways to trigger neuronal death (Lipton et al., 1993; Chen et al., 2013; Li et al., 2013). First, nNOS can produce the neurotoxic molecule NO, which can subsequently form the highly reactive oxidant peroxynitrite to cause DNA damage (Lipton et al., 1993). Second, nitric oxide synapse 1 adaptor protein (NOS1AP) was found to directly interact with NOS and is coupled to the activation of pro-death MAPK kinase p38. Peptide inhibiting NOS1AP and nNOS interaction is neuroprotective in-vitro and in-vivo in a model of neonatal hypoxia-ischemia (Li et al., 2013; Li et al., 2015). Third, Dexras is coupled to nNOS and leads to downstream iron transport through DMT1 and neurotoxicity. Dexras KO mice are protected from NMDA-induced toxicity of retinal ganglion cells (Chen et al., 2013). Taken together, the GluN2B-PSD95-nNOS complex initiates a potent cell death program by triggering multiple pro-death signaling pathways. GluN2B-containing NMDAR activation also induces the formation the GluN2B-DAPK1-p53 complex (Figure 1.4 B). Tu et al. first identified that the association between GluN2B-DAPK1 promotes neuronal death, and disrupting this association using a TAT-NR2BCT peptide protects mice against stroke damage in-vivo (Tu et al., 2010). Subsequently, researchers found that the DAPK1-p53 interaction is critical for propagating the death signal, and
disrupting the interaction using TAT-p53DM is highly neuroprotective against OGD induced apoptosis and necrosis (Pei et al., 2014). However, recently, one group failed to replicate the interaction between DAPK1 and GluN2B and claimed that TAT-NR2B\textsuperscript{CT} functions as an open channel blocker for GluN2B-containing NMDAR to exert neuroprotective effects (McQueen et al., 2017). The controversy on the GluN2B-DAPK1-p53 complex remains unresolved.

Phosphatase and tensin homolog deleted on chromosome TEN (PTEN) is a dual lipid/protein phosphatase that interacts with GluN2B-containing NMDAR but not GluN2A-containing NMDAR, and is directly bound to the GluN1 subunit CTD (Ning et al., 2004). siRNA knockdown of PTEN is neuroprotective against OGD induced neuronal death on hippocampal CA1 neurons (Ning et al., 2004). Moreover, PTEN nuclear translocation is a critical step in ischemic neuronal injury. Using a peptide K13 that flanks the critical residue for PTEN nuclear translocation is strongly neuroprotective in-vitro and in-vivo (Zhang et al., 2013) (Figure 1.4 A).

In summary, nNOS, DAPK and PTEN constitute the key proteins in the traditional “big three” death signaling complexes associated with GluN2B-containing NMDAR activities, inhibiting each NDC in isolation is sufficient to produce potent neuroprotective effects. However, it is less known whether each NDC is independent or interacts with one another. Future studies will need to determine whether each NDC is activated in succession or in parallel and how to most efficiently inhibit multiple death signaling pathways to achieve the best neuroprotective efficacy.
Figure 1.4 Neuronal Death-Signaling Complexes (NDCs) associated with NMDARs.

(A) During cerebral ischemia, overactivated GluN2B-containing NMDARs cause phosphatase and tensin homolog deleted on chromosome ten (PTEN) nuclear translocation and subsequent neuronal death. Similarly, an influx of calcium through NMDARs induces phosphorylation of death-associated protein kinase 1 (DAPK1) and its recruitment to the C-terminal tail of GluN2B-containing NMDARs. Activated DAPK1 further promotes p53 nuclear translocation to induce neuronal death. (B) Under ischemic conditions, postsynaptic density protein 95 (PSD95)-GluN2B-neuronal nitric oxide synthase (nNOS) complex formation is required for the massive GluN2B-containing NMDAR-dependent NO production. Moreover, the GluN2B-PSD95-dual leucine zipper kinase (DLK)-c-Jun N-terminal kinase (JNK) pathway is activated by hyperfunctioning NMDARs and contributes to neuronal death during ischemia. (C) Excess glutamate release during ischemia triggers the activation of NMDARs and their downstream targets SRC family kinase (SFK) and pannexin-1 in a calcium-independent manner. Pannexin-1 activation leads to ionic dysregulation and, consequently, neuronal death.
Several other NDCs that assemble at the CTD of the GluN2B subunit have emerged recently. For example, a NMDAR-Src-Panx1 complex was discovered to be activated during ischemia. Blocking the Y308 site at the C-terminal of Panx1 using an interfering peptide can reduce the infarction size following MCAo (Weilinger et al., 2016a) (Figure 1.4 C). Interestingly, this complex is dependent on the metabotropic function of NMDAR, meaning that it can be triggered by glutamate binding to NMDAR alone and is independent of the ion influx (Weilinger et al., 2016a). These pieces of evidence suggest that calcium overload is not required for NMDAR related neuronal toxicity and emphasize the importance of targeting calcium independent pathways during a stroke. Moreover, Dual leucine zipper kinase (DLK) is associated with PSD95 and mediates excitotoxicity. DLK knockout attenuated JNK activation following in-vivo injection of kainic acid and is significantly protected from hippocampal neurodegeneration (Pozniak et al., 2013). Recently, a potent DLK selective inhibitor, GNE-3511, was discovered and was found to be neuroprotective in various models of neurodegeneration and neurotoxicity (Patel et al., 2014; Larhammar et al., 2017; Patel et al., 2017). NMDARs are even reportedly to physically interact with TRPM4 and activate downstream signaling pathways. And disrupting that physical coupling by specifically designed compounds effectively improved mitochondrial dysfunction and against excitotoxic cell death in both cultured neurons and in mouse models of ischemia (Yan and Bengtson, 2020). Based on these studies, we can expect that inhibiting any of the NDCs can potently protect neurons from excitotoxicity in-vitro and in-vivo. Therefore, developing potent and specific neuroprotective therapeutics targeting these NDCs might lead to novel treatments of stroke with clinical success.

Further downstream of NDCs, several death-promoting molecules are activated to trigger neuronal death (see (Wu and Tymianski, 2018b) for a detailed review of these signaling
pathways). For example, calcium dependent cysteine protease calpain is activated following pro-
death NMDAR activities and cleaves sodium-calcium exchanger (NCX3) (Bano et al., 2005),
Kinase D-interacting substrate of 220kDa (kidins220) (López-Menéndez et al., 2009), the striatal
enriched protein tyrosine phosphatase (STEP) (Xu et al., 2009), metabotropic glutamate receptor
1 (mGluR1) (Xu et al., 2007), and drebrin (Chimura et al., 2015). Calpain inhibitor (SNJ-1945),
peptide Tat-K which interferes with calpain degradation of kidins220, and Tat-STEP that
interferes with calpain degradation of STEP all showed therapeutic potential against stroke
damage using the MCAo model (Koumura et al., 2008; Xu et al., 2009; Gamir-Morrala et al.,
2015). Similarly, the mitogen-activated protein kinase (MAPK) family protein p38 MAPK and c-
Jun amino terminal kinase (JNK) are activated during excitotoxicity (Kawasaki et al., 1997).
Novel evidence suggested that JNK activities might be downstream of the DLK complex, and
inhibiting either protein is neuroprotective against neurotrophin deprivation (Larhammar et al.,
2017). Moreover, SREBP is a transcription factor activated downstream of pro-death NMDAR
activation and regulates cholesterol and lipid biosynthesis. An interference peptide, Indip, was
developed to block the nuclear translocation of SREBP and was shown to be neuroprotective in-
vitro and in-vivo (Taghibiglou et al., 2009). Lastly, the endocytosis of ionotropic kainic acid
(KA) and AMPA-type glutamate receptors are critical steps during excitotoxicity (Wang et al.,
2004; Zhu et al., 2014). Blocking the endocytosis of each receptor family using an interference
peptide for AMPAR or a point mutation on the phosphorylation site of KA receptor is
neuroprotective during a stroke (Wang et al., 2004; Zhu et al., 2014). These downstream targets
may be particularly important during the late phase of excitotoxicity and may achieve a very
long therapeutic time window of up to 12 hrs (Borsello et al., 2003).
1.2.1.6 Treatments targeting NMDAR signaling cascades

Since NSCs and NDCs can be activated for hours or even days after the initial NMDA activation during ischemic stroke, targeting NSC and NDC might effectively treat stroke with a prolonged therapeutic time window (Zhang et al., 2013; Pei et al., 2014; Wu and Tymianski, 2018b). To enhance NSC signaling, Jo et al. developed a small molecule activator of the prosurvival kinase Akt known as SC79 through in-vitro high-throughput screening (Jo et al., 2012). They found that SC79 attenuated glutamate induced neuronal death in-vitro and the infarct volume in-vivo following permanent MCAo (Jo et al., 2012). Likewise, neuroprotectin D1 (NPD1) is an endogenous molecule that has neuroprotective effects post stroke (Bazan, 2006). Application of a NPD1 precursor DHA following ischemic damage in rats upregulates the neuronal survival molecule iduna, reduces infarct volume and rescues their neurological score (Belayev et al., 2017). On the other hand, blocking NDC signaling has already resulted in the most successful of all neuroprotective treatments. TAT-NR2B9c (also referred to as NA-1) is a cell penetrating interfering peptide that binds to the PDZ domains of PSD95. It was shown to be strongly neuroprotective against ischemic stroke in both rodent models and in a non-human primate model of stroke (Aarts et al., 2002b; Cook et al., 2012). Most promisingly, NA-1 completed the phase II clinical trial against the treatment of iatrogenic stroke after endovascular aneurysm repair (Hill et al., 2012) and is currently undergoing two phase III clinical trials FRONTIER (NCT02315443) and ESCAPE-NA1 (NCT02930018) (Hill et al., 2020; Zhou, 2021) (Figure 1.5).
Figure 1.5 Diverse classes of treatment have been developed to protect neurons from NMDAR-mediated excitotoxicity based on recently identified neuronal survival and death-signaling pathways. These include molecules that modulate the channel activities of selected NMDAR subtypes, interfering peptides that inhibit intracellular death-signaling protein complexes and enzyme activators and inhibitors. Treatments enhancing the functions of their targets are colored in green, whereas protein or protein complex inhibitors are colored in red.

1.2.2 Allosteric modulation of NMDARs

1.2.2.1 Structural basis for allosteric modulation

As mentioned previously, NMDA receptors are heterotetrameric assemblies that consist of two GluN1 and two GluN2 subunits which require the binding of the agonist glutamate and co-agonist glycine or D-serine (Benveniste and Mayer, 1991; Clements and Westbrook, 1991)
(Figure 1.6 Right). Synaptic NMDA receptors are activated by the synaptic release of glutamate as glycine or d-serine is thought to be constantly present in the extracellular space (Billups and Attwell, 2003; Wolosker, 2007; Mothet et al., 2015). The Papouin group has suggested that D-serine is the dominant co-agonist at synapse while glycine is the main co-agonist at extrasynaptic sites. Glycine and d-serine bind to the two binding sites of GluN1 subunits, while glutamate binds to the two binding sites of GluN2 subunits (Papouin et al., 2012).

Recent studies of crystal or cryo-EM structures of intact or individual domains of NMDA receptors have provided a structural framework based on which we can look at the biophysical properties of the receptors and allosteric modulation. NMDA receptors share a similar architecture as other glutamate receptors: an extracellular amino-terminal domain (ATD), a bilobed agonist-binding domain (ABD), a pore-forming transmembrane domain (TMD), and an intracellular carboxyl-terminal domain (CTD) (Figure 1.6 Left). The TMD consist of three transmembrane helices (M1, M3, and M4) and a re-entrant loop (M2). The re-entrant loop M2 forms the intracellular portion of the ion channel pore and the third transmembrane segment M3 forms the extracellular part of the pore. The residues in the pore region that control ion permeation are highly conserved, despite the number of NMDA receptor subtypes. For instance, the residue at the apex of the re-entrant M2 loop in NMDARs determines the Mg$^{2+}$ blockade (Wollmuth, 2018). The ATDs of GluN1 and GluN2 subunits form a back-to-side bilobed structure which provides a lot of sites for modulating NMDAR functions (Yuan et al., 2009; Farina et al., 2011).
The ABDs also form bilobed structures that have an upper lobe and a lower lobe with a pocket for the agonist binding in-between. The glycine-binding pocket in GluN1 is smaller and more hydrophobic in comparison with the glutamate-binding pocket in GluN2 (Furukawa and Gouaux, 2003; Yao et al., 2008; Yao et al., 2013). Residues within the glutamate-binding pocket that interact with agonists or other competitive antagonists are highly conserved among GluN2 subunits (Lind et al., 2017; Romero-Hernandez and Furukawa, 2017). The TMDs are symmetrically arranged surrounding the ion channel pore while the extracellular portion of the receptor forms a dimer-of-dimer pose. Based on published crystal structures, extensive contacts
between the two GluN1/2 LBDs and the ATDs are observed, giving the NMDA receptor a more compact appearance compared to other glutamate receptors and produces numerous protein-protein interfaces where modulators can bind. Therefore, the close intra- and inter-domain contacts create great potential for allosteric interactions between subunits and allosteric modulation by small molecules (Hansen et al., 2017).

1.2.2.2 Positive, negative and dual allosteric modulators of NMDARs

Given the extensive intra- and inter-domain contacts inherent to NMDARs as described above, many agents have been reported that modulate NMDAR function, including both endogenous and exogenous molecules (Figure 1.7). For example, extracellular Zn\(^{2+}\) is an endogenous molecule that binds to the GluN2A and GluN2B ATDs (higher affinity to GluN2A than GluN2B) and negatively modulates NMDARs in a voltage-independent manner (Williams, 1996; Choi and Lipton, 1999; Karakas et al., 2009). In addition, extracellular protons can also strongly inhibit NMDA receptor function with an IC50 of around 50 nM. However, the extent to which NMDA receptors are inhibited by protons depends on the GluN2 subunit composition: GluN2A-, GluN2B-, and GluN2D-containing NMDARs are inhibited to a greater extent than GluN2C-containing receptors near physiological pH (7.2 – 7.4) (Traynelis et al., 1995; Low et al., 2003). Endogenous neurosteroids such as pregnenolone are also able to potentiate GluN1/GluN2A and GluN1/GluN2B while inhibit GluN1/GluN2C and GluN1/GluN2D NMDARs (Horak et al., 2006). In addition, 24(S)-hydroxycholesterol, a major brain cholesterol metabolite, is demonstrated to be a pan-potentiator that augments the signal of all NMDAR subunits (Paul et al., 2013).

Apart from endogenous modulators, other reagents have also been discovered or developed to allosterically modulate NMDA receptor functions. Polyamines, including spermine
and spermidine, are reported to act as positive allosteric modulators (PAMs) and selectively enhance GluN2B through binding to the negatively charged residues in the lower lobes of GluN1 and GluN2B ATDs (Mony et al., 2011). One more interesting thing needs to be noted is that by binding to the spermine binding site, extracellular Mg$^{2+}$ could increase the receptor's affinity for glycine of GluN1-a/GluN2B and potentiate the NMDA response (Paoletti et al., 1995; Wang and MacDonald, 1995; Lu et al., 1998). In contrast, ifenprodil and Ro 25–6981 bind the subunit interface between GluN1 and GluN2B ATDs and inhibit GluN2B functions as negative allosteric modulators (NAM) (Williams, 1993; Fischer et al., 1997; Karakas et al., 2011). New generations of GluN2B-selective NAMs that bind at the ifenprodil site have recently been synthesized with improved potency and selectivity (CP-101,606) (Chenard et al., 1995) or with add-on applicable features such as pH-sensitivity to the context (Yuan et al., 2015) and better bioavailability (Stroebel et al., 2016).

NVP-AAM077 is a competitive antagonist that binds to the glutamate binding site of NMDAR LBDs. While NVP-AAM077 has been considered selective for GluN2A over GluN2B since its discovery (Auberson et al., 2002), subsequent studies have found its 5-fold selectivity is insufficient to distinguish synaptic responses from GluN2A- and GluN2B-containing NMDARs unless it is used carefully at a very low concentration (Frizelle et al., 2006; Neyton and Paoletti, 2006; Bartlett et al., 2007). TCN-201 and MPX are developed as novel GluN2A-selective NAMs that bind to the LBD interface between GluN1 and GluN2A subunits (Bettini et al., 2010; Hansen et al., 2012; Volkmann et al., 2016). PAMs for GluN1/GluN2A have also been reported recently, including several structurally-related compounds, referred as GNE compounds, and have been demonstrated to bind to the LBD heterodimer interface between GluN1 and GluN2A subunits similarly to the GluN2A NAMs mentioned above (Hackos et al., 2016; Volgraf et al.,
Meanwhile, our lab discovered a GluN1/GluN2A specific PAM through in-silico screening, Npam43, which has demonstrated satisfying neuroprotective effects against ischemic stroke. Npam 43 is reported to bind to the ATD interface between GluN1 and GluN2A subunits based on site-directed mutagenesis analysis (Axerio-Cilies, 2016). A series of quinazolin-4-ones (QNZ) have been discovered as NAMs of NMDARs that are selective for GluN1/GluN2C- and GluN1/GluN2D NMDARs and bind to the lower lobe of the GluN2C/D LBD (Hansen and Traynelis, 2011). A series of tetrahydroisoquinoline PAMs, such CIQ, that are highly selective for GluN2C/D-containing NMDA receptors have also been found where the potentiation by CIQ caused by binding to residues in the TM1 helix and a short preM1 helix in the GluN2C/D subunit (Mullasseril et al., 2010; Santangelo Freel et al., 2013, 2014). Another compound, PYD-106, has better selectivity for GluN2C over GluN2D and binds at the interface between the lower portion of the ATD and the upper lobe of the GluN2C LBD (Khatri et al., 2014). A positive allosteric modulator (PAM), Npam59, that specifically potentiates both GluN1/GluN2A and GluN1/GluN2B has been characterized following its discovery (Axerio-Cilies, 2016; Li, 2017) and was found to boost the d-amphetamine-induced release of dopamine in the nucleus accumbens (NAc) in vivo upon administration (Li, 2017).

NAMs, PAMs, and dual allosteric modulators (DAMs) of NMDARs have attracted tons of academic and industrial attention because of their vast potential in suppressing or potentiating NMDAR functions without altering the binding of agonists or co-agonists, unlike classic glutamate site competitive antagonists, such as AP5 (Morris, 1989). Allosteric modulations also allow the manipulation of specific subtype of NMDA receptor, sparing the physiological function mediated by other subtypes. For example, boosting the GluN1/GluN2A function is thought to be beneficial (Hanson et al., 2020) while overstimulating GluN1/GluN2B is
detrimental (Liu et al., 2007; Vieira et al., 2016). Therefore, the ideal situation is to potentiate GluN1/GluN2A and inhibit GluN1/GluN2B functions but not complete elimination of GluN1/GluN2B functions to preserve their physiological roles in CNS.

![Diagram of NMDARs with binding sites labeled](image)

**Figure 1.7 Known or predicted binding sites of agonists, co-agonists, antagonists, positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) on NMDARs.**

Illustration modified from the crystal structure of GluN1/GluN2B with bond ifenprodil (green sticks), glutamate (green balls) and glycine (yellow balls) (Protein Data Bank ID. 5IOV; (Zhu et al., 2016)).


1.2.2.3 Channel pore blockers of NMDARs

The ion channel pore of NMDARs consist of intracellular and extracellular vestibules that are divided by a narrow space. This narrow space is located at the top of the membrane reentrant loop M2 (Q/R/N site), and is referred to as an ‘ion-selective filter’ that determines the permeability of calcium, single channel properties, and channel blockers (Wollmuth and Sobolevsky, 2004; Glasgow et al., 2015).

The most renowned channel blocker is magnesium (Mg$^{2+}$) which voltage-dependently blocks NMDARs. Blockade by Mg$^{2+}$ can be reduced when neuronal depolarization happens which allows the activation of NMDARs following the binding of glutamate and glycine (Perszyk et al., 2020). The concentrations of Mg$^{2+}$ that induce half-maximal inhibition (IC50) vary by NMDAR subtypes, with stronger blockade for GluN1/GluN2A (2 µM) and GluN1/GluN2B (2 µM) but relatively weaker blockade for GluN1/GluN2C (14 µM) and GluN1/GluN2D (10 µM) (Kuner and Schoepfer, 1996; Qian et al., 2005; Siegler Retchless et al., 2012). Similar to Mg$^{2+}$, ketamine, phencyclidine [PCP], and MK-801 bind to the channel pore when it is opened following receptor activation but are trapped in the channel pore as it closes (Sobolevsky and Yelshansky, 2000; Poulsen et al., 2015). As such, these blockers are often referred to as ‘open channel blockers’

Unlike the open channel blockers mentioned above, the channel blockers amantadine and memantine act in a different pattern when blocking the NMDAR channel pore. Amantadine and memantine can obstruct the channel pore closure but not completely prevent it, hence are often termed partial trapping blockers (Blanpied et al., 1997; Chen and Lipton, 1997; Mealing et al., 1999; Johnson et al., 2015). Interestingly, as partial trapping blockers, ketamine and memantine have around an 8-fold preference for GluN1/GluN2C and GluN1/GluN2D over GluN1/GluN2A.
and GluN1/GluN2B in the presence of the physiological concentration of Mg²⁺ (Kotermanski and Johnson, 2009).

Distinct from the open channel and partial trapping blockers, a third type of NMDAR channel pore blocker has been demonstrated to bind to a closed channel (e.g., dextromethorphan [DMX]) (Netzer et al., 1993; LePage et al., 2005). The binding site of DMX may be shallower in comparison with the binding of MK801 (LePage et al., 2005).

NMDAR channel blockers have been preclinically demonstrated to have neuroprotective effects against excessive NMDAR activation including stroke, epilepsy, and traumatic brain injury. Disappointingly, subsequent clinical trials have not been successful due to their dose-limiting side effects, patient heterogeneity, and narrow temporal windows for intervention (Ikonomidou and Turski, 2002; Farin and Marshall, 2004; Muir, 2006). However, NMDAR channel blockers that bind with high affinity, including ketamine and PCP, are typically dissociative anesthetics in clinical use have attracted extensive interest for the treatment of major depressive disorders (MDDs) due to recent encouraging evidence of the antidepressant-like effects of NMDAR antagonists (Niciu et al., 2014; Abdallah et al., 2015; Yang et al., 2018). More recently, dextromethorphan (DXM), a drug previously prescribed to treat cough, is well tolerated and exhibits rapid-onset clinically significant antidepressant effects (Majeed et al., 2021). Lastly, memantine is an approved NMDAR channel blocker for the treatment of moderate to severe Alzheimer’s disease, although the mechanism is not well understood (Cosman et al., 2007; Cappell et al., 2010).
1.2.3 **Hypothesis and specific aims**

Based on both the location and subtype NMDAR hypotheses, GluN2A-potentiating and GluN2B-inhibiting NMDAR dual allosteric modulators (Ndam) have promising potential as a new class of stroke therapeutics. Through previous virtual screening and in silico drug design, a lead compound 813 was found to function as an Ndam that potentiates GluN2A-containing NMDARs and at the same time inhibits GluN2B-containing NMDARs, albeit with very low potency. We believe that such a dual functioning compound, if its efficacy can be further improved, may have great potential for treating excitotoxic neuronal injuries. Therefore, I hypothesize that neuroprotection against excitotoxic neuronal damages may be achieved by simultaneously potentiating synaptic GluN1/GluN2A and inhibiting extra-synaptic GluN1/GluN2B. Chapter 3 in this thesis is designed to test this hypothesis through the following specific aims:

1. Identification and optimization of drug candidates to find a lead compound using structure-based in-silico drug design and in-vitro functional screening.
2. Assessment of the neuroprotective effects of the lead compound in vitro.
3. Determination of the in-vivo blood-brain barrier (BBB) penetrating ability of the lead compound.
4. Investigation of the neuroprotective effects of the lead compound in an in vivo stroke model and its effects in facilitating neurological function recovery.

This proposed study will not only provide supporting evidence for the subcellular ‘NMDAR location’ and ‘NMDAR subtype’ hypotheses in excitotoxicity, but might also yield a novel therapeutic intervention against ischemic stroke.
1.3 Overview of the GABA<sub>A</sub>R

1.3.1 GABA<sub>A</sub>R structure and function

In contrast to NMDA receptors that mediate neuronal excitation, type A γ-aminobutyric acid receptors (GABA<sub>A</sub>Rs) are the major inhibitory receptors in the mammalian central nervous system (CNS) (Olsen and Sieghart, 2009). GABA<sub>A</sub>Rs are heteropentameric chloride channels that contain five subunits encoded by 19 different genes (α1-6, β1-3, γ1-3, δ, ε, π, ρ1-3, and θ) (Barnard et al., 1998; Olsen and Sieghart, 2008). The assembly of GABA<sub>A</sub>Rs as heteropentamers yields heterogeneous structures which gives rise to their varying pharmacological profiles (Olsen and Sieghart, 2008). A typical GABA<sub>A</sub>R in the brain consists of two α subunits, two β subunits and one subunit of either γ, δ, ε, θ or π (Figure 1.8) (Sieghart and Sperk, 2002). The α/β-containing GABA<sub>A</sub> receptors are the most abundantly expressed in the brain (>95%) with the most common isoform being α1β2γ2 that constitutes 60% of all GABA<sub>A</sub>Rs (Möhler, 2006). Expression of recombinant fully functional GABA<sub>A</sub>Rs capable of GABA-dependent channel gating requires the presence of all α, β, and γ subunits in a heterologous cell line (Sigel et al., 1990).

The overall architecture of the human GABA<sub>A</sub>R, as determined by X-ray crystallography, shows that each GABA<sub>A</sub>R subunit contains a large extracellular domain, four hydrophobic transmembrane domains (TM1–4), and an intracellular loop between TM3 and TM4 (Ernst et al., 2005; Miller and Aricescu, 2014). The transmembrane domain TM2 is reported to line the channel pore (Olsen and Tobin, 1990) and the intracellular loop is the target for protein interactions and post-translational modifications (Brandon et al., 2000; Saliba et al., 2012; Nani et al., 2013). The extracellular domains contain a positively charged ring that is thought to be responsible for the receptor’s selectivity for chloride anion (Miller and Aricescu, 2014). In
addition, these domains provide binding sites not only for the endogenous ligand GABA but other various modulators, such as benzodiazepines (BZDs) (Enna and McCarson, 2013; Sigel and Ernst, 2018). In the major isoforms in native GABA_ARs (αβγ-containing GABA_ARs), the extracellular domain of each subunit forms a pseudo-symmetrical ring, thereby creating two binding sites for GABA at the α/β interfaces (Ernst et al., 2003; Kash et al., 2004) (Figure 1.8).

As a GABA-gated chloride channel, activation of GABA_AR triggers an influx of chloride ions which hyperpolarize the postsynaptic membrane and reduces the probability of firing an action potential (AP) in the target cell of mature neurons (Kaila, 1994). There are two forms of GABA_AR-mediated inhibition: phasic inhibition and tonic inhibition. The phasic inhibition is a fast and transient inhibition by synaptic GABA_AR transmissions (Cherubini, 2012). The tonic inhibition is a slow but persistent inhibition caused by activating extrasynaptic GABA_ARs (Farrant and Nusser, 2005; Brickley and Mody, 2012). At the early stages of development, GABA_ARs have excitatory functions resulting from a reversed chloride membrane gradient (Obata et al., 1978; Ben-Ari et al., 1989) and undergo a developmental switch towards maturation (Owens and Kriegstein, 2002; Cherubini et al., 2011).
Figure 1.8 Molecular architecture of a typical GABA\textsubscript{A}R.

(Left) Side view of GABA\textsubscript{A}R showing the typical extracellular domain (ECD) and transmembrane domain (TMD) with known or predicted binding sites of GABA, PAMs (BZDs, barbiturates, general anesthetics, neurosteroids, ethanol, glutamate), NAMs (flumanzenil and bemepride) on GABA\textsubscript{A}Rs. (Right) Top view of GABA\textsubscript{A}R showing the pentameric arrangement of $\alpha$ (red)/$\beta$ (blue)/$\gamma$ or $\delta$ (green) subunits. Illustration created using the crystal structure of human GABA\textsubscript{A}R (Protein Data Bank ID: 6X3U; (Kim et al., 2020)).

1.3.2 Allosteric modulation of GABA\textsubscript{A}Rs

GABA\textsubscript{A}Rs play critical roles in maintaining the excitation/inhibition (E/I) balance in the CNS; hence they have been considered promising therapeutic targets for treating related neurological disorders. GABA\textsubscript{A}R allosteric modulators can be categorized into NAMs and PAMs. Despite their endogenous and exogenous sources, GABA\textsubscript{A}R PAMs are exemplified by alcohol, benzodiazepines (BZDs), neurosteroids, barbiturates, general anesthetics and glutamate while NAMs include flumazenil (FLZ), bemepride, thiocolchicoside (TCC) (Zaitseva, 1961;
BZDs like diazepam and alprazolam exert tranquilizing effects and are clinically used to treat anxiety, sleep disorders, seizure disorders, muscle spasms, and some forms of depression (Möhler et al., 2002). There are two binding sites for BZDs on the GABA~A~R. The classic high-affinity binding site for benzodiazepines on GABA~A~R is located at the α1+/γ2- interface that is homologous to the GABA-binding position that is located between α1-/β2+ subunits in this receptor. The second low-affinity binding pocket for BZDs locates at the α+/β- interface of GABA~A~R (Sigel and Ernst, 2018). The binding of BZDs to the GABA~A~Rs causes conformational changes of the receptor and increases its binding affinity for GABA (Sieghart, 1994; Johnston, 1996). Single channel recording studies have revealed that benzodiazepines enhance GABA~A~R function primarily through increasing channel open frequency but not open time duration (Vicini et al., 1987).

Barbiturates are a group of sedative drugs used in clinics and act as GABA~A~R PAMs. Barbiturates work as a modulator at <10 μM, yet also directly activate the GABA~A~R at a high concentrations (usually >100 μM) (Feng et al., 2004; Muroi et al., 2009; Fisher and Fisher, 2010). In contrast to benzodiazepines, barbiturates boost the single channel property of the GABA~A~R by increasing the channel open time duration without changing the open frequency (Twyman et al., 1989). Given the double actions of barbiturate, it is postulated that barbiturate may have two binding sites on the GABA~A~R. Indeed, two binding sites of barbiturate on GABA~A~R were reportedly located between the interfaces of α1+/β3- and γ2+/β3- subunits of the receptor’s transmembrane domain (TMD) (Savechenkov et al., 2012).
Many anesthetics including propofol, etomidate and isoflurane, positively modulate the GABA\(_A\)R function via increasing the receptors’ binding affinity for lower concentrations of GABA, decreasing the rate and extent of desensitization and slowing down the receptors’ deactivation (Nakahiro et al., 1989; Orser et al., 1998; Krasowski et al., 2001). At higher concentrations, etomidate, propofol, and barbiturates can also directly activate GABA\(_A\)Rs in the absence of GABA (Garcia et al., 2010). It has been proposed that the binding site for propofol, etomidate and pentobarbital is located at the \(\alpha 1/\beta 2\) interface of the transmembrane domain (TMD) (Maldifassi et al., 2016).

Electrophysiological studies have shown ethanol can potentiate the GABA\(_A\)R functions in neurons (Aguayo, 1990; Nakahiro et al., 1991). Single channel recordings reveal that ethanol increases both channel opening frequency and mean open time of the GABA\(_A\)R (Tatebayashi et al., 1998). The positive allosteric modulation action of alcohol is responsible for symptoms like anxiolytic, sedation, hypnosis and motor impairments depending on the brain regions being affected (Buck, 1996; Liang and Olsen, 2014). A predicted ethanol binding pocket was proposed recently to be at the \(\alpha/\beta\) interfaces of the \(\alpha\beta3\delta\) GABA\(_A\)R extracellular domain (ECD) (Wallner et al., 2014).

Neurosteroids are steroids or steroid metabolites that can directly regulate neuronal activity through binding to target receptors without entering the nucleus and regulating transcription and expression (Liang and Olsen, 2014). The modulation effects of neurosteroids vary a lot with the type of neurosteroids and the subunit composition of the GABA\(_A\)R. The most well-known neurosteroids are progesterone metabolites 5\(\alpha\)-pregnan-3\(\alpha\)-ol-20-one (3\(\alpha,5\alpha\)-THPROG), 5\(\beta\)-pregnan-3\(\alpha\)-ol-20-one (3\(\alpha,5\beta\)-THPROG), and the deoxycorticosterone metabolite 3\(\alpha,5\alpha\)-tetrahydrodeoxycorticosterone (3\(\alpha,5\alpha\)-THDOC). They exert their effects either as positive
allosteric modulators at lower concentrations or directly activate GABA\textsubscript{A}Rs at higher concentrations (Belelli et al., 1990; Lambert et al., 1995). The binding site for neurosteroids is located at the transmembrane (TM) domains of the $\alpha$-subunit and both open frequency and duration of the GABA\textsubscript{A}R channel are increased upon the binding of neurosteroids (Callachan et al., 1987; Puia et al., 1990; Zhu and Vicini, 1997). However, higher concentrations of steroids bind to a site between $\alpha$ and $\beta$ subunits that differ from the positive modulation site (Hosie et al., 2006).

Glutamate is an excitatory neurotransmitter in CNS and agonist for several glutamate receptors including NMDA receptors (NMDARs), AMPA receptors (AMPAR) and metabolic glutamate receptors (mGluRs). Early research has revealed that glutamate and its analogs (quisqualate, kainate and NMDA) can reversibly potentiate GABA\textsubscript{A}R-mediated responses in hippocampal neurons (Stelzer and Wong, 1989). Our lab has also found that glutamate is an allosteric modulator that act on the glycine receptor (Liu et al., 2010a). More recently, a novel glutamate-binding site at the $\alpha+/\beta$- interface of the extracellular domain of GABA\textsubscript{A}R has been identified. Glutamate and analogs are reported to potentiate both the synaptic GABA\textsubscript{A}R-mediated phasic inhibition and the extrasynaptic GABA\textsubscript{A}R-mediated tonic inhibition upon the activation of this glutamate binding site (Wen, 2016).

Flumazenil (FLZ) has become known as a benzodiazepine (BZD) inhibitor and is now used in clinic practice to treat benzodiazepine dependence. The crystal structure of human GABA\textsubscript{A}R in complex with GABA plus flumazenil has already been solved recently, revealing the binding site of flumazenil being at the $\alpha1/\gamma2$ interface of the extracellular domain of the receptor. Chronic benzodiazepine use, even at therapeutic doses, is reported to be associated with the development of physical dependence characterized by moderate to severe withdrawal
symptoms upon cessation including emergent anxiety attack and symptomatic depression (Petursson and Lader, 1981; Ashton, 1991; de las Cuevas et al., 2000). Flumazenil is able to alleviate persistent withdrawal symptoms in patients who have been benzodiazepine free for up to two years (Lader and Morton, 1992).

Bemegride has an antagonistic action on the GABAA receptor through suppressing both GABA- and pentobarbitone-evoked whole-cell currents, thus making it a treatment option for hypnotic overdose (Mistry and Cottrell, 1990). So the predicted binding of bemegride might be near the binding sites of barbiturates. It is also considered as a central nervous system stimulant due to its negative modulation effects (Faingold et al., 1985).

Thiocolchicoside (TCC) is widely used as a muscle relaxant to treat myofascial spasm of muscles (Yilmaz et al., 2019). It has also been shown to interact with GABAARs and strychnine-sensitive glycine receptors in the rat central nervous system. Moreover, TCC manifests convulsant activity and epileptogenic effects in animals and humans possibly through inhibiting phasic and tonic GABAAR-mediated currents of Purkinje cells and granule neurons in a concentration-dependent manner (Carta et al., 2006; Mascia et al., 2007). However, the binding site of TCC has not been investigated yet. As negative allosteric modulators of GABAARs, flumazenil (FLZ), bemegride and TCC have been recently proposed as a potential therapeutic option for the management of GABAAR gain-of-function related neurological disorders (Chen et al., 2022).

1.3.3 GABAAR dysfunction and neurological disorders

A disturbance of E/I induced by impaired GABAAR functions can trigger the occurrence of epilepsy (Ben-Ari et al., 2007; Kaila et al., 2014). Epilepsy is a neurological disorder characterized by frequent and unexpected seizures caused by abnormal brain electric discharge
and leads to loss of consciousness and abnormal behaviors (Jacobs et al., 2009). Mutations in GABA\textsubscript{A}R subunit genes are found to be closely associated with the pathogenesis of epilepsy since the mutation-caused impairments in the gating properties of the GABA\textsubscript{A}R channel, receptor protein expression, or receptor trafficking (Macdonald et al., 2010). The extent of disorder severity varies depending on the type of mutation (nonsense, missense, or frameshift), the affected region of the encoded protein (intra-/extracellular or transmembrane) and the affected specific subunit (Hirose, 2014; Braat and Kooy, 2015). For example, Dravet syndrome, also called severe myoclonic epilepsy in infancy (SMEI), is a form of pediatric epilepsy that occurs in 1 year-old children can result from mutations in genes encoding the \( \alpha_1, \beta_1, \beta_2, \) and \( \gamma_2 \) subunits of GABA\textsubscript{A}Rs (Carvill et al., 2014; Mele et al., 2019). GABA\textsubscript{A}R mutations related to epilepsy are also linked with abnormal trafficking of the receptors and their expression on the cell membrane (Kang et al., 2015; Mele et al., 2019). Given its important role in the pathogenesis of epilepsy, GABA\textsubscript{A} receptors have attracted mounting interest as targets for antiepileptic drugs (Schipper et al., 2016; Palma et al., 2017; Janković et al., 2021).

Apart from epilepsy, evidence also indicates that dysfunction of GABA\textsubscript{A}Rs underlies the pathogenesis of neurodevelopmental disorders such as impaired attention and social behavior, Rett syndrome, and Autism spectrum disorder (Braat and Kooy, 2015; Ghit and Assal, 2021). Autism spectrum disorder (ASD) is characterized by three behavioral features: impaired communication, social deficits, and repetitive behaviors. Several studies have demonstrated that an imbalance in the glutamatergic/GABAergic signaling pathways in addition to the neuroinflammation processes, is associated with ASD pathophysiology and is present in ASD mice models (El-Ansary and Al-Ayadhi, 2014). Previous studies have reported the presence of molecular-level cortical abnormalities related to GABA\textsubscript{A}R dysfunction in the brains of ASD. The
excitation and inhibition imbalance caused by dysfunction of GABA$_A$R is one of the characteristic features behind the behavioral deficits in the Autism spectrum (Pizzarelli and Cherubini, 2011).

Several studies have shown that inhibiting cortical GABA$_A$R leads to impaired attention, social behavior, and decision-making (Sigel et al., 1990; Piantadosi et al., 2016; Auger et al., 2017; Paine et al., 2017). Mice models carrying impaired 5-alpha GABA$_A$R are presented with behavioral deficits similar to those symptoms associated with attention and social disorders (Paine et al., 2020).

Rett syndrome is caused by loss-of-function mutations of the methyl-CpG-binding protein 2 (MECP2) gene and is characterized by behavioral regression accompanied by a loss of acquired skills. Associated autistic-like features also involve social withdrawal, lack of eye contact, and indifference to the surrounding environment, intellectual disability, epileptic seizures breathing abnormalities, and hypotonia (Neul et al., 2010). GABA$_A$ receptor binding has been reported to be significantly increased in the caudate at a young age which is measured by a $^3$H-muscimol receptor autoradiography of basal ganglia slices (Blue et al., 1999). Electrophysiological recordings in Mecp2-deficient mice have shown reduced basal inhibitory rhythmic activity in the CA3 region of the hippocampus and decreased phasic inhibition in the ventrolateral medulla and in the locus coeruleus (Medrihan et al., 2008; Zhang et al., 2008; Jin et al., 2013). Although GABA$_A$Rs are indirectly implicated in the pathophysiology of neurodevelopmental disorders, clear strings of evidence still warrant future investigations.

1.3.4 **Hypothesis and specific aims**

Since GABA$_A$R mutations can cause receptor dysfunctions and subsequent neurological disorders as mentioned above, studying clinically identified GABA$_A$R mutations becomes very
intriguing and significant. In Chapter 5 of this thesis, we investigated two de novo GABA_{A}R mutations identified in pediatric patients manifested with epileptic encephalopathy and neurodevelopmental disorders, respectively. The two mutations are located at the same residue T292 (T292S and T292I) of the α1 subunit of GABA_{A}R. Considering allosteric modulators are widely used in the treatment and management of GABA_{A}R related disorders, we hypothesize that selected allosteric modulators of GABA_{A}Rs can rescue the dysfunction caused by these mutations based on the characterization and profiling of the functional alterations of GABA_{A}Rs resulting from the two mutations. To achieve this goal, the following aims were designed:

2. Assess the impact of both mutations on GABA_{A}R total and surface expression levels via immunoblotting and surface biotinylation.
3. Quantify T292S and T292I GABA_{A}R single channel properties using cell-attached single channel recordings.
4. Assess changes in mutant GABA_{A}R-gated tonic and leak currents.
5. Screen and test known allosteric modulators of GABA_{A}Rs in to rescue the dysfunctions caused by T292S and T292I mutations.

This work not only highlights the importance of functionally characterizing each individual GABA_{A}R mutation, but also explores precision medicine that is tailored for individual mutations based on the functional features of GABA_{A}R mutations.
Chapter 2: Materials and Methods

2.1 Virtual screening, analog search and docking for screened modulators of diheteromeric GluN1/GluN2A and GluN1/GluN2B NMDARs

Analog search is performed to identify structurally similar compounds that are commercially available from current chemical suppliers because similar compounds should have similar biological properties. The active compound (Ndam813) was used as a template to search in a database of purchasable chemicals in an effort to find a subclass of compounds with enhanced activity.

Using a previously defined docking mode of Npams of NMDA receptor modulators, I docked purchasable chemical substances analogous to Nd813 (Axerio-Cilies, 2016). The compounds screened were ranked based on the comprehensive docking scores, which dictated which compound could enter the next round of in vitro functional tests. Compounds tested in vitro would be fed back to assist the depiction of the molecular fingerprint of each chemical structure. The molecular fingerprint is defined by determining which position in a molecular structure accounts for the presence or absence of certain features in a molecule (Williams, 2006). This process generated around 40 compounds available from a commercial chemical supplier. These purchased compounds combined with the chemically synthesized compounds designed in-silico, were tested by whole-cell voltage clamp recordings to eventually identify a lead compound that potentiated GluN1/GluN2A and inhibited GluN1/GluN2B simultaneously with satisfying potency and efficacy.

2.2 Chemicals

Analogs of Npam813 were purchased from Hit2Lead. N-methyl-D-aspartate (NMDA), D-2-amino-5-phosphonovaleric acid (APV), α-amino-3-hydroxyl-5-methyl-4-isoxazole-
propionate (AMPA), 6-cyano-7-nitroquinoxaline-2,3-dione (DNQX), Ro 25-6981 maleate, 
PEAQX tetrasodium salt (NVP-AAM077), diazepam, flumazenil, verapamil hydrochloride were 
purchased from Tocris. Glutamate, gamma-Aminobutyric acid (GABA), hydrogen peroxide 
(H₂O₂) solution and Kolliphor EL were purchased from Sigma-Aldrich. Tetrodotoxin citrate 
(TTX) and bicuculline were purchased from Hellobio. Picrotoxin was purchased from Abcam, 
bemegride and thiocolchicoside (TCC) were purchased from Cedarlane.

2.3 Chemical synthesis of N’-[(E)-(5-bromo-2,3-dihydroxyphenyl)methylidene]-3-
chlorobenzohydrazide (Ndam830)

Step1: 5-bromo-2,3-dihydroxybenzaldehyde (5.2 mmol) was dissolved in acetic acid (20 
ml) and N-chlorosuccinimide (NCS) or N-bromosuccinimide (11 mmol) was added. The 
reaction mixture was stirred overnight at 80°C, and then cooled to room temperature. Water and 
CH₂Cl₂ were then added, the phases were separated and the water phase was further extracted 
with CH₂Cl₂, dried over MgSO₄ and evaporated under vacuum. The crude product was purified 
by flash chromatography (CH₂Cl₂/hexane) to yield pure product as a yellow solid. Step2: 
dissolve equal molar amounts of 3-chlorobenzohydrazide and aldehyde from step 1 in THF. Add 
2 equivalents MgSO₄ and heat to reflux for 1 h. The product might precipitate. If not, the 
reaction was checked by TLC or NMR to ascertain consumption of the aldehyde. If the product 
precipitated, it was recovered by filtration and washed with water (2x2ml) to remove residual 
MgSO₄. If the product did not precipitate the reaction mixture was diluted with water until the 
product precipitated. The mixture was acidified to pH 3 with diluted HCl to remove residual 
hydrazine and then filtered. Extraction was avoided. If no precipitation was observed, the solvent 
was evaporated in vacuum and the compound was purified by flash column chromatography. 
The final compound was N’-[(E)-(5-bromo-2,3-dihydroxyphenyl)methylidene]-3-
chlorobenzohydrazide (Ndam830). (See appendix A for $^1$H-NMR and mass spectroscopy of synthesized Ndam830).

2.4 Plasmids

Wild-type diheteromeric rat EGFP-GluN1-1a, GluN2A, GluN2B and engineered triheteromeric EGFP-GluN1-1a, GluN2A-C1 and GluN2B-2AC2 plasmids were gifts from Dr. Hansen Kasper at the University of Montana. Enhanced green fluorescent protein (EGFP) was inserted between the CMV promoter in pCI-neo and the ORF of GluN1 without fusing them together (Hansen et al., 2014). C1 and C2 are peptides derived from the GABA$_B$ receptor C-terminal domains that prevent surface homomeric GABA$_B$ receptor expression. The selective surface expression of triheteromeric GluN1/GluN2A/GluN2B was achievable because the GluN2A-C1 and GluN2B-2AC2 contain two peptide tails C1 and C2 which only allow the receptors assembled with both C1 and C2 in the endoplasmic reticulum (ER) can be delivered to the cell surface (Hansen et al., 2014). Wild-type diheteromeric GluN2C, GluN2D were produced by Dr. Peter Seeburg's lab at Heidelberg University and were kindly sent to us upon request by Dr. Stephen F. Traynelis at Emory University.

The cDNAs encoding rat GABA$_A$R $\alpha_1$, GABA$_A$R $\beta_2$, GABA$_A$R $\gamma_2$, and EGFP were separately cloned into the pcDNA3.0 vector (Invitrogen). Using the plasmid with the wild-type rat GABA$_A$R $\alpha_1$ cDNA as the template, PCR mediated QuickChange site-directed mutagenesis was performed with a high fidelity Hot Start DNA polymerase (KAPA Biosystems, Cat: KM2605) to construct mutant variants of the GABA$_A$R $\alpha_1$ (c.875 C>G and c.875 C>T) subunit. (point mutation primers: Sense 5’-cgaccgttctgagcatgacaacctt-3’, reverse 5’-aaggttgtcatgctcagaacggtcg-3’ and sense 5’-cgaccgttctgagcatgacaacctt-3’, reverse 5’-aaggttgtcatgctcagaacggtcg-3’ for T292S and T292I mutations, respectively). The mutations were
confirmed by sequencing and the resulting plasmids encoding mutant GABA\textsubscript{A}R \(\alpha\)1 were referred to as \(\alpha1\text{T292S}\) (T292S) and \(\alpha1\text{T292I}\) (T292I).

### 2.5 Primary culture of cortical and hippocampal neurons

Cultured hippocampal and cortical neurons were prepared from the brains of D18-19 fetal SD rats. Brain tissues were digested with a 0.05% trypsin-EDTA solution (Invitrogen) for 15-30 min in a 37 °C incubator, and then triturated with pipette tips to make a single cell suspension. The cell suspension was then centrifuged at 2500g for 60 s and the cell pellets was re-suspended in Neurobasal media containing 0.5mM GlutaMAX-1 and 2% B27 supplement. Following this step, the cells were seeded on poly-D-lysine-coated 24-well coverslips at a density of 1.5 \(\times 10^5\) cells/well hippocampal neurons. Cortical neurons were seeded on poly-D-lysine-coated 24-well without coverslips at the bottom of the well at a similar density. The cultures were maintained in a humidified incubator with 5% CO\textsubscript{2} at 37 °C. Media change was performed every 4 days afterward. Hippocampal neurons were used for electrophysiological recordings 14-16 days after plating. Cortical neurons were used for LDH assays 14 days after plating.

### 2.6 HEK293 cell culture and transfection

Human Embryonic Kidney 293 (HEK293) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The cells were maintained at 37°C with 5% CO\textsubscript{2}.

For western blot experiments investigating the expression of the GABA\textsubscript{A}R \(\alpha\)1 subunits, cells were grown to 70% confluency in poly-L-lysine-coated 6-well plates and co-transfected with plasmids encoding \(\alpha1:\beta2:\gamma2\) (1 \(\mu\)g:1 \(\mu\)g:0.5 \(\mu\)g), \(\alpha1\text{T292S}:\beta2:\gamma2\) (1 \(\mu\)g:1 \(\mu\)g:0.5 \(\mu\)g), or \(\alpha1\text{T292I}:\beta2:\gamma2\) (1 \(\mu\)g:1 \(\mu\)g:0.5 \(\mu\)g). The transfection was performed using lipofectamine 2000
reagent (Invitrogen) according to the manufacturer’s instructions. Transfected HEK293 cells were cultured for 40 hours and then used for downstream western blotting analysis and surface biotinylation assays.

For electrophysiology experiments, cells were grown to 70-90% confluency in 6-well plates. EGFP (0.2μg) plasmid was co-transfected with the GABAAR subunits to help in visualizing the successfully transfected cells during electrophysiology recording. The transfection of recombinant NMDAR subunits were GluN1:GluN2A (1ug:1ug), GluN1:GluN2B (1ug:1ug) and GluN1:GluN2A-C1:GluN2-2AC2 (1ug:0.5ug:0.5ug). Cells were maintained in 6-well plates for 5 hours before being re-plated onto 12mm glass coverslips coated with poly-L-lysine and were cultured for an additional 18-30 hours before recording.

2.7 Western blot and surface biotinylation

Transfected HEK293 cells in 6-well cell culture plate were washed with ice-cold PBS for three times and lysed with 0.5ml of 1% SDS TBS buffer containing a protease inhibitor cocktail (Bimake) at 4 °C for 30 min. Cells were then harvested and homogenized using needles with gauge sizes from 18G to 23G to 26G, progressively. The supernatant was collected after centrifugation (13,000 rpm, 4 °C, 10 min) and the protein concentration in each sample was measured using the Pierce BCA protein assay (Thermo scientific, REP233228). Samples containing equal an amount of total protein were treated with 6X Laemmlli sample buffer containing 9% beta-mercaptoethanol and boiled at 55 °C for 5 min before loading onto 10% SDS-PAGE gels. The proteins in the gels were transferred onto a PVDF membrane (EMD Millipore). Rabbit anti-GABA A receptor α1 polyclonal antibody (1:1000, EMD Millipore, Cat. #06-868) was used to detect the WT and variant GABAAR α1 subunits. HSP90 (mouse monoclonal antibody 1:4000, BD, Cat. #610418) served as the loading controls. The HRP-
conjugated anti-mouse or anti-rabbit secondary antibodies (Thermo Fisher, Cat. # 31430 and 31460) were used at a ratio of 1:5000. The primary antibodies were incubated overnight in a 4 degree cold room. Secondary antibodies were incubated at room temperature. The signal was detected using an ECL detection system (Millipore, Immobilon Crescendo Western HPP Substrate, Cat#WBLUR0500) via Bio-Rad ChemiDoc MP imaging system.

In the surface biotinylation assay, cells in 6-well cell culture plate were washed 3 times on ice with ice-cold PBS 40 hours past-transfection, then 0.5 ml of PBS with membrane-impermeable EZ-Link Sulfo-HNS-LC-Biotin (1 mg/ml, Thermo Scientific) was added into each well and kept at 4 °C for 30 min to label surface membrane proteins. To quench the biotin reaction, cells were washed on ice with 1 ml of 100 mM glycine dissolved in ice-cold PBS for three times (10 min each time) at 4 °C. Then the biotinylated cells in each well were harvested using 0.5ml of 1% SDS lysate buffer (1% SDS in 1XTris Buffered saline, pH 7.6) at 4 °C for 30 min and homogenized using needles with gauge size from G18 to G23 to G26, progressively. Supernatants were collected after centrifugation (13,000 rpm, 4 °C, 10min). Protein concentration in each sample was measured using the Pierce BCA protein assay (Thermo scientific, REP233228). Each sample with an equal amount of biotin-labeled membrane proteins was rotationally incubated with 30ul of High Capacity Streptavidin Agarose Resin (beads) (Thermo Scientific, REF20359) at 4 °C overnight, after which the beads were pulled down and washed with PBS for 3-4 times via centrifugation (500g, 4 °C, 2 min). The beads were suspended by 45 ul 2X sample buffer and boiled at 55 °C for 5 min. Boiled samples were then centrifuged at 1000 rpm for 2 minutes. Finally, the supernatants were loaded onto 10% SDS-PAGE gels. 

Na⁺/K⁺ ATPase (abcam: mouse monoclonal antibody alpha1 sodium potassium ATPase ([464.6] 1:1000) served as a loading control for biotinylated membrane proteins and β-actin (Sigma: 52
mouse monoclonal antibody 1:3000, Cat, #A2228) served as a loading control for the cytoplasmic proteins.

2.8 Electrophysiology

The recordings were conducted and low-pass filtered at 2 kHz using a MultiClamp 700A amplifier (Axon instruments) and digitized at 20 kHz using Digidata 1440A. The recordings were performed using the Clampex 10.7 software (Axon instruments) and the data were analyzed offline using Clampfit 10.7 (Axon instruments).

2.8.1 Electrophysiology in HEK293 cells expressing recombinant NMDARs and neurons

Whole-cell voltage clamp recordings were performed using an extracellular recording solution containing (in mM) 140 NaCl, 5.4 KCl, 2 MgCl₂, 1.3 CaCl₂, 10 HEPES, and 33 glucose (pH = 7.4, 310-320 mOsm). Recording pipettes (3-5 MΩ) were filled with the intracellular solution that contained (in mM): Cs-gluconate, 0.1 CaCl₂, 2 MgCl₂, 10 HEPES, 10 BAPTA, and 4 ATP (K) (pH = 7.2, 290–300 mOsm). Glutamate (10 μM) and glycine (1μM) were used to induce basal NMDAR-gated currents through using a fast step perfusion system (Warner Instruments). Neuronal NMDAR, GABAₐR or AMPAR gated-currents were induced by the application of NMDA, GABA, AMPA through perfusion fast-step. GABAₐR gated-currents were recorded using intracellular solution that contained (in mM): 140 CsCl, 0.1 CaCl₂, 2 MgCl₂, 10 HEPES, 0.5 EGTA and 4 ATP (K). DNQX (20 μM) and TTX (0.5 μM) were added in the extracellular solution to minimize the activation of other ionotropic glutamate receptors and voltage-gated sodium channels, respectively. Ro 25-6981 (0.5 μM) and NVP-AAM077 (0.2 μM) were used to isolate the GluN2A-containing NMDAR-gated current and GluN2B-containing NMDAR-gated current, respectively. The effects of tested compounds on NDMARs were fed back to the SAR to guide further modifications of the lead compounds. The percentage response
of compounds screened was calculated by the equation: \( \% \text{ Activity} = 100 \times \frac{\text{AUC}_{\text{compound}} - \text{AUC}_{\text{baseline}}}{\text{AUC}_{\text{baseline}}} \)

where the \% Activity was based on the area under the curve (AUC) for the compound (induced by 10\(\mu\)M glutamate/1\(\mu\)M glycine plus the compound) and AUC of the agonists alone (10\(\mu\)M glutamate/1\(\mu\)M glycine). We also adopted a washout strategy to determine the time required to wash away the effect of the drug by re-perfusing the agonist and allowing the signal to return back to baseline.

### 2.8.2 Electrophysiology for \(\text{GABA}_{\text{A}}\)Rs

Electrophysiology experiments, including whole-cell voltage clamp recordings and cell-attached single channel recordings, were performed at room temperature on HEK293 cells transfected with the WT, T292S, or T292I \(\text{GABA}_{\text{A}}\)R subunits.

Whole-cell voltage clamp recordings were performed using an extracellular recording solution containing (in mM) 140 NaCl, 5.4 KCl, 2 MgCl\(_2\), 1.3 CaCl\(_2\), 10 HEPES, and 33 glucose (pH = 7.4, 310-320 mOsm). The thin-walled borosilicate glass patch pipettes (World Precision Instruments) were pulled to 3-5 M\(\Omega\) resistance using a model P-97 micropipette puller (Sutter Instruments). During the recording, the glass patch pipettes were filled with internal solution containing (in mM) 140 CsCl, 0.1 CaCl\(_2\), 2 MgCl\(_2\), 10 HEPES, 0.5 EGTA and 4 ATP (K) (pH = 7.2, 290–300 mOsm). For the GABA dose-response, the holding voltage was set at -60mV, and \(\text{GABA}_{\text{A}}\)R-gated currents were induced by applying various concentrations of GABA (0.1 \(\mu\)M -1000 \(\mu\)M, 2s) using a fast step perfusion system (Warner Instruments). For the \(\text{GABA}_{\text{A}}\)R current-voltage (I/V) relationship experiment, the holding voltages were set at (in mV) -80, -60, -40, -20, 0, +20, +40, +60, +80 while applying a high concentration of GABA (1 mM, 1s).
Tonic and leak currents assay was performed using a bath solution exchange protocol. Specifically, cells were patched and then recorded using a gap-free program, 10 μM GABA, GABA-free ECS, 20 μM bicuculline and 20 μM bicuculline plus 100 μM picrotoxin were bath applied sequentially. The application of each solution lasted for at least 60 s to allow enough time for solution exchanges in the chamber. The initial application of GABA was meant to confirm the success of the transfection of recombinant GABA_ARs.

Cell attached single channel recordings were conducted using the same protocol as before (Bai et al., 2019). The patch electrodes were fire polished to a resistance of 10-20 MΩ and filled with the extracellular recording solution with 3 μM or 1 mM GABA (pH = 7.4, 310-320 mOsm), and the holding potential was +100 mV. Single channel events were detected using the 50% amplitude threshold detection method and was visually inspected before being accepted. Single channel open probability was determined by the fraction of open time over the total amount of analyzed time (120 s for each recording), and the channel mean open time was determined by the total amount of open time divided by the number of channel open events.

2.9 NMDA-induced excitotoxicity and H2O2-induced oxidative stress

Primary cultures of mature cortical neurons (DIV 14) were used in this study. Before NMDA treatment, half of the conditioned medium was taken out and saved in a 50 mL falcon tube. For the NMDA-induced excitotoxicity experiment, cortical neurons were pretreated with Ndam830 or vehicle for 1 hour followed by wash-out with fresh medium and the addition of 100 μM freshly dissolved NMDA together with Ndam830. One hour later, NMDA and compounds were washed away and conditioned medium containing the compounds was added back. Cortical cultures were assessed for NMDA-induced excitotoxicity 20-24 hours later by measuring lactate dehydrogenase (LDH) release. The same procedures as described above were also applied for the
H$_2$O$_2$-induced oxidative stress assay, except that cytotoxic insults were induced by a 30-min treatment of 300 μM H$_2$O$_2$. Extracellular LDH level was measured using an in vitro assay kit purchased from Sigma/Roche (cat. 11644793001). The cell death rate was expressed as a ratio (%) of the absorbance at 490 nm of the treated groups and that of the control group.

2.9.1 Formulation of Ndam830

For the pharmaceutical formulation of Ndam830, it was prepared in DMSO together with the mixture of cremophor EL and saline. Ndam830 was initially dissolved in DMSO (1-2% v:v) and then mixed with a combination of cremophor EL (2-3% v:v) and saline. This formulation (DMSO, cremphor El, saline) was used for animals involved in the in-vivo experiments.

2.9.2 Cerebrospinal fluid (CSF) extraction and HPLC-ECD analysis

Cerebrospinal fluid (CSF) samples were collected 30 min after tail vein i.v. injection of formulated Ndam 830 (5 mg/kg) through puncturing through the cisterna magna of a rat with a glass pipette. Clean CSF collected from the vehicle control and treated groups were sent for high-performance liquid chromatography electrochemical detection (HPLC-ECD) analysis. Standard samples of Ndam 830 (in aCSF) containing 1, 2.5, 5, 10 μM were run were used to create the standard curve (the limit of detection on HPLC machine for Ndam830). These concentrations were run in triplicate. Area under the peak curve of the compound was measured to quantify the concentration of Ndam830. Rough quantification of Ndam830 obtained from treated rats was based on the standard curve generated.

2.10 Middle cerebral artery occlusion (MCAo) model

MCAo for pre-operative administration of Ndam830: Transient focal cerebral ischemia was induced using the suture occlusion technique as described previously (Chen et al., 2017). Male Sprague-Dawley rats weighing 250–300 g were anesthetized with 4% isoflurane in 70%
N\textsubscript{2}O and 30\% O\textsubscript{2} with a mask. A midline incision was made in the neck, the right external carotid artery (ECA) was exposed and dissected, and a 3\textendash{}0 monofilament nylon suture was inserted from the ECA into the right internal carotid artery to occlude the origin of the right middle cerebral artery (MCA) (approximately 22 mm). After 90 minutes of occlusion, the suture was removed to allow instant reperfusion, the ECA was then ligated, and the wound was closed. Rectal temperature was maintained at 37.0 ± 0.5 °C using a heating pad and heating lamp. Rats were divided into two groups: Ndam830 (5 mg/kg, I.V) and saline/vehicle (DMSO/cremophor EL/saline, I.V). Ndam830 and saline/vehicle were administered 1 day before and 1 h prior to stroke onset on surgery day. One dose of Ndam830 was given to rats daily for 6 days after MCAo surgery until being sacrificed for 2,3,5-Triphenyltetrazolium chloride (TCC) staining. This experiment was done at Tongji Medical College, Huazhong University of Science and Technology, Wuhuan, China and was approved by the Ethical Committee for Animal Research at Tongji Medical College, Huazhong University of Science and Technology.

MCAo for post-operative administration of Ndam830: Transient three-vessel occlusion model in rats was adopted to mimic human ischemic stroke as the majority cases of human ischemic stroke result from an occlusion of the middle cerebral artery (MCA) (Chen et al., 1986; Shyu et al., 2008). Adult male Sprague Dawley rats weighting around 200 g were anesthetized with chlora hydrate (0.4 g/kg; i.p.). A craniotomy, 2 mm in diameter, was drilled 1 mm rostral to the anterior junction of the zygoma and the squamosal bone using a surgical microscope and the middle cerebral artery (MCA) was exposed and then ligated with a square knot using a 10-0 nylon suture. The bilateral common carotid arteries (CCAs) were then clamped with nontraumatic arterial clips. Successful surgery was assessed by observing a drop in the regional cerebral blood flow which was monitored by a laser Doppler flowmeter (PF-5010, Periflux.
system; Perimed AB). The core body temperature was maintained at 37.0 ± 0.5°C with a heating pad and monitored with a thermometer probe (Hewlett-Packard Model 21090A probe). Blood pressure and blood gas levels were also monitored during the experiment. After 90 min of ischemic insults, the suture and clips were removed to allow instant reperfusion. Rats were divided into two major groups: Ndam830 (1 and 3 mg/kg, I.V) or saline/vehicle (DMSO/cremophor EL/saline, I.V). The first bolus of Npam-830 was administered at 3 hours post-stroke onset. To achieve the optimal outcome, Ndam-830 was administered one dose consecutively for either 3 days or 7 days, respectively. Rats were then allowed to recover for different periods of time until additional experiments including TCC staining and neurological function behavioral tests. This experiment was done at China Medical University Hospital and was approved by the Ethical Committee for Animal Research at China Medical University Hospital, Taiwan, China.

2.11 Triphenyltetrazolium chloride (TTC) staining

Three days after cerebral ischemia, animals were intracardially perfused with saline. The brain tissue was removed, immersed in cold saline for 5 min, and sliced into 2.0-mm-thick sections (6-8 slices per rat). The brain slices were incubated in 20 g/L TTC (Research Organics Inc), dissolved in saline for 30 min at 37°C, and then transferred to a 5% formaldehyde solution for fixation. The area of infarction in each slice was measured with a digital scanner as described previously (Wang et al., 1997). The volume of infarction was obtained from the product of average slice thickness (2mm) and by examining infarcted areas in all brain slices. To minimize any artifacts induced by post-ischemic edema in the infarcted tissue, the area of infarction was also calculated as previously described (Chou et al., 2004). To measure the infarcted area in the
right cortex, we subtracted the non-infarcted area in the right cortex from the total cortical area of the left hemisphere.

2.12 Neurological behavioral tests

Three typical sensorimotor deficit assessments were designed to evaluate the functional recovery of neural circuits damaged resulting from the ischemic insult. There were three parameters of vertical movement (Chang et al., 2003; Shyu et al., 2008). Vertical movement of the ischemic rats was monitored using the VersaMax Animal Activity Monitor purchased from Accuscan Instruments (Chang et al., 2003). The monitor consist of 16 horizontal X-Y and 8 vertical Z infrared sensors that are spaced 87 cm apart. The vertical sensors were set to be 10 cm from the floor of the chamber. At room temperature, the ischemic rats were placed in the recording chambers at night, and the number of vertical movements, vertical activity and vertical movement time (in seconds) were automatically recorded when the infrared beams were broken by their movements during a 2-hour experimental time. The length of their vertical movement indicated the recovery of locomotor abilities impaired by ischemic insults.

2.13 Data analysis

Data were presented as mean ± SEM (n = number of experiments or number of cells). The two-tailed Student’s t test was used for comparison of two groups. Comparison of three or more groups was done using one-way ANOVA test followed by Bonferroni’s post hoc analysis. P values less than 0.05 were considered statistically significant. In the figures, * represents P<0.05, ** represents P<0.01, **** represents P< 0.0001. Concentration–response curves were created by fitting data to Hill equation: I = I_{max}/[1+(EC50/[A])^n], where I is the current, I_{max} is the maximum current, [A] is a given concentration of agonist, n is the Hill coefficient. Whole-cell
peak currents, area under curve (AUC), channel kinetics, and single-channel currents were analyzed by Clampfit 10.7.
Chapter 3: Development of dual allosteric modulators of NMDARs for stroke

3.1 Introduction

Stroke is a cerebrovascular disorder characterized by the sudden onset of symptoms and clinical signs such as unilateral numbness or weakness in the face, arm, or leg that are caused by disruption of blood supply (Yew and Cheng, 2015). It is a leading cause of mortality and disability and an economic burden in both developing and developed countries worldwide (Mozaffarian et al., 2016). There are mainly two types of strokes, including ischemic and hemorrhagic stroke with the ischemic form accounting for around 80% cases (Grysiewicz et al., 2008; Mozaffarian et al., 2016). For ischemic stroke, the most commonly adopted interventions are intravenous thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) and interventional treatment with endovascular thrombectomy (Prabhakaran et al., 2015; Chamorro et al., 2016). These two methods are only applicable to a small portion of stroke patients due to limited therapeutic windows (4.5 hours and 6 hours respectively) (Hurford et al., 2020; Phipps and Cronin, 2020) and the majority of stroke patients do not have good pharmacological treatments so far. Therefore novel stroke therapies are urgently needed.

The mechanisms mediating stroke damage are multifactorial, but NMDAR-mediated excitotoxicity plays a major role (Simon et al., 1984; Rothman and Olney, 1995; Lee et al., 1999). Among several theories about the roles of NMDARs in the pathogenesis of excitotoxic neuronal injuries, the subcellular ‘NMDAR location’ and ‘NMDAR subtype’ hypotheses have been gaining more supporting evidence as activating synaptic, predominantly GluN2A-containing NMDARs, is pro-neuronal survival, whereas activating extrasynaptic, preferentially GluN2B-containing, NMDARs induces neuronal death (Liu et al., 2007; Chen et al., 2008; Lai and Wang, 2010). There is also evidence suggesting the contrary, with synaptic NMDARs
mediating neuronal death and extrasynaptic NMDARs mediating neuronal survival (Papouin et al., 2012; Wroge et al., 2012). Alternatively, some studies report that activating synaptic or extrasynaptic NMDARs alone is pro-survival while activating both induces cell death (Chen et al., 2014).

Based on both location and subtype NMDAR hypotheses, we propose that the GluN2A potentiating and GluN2B inhibiting NMDAR dual allosteric modulators (Ndam) have promising potential as a new class of stroke therapeutics. Through virtual screening and in silico drug design, a lead compound Ndam813 was found to function as an NMDAR dual allosteric modulator (Ndam) that potentiated GluN2A-containing NMDARs and at the same time inhibited GluN2B-containing NMDARs, albeit with very low potency (Axerio-Cilies, 2016). We wondered whether further improving the efficacy of such a dual functioning compound could treat excitotoxic neuronal injuries in vitro and in vivo. Briefly, by employing structure-based in-silico drug design and in-vitro functional screening, we successfully optimized the candidate compound Ndam813 into a new compound Ndam830 with better potency and efficacy. Ndam830 protected cortical neurons from NMDA-induced excitotoxicity and H2O2-induced oxidative stress in vitro. Ndam830 also reduced the infarct volume in in vivo MCAo models when given pre- and post-operatively. Moreover, post-stroke administration of Ndam830 could accelerate the neurological function recovery in rats. This study will not only provide supporting evidence for the subcellular ‘NMDAR location’ and ‘NMDAR subtype’ hypotheses in excitotoxicity, but might also yield a novel therapeutic intervention against ischemic stroke.
3.2 Results

3.2.1 Identification and optimization of drug candidates to find a lead compound using virtual screening, structure-based in-silico drug design, and in-vitro functional screening

Ndam813 was previously identified as a potential dual NMDAR allosteric modulator, albeit with very low efficacy, in our lab through virtual screening and in-silico drug design. To develop a higher and more specific dual modulator, we then used a computer-assisted in-silico screening assay to screen commercial chemical libraries to select chemicals that are analogs of Ndam813. Following this, we tested these selected chemicals via electrophysiological recordings on HEK293 cells expressing recombinant NMDARs to determine their effects on diheteromeric GluN1/GluN2A and GluN1/GluN2B combinations. Each compound was tested at least three times co-applied with 10 μM glutamate and 1 μM glycine and area under curve and peak amplitude were measured. The effects of the tested compounds on NMDARs were compiled for the compounds that showed activity. This in turn was used to establish a structure-activity relationships (SAR) analysis and to identify a pharmacophore that could be used as a base for further chemical optimization. We implemented compound docking for these positive hits using published NMDAR structural models (PDB ID: 6IRA and 5IOU) to establish the binding orientation for each of the compounds. This in combination with simulated compound dockings using structure models of NMDARs (PDB ID: 6IRA and 5IOU) informed us on possible further chemical synthetic optimizations that could be done to increase the potency of our lead compounds (Figure 3.1).
Figure 3.1 Drug screening and Ndam830 docking model.

(A) Drug discovery pipeline of procedures for identifying Ndam813 and the subsequent procedures of optimization of Ndam813 into Ndam830 and Ndam844. (B) Docking Ndam830 in the putative binding pocket formed at the interface between the NTDs of GluN1 and GluN2. Illustration modified from the crystal structure of GluN1/GluN2B (Protein Data Bank ID. 5IOV; (Zhu et al., 2016)). (C) Summary of percentage of the potentiation or inhibition of Ndam813 analogs when co-applied with 10 μM glutamate and 1 μM glycine on recombinant diheteromeric GluN1/GluN2A. (D) Summary of percentage of the potentiation or inhibition of Ndam813 analogs when co-applied with 10 μM glutamate and 1 μM glycine on recombinant diheteromeric GluN1/GluN2B. The hit compounds Ndam830 and Ndam844 are indicated by * and #, respectively.
Compounds were tested at concentrations of their optimal solubility on HEK293 cells expressing recombinant diheteromeric GluN1/GluN2A and GluN1/GluN2B. We found that the potency of the compounds on diheteromeric GluN1/2A was positively correlated with the hydrophobicity of the functional groups at F1-F6 on ring1 (R1) and ring2 (R2) (Table 3.1, 813-817, 823,824 and 830). The two hydroxyl groups (–OH) at the (2) ortho and (3) meta positions of R1 were essential for the dual modulating effects, as changing the positions or replacing them with other functional groups compromised the effectiveness and modulation types (Table 3.1, 818,821-823,826,827,835-845,846,847). However, the modulation types were also altered when the hydrophobicity of the functional groups at F1-F6 on ring1 (R1) and ring2 (R2) became too strong (Table 3.1, 819,820,829,831,851 and 853). One interesting finding was when one hydroxyl groups (–OH) at the meta was replaced by a methoxy group (-OCH₃), the GluN1/GluN2A potentiating and GluN1/GluN2B inhibiting NMDAR dual allosteric modulation could be transformed into a new modulating pattern that resulted into a compound that could potentiate both GluN1/GluN2A and GluN1/GluN2B combinations (Table 3.1, 828 and 835-845). These structure-activity relationships (SAR) analysis from all the drugs tested together with the in-vitro functional screenings led to the discoveries of Ndam830 (potentiates GluN1/GluN2A but inhibits GluN1/GluN2B) and Ndam844 (potentiates both GluN1/GluN2A and GluN1/GluN2B). These two allosteric modulators of NMDARs were found to be potent but had distinct modulating patterns (Figure 3.1 A, C&D). Ndam830 will be discussed in this chapter and the characterization of Ndam844 will be described in Chapter 4.
Table 3.1 Summary of analogs of Ndam813 tested electrophysiologically on diheteromeric GluN1/GluN2A and GluN1/GluN2B.

+ indicates the percentage of potentiation, - indicates the percentage of inhibition.

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F1-6: functional group sites, R1: ring 1, R2: ring 2
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3.2.2 Characterization of Ndam830 on recombinant diheteromeric GluN1/GluN2A, GluN1/GluN2B and triheteromeric GluN1/GluN2A/GluN2B overexpressed in HEK293 cells

Following the identification of Ndam830 in the initial screening process, we ran tests on recombinant diheteromeric GluN1/GluN2A, GluN1/GluN2B and triheteromeric GluN1/GluN2A/GluN2B overexpressed in HEK293 cells. Results showed that Ndam830 (10 μM) could potentiate diheteromeric GluN1/GluN2A by around 2.5 folds (365.6±72.73% of baseline, P<0.0001), potentiate triheteromeric GluN1/GluN2A/GluN2B by ~1.5 folds (268±68.06% of baseline, P<0.0001) and inhibit diheteromeric GluN1/GluN2B by ~50% (50.71±12.63% of baseline, P<0.0001) (Figure 3.2, A&B, D&E, G&H). We then proceeded to systematically characterize it in order to have better knowledge of its functional properties. We observed that Ndam830 was able to potentiate diheteromeric GluN1/GluN2A and triheteromeric GluN1/GluN2A/GluN2B in a dose-dependent manner with an EC50 of 0.5126 μM and 0.4547 μM, respectively (Figure 3.2, C&I). However, Ndam830’s action when tested in a full dose response on diheteromeric GluN1/GluN2B, showed a variation: it slightly potentiated diheteromeric GluN1/GluN2B at concentrations below 5 μM but significantly inhibited the receptor at concentrations higher than 10 μM (Figure 3.2, F). This may indicate that the binding of Ndam830 to the pocket on diheteromeric GluN1/GluN2B increases in a concentration dependent manner but triggers different conformational changes. Another possibility is that there are two possible binding sites on the diheteromeric GluN1/GluN2B. At low concentrations it binds to only one site but at higher concentrations it binds to two sites at the same time and causes distinct conformational changes, respectively. Moreover, we noticed that the dissociations
of Ndam830 bond diheteromeric GluN1/GluN2A and triheteromeric GluN1/GluN2A/GluN2B may differ a lot from that of diheteromeric GluN1/GluN2B. This observation was made when we adopted a 40-sec wash out strategy the modulation effect mediated by Ndam830 on diheteromeric GluN1/GluN2A (306.9±48.78 of baseline, P<0.0001) and triheteromeric GluN1/GluN2A/GluN2B were lingering (268±68.06 of baseline, P<0.0001) (Figure 3.2, A, B, G&H) while the effect on diheteromeric GluN1/GluN2B quickly went back to baseline within 40s (103.5±5.055% of baseline) (Figure 3.2, D&E).

Figure 3.2 Effects of Ndam830 tested on recombinant NMDARs overexpressed in HEK293.

(A) Representative response of baseline trace stimulated by glutamate (10 μM) and glycine (1 μM) (black), glutamate (10 μM) and glycine (1 μM) plus Ndam830 (10 μM) (red) and glutamate (10 μM) and glycine (1 μM) wash (gray) of diheteromeric GluN1/GluN2A. (B) Quantified results of the effects of Ndam830 on diheteromeric GluN1/GluN2A, Ndam830 significantly potentiated glutamate and glycine induced current on diheteromeric GluN1/GluN2A (365.6±72.73% of baseline, n=8, P<0.0001). 40-sec wash with glutamate and glycine did not
caused significant reduction of signal compared with Ndam830 group (306.9±48.78% of baseline, n=6, ns). (C) Concentration-response curve of Ndam830 potentiation of glutamate and glycine induced current on diheteromeric GluN1/GluN2A (EC50=0.5126 μM, n=6). (D) Representative response of baseline trace stimulated by glutamate (10 μM) and glycine (1 μM) (black), glutamate (10 μM) and glycine (1 μM) plus Ndam830 (10 μM) (red) and glutamate (10 μM) and glycine (1 μM) wash (gray) of diheteromeric GluN1/GluN2B. (E) Quantified results of the effects of Ndam830 on diheteromeric GluN1/GluN2B, Ndam830 significantly inhibited glutamate and glycine induced current on diheteromeric GluN1/GluN2B (50.71±12.63% of baseline, n=14, P<0.0001). 40-sec wash with glutamate and glycine caused the signal to go back to baseline level (103.5±5.055% of baseline, n=12). (F) Concentration-response curve of Ndam830’s effects of glutamate and glycine induced current on diheteromeric GluN1/GluN2B (n=6). (G) Representative response of baseline trace stimulated by glutamate (10 μM) and glycine (1 μM) (black), glutamate (10 μM) and glycine (1 μM) plus Ndam830 (10 μM) (red) and glutamate (10 μM) and glycine (1 μM) wash (gray) of triheteromeric GluN1/GluN2A/GluN2B. (H) Quantified results of the effects of Ndam830 on triheteromeric GluN1/GluN2A/GluN2B, Ndam830 significantly potentiated glutamate and glycine induced current on triheteromeric GluN1/GluN2A/GluN2B (268±68.06% of baseline, n=10, P<0.0001). 40-sec wash with glutamate and glycine did not caused significant reduction of signal compared with Ndam830 group (239.1±67% of baseline, n=10, ns). (I) Concentration-response curve of Ndam830 potentiation of glutamate and glycine induced current on triheteromeric GluN1/GluN2A/GluN2B (EC50=0.4547 μM, n=6). ****P< 0.0001, ns=not significant, Tukey’s multiple comparisons after one-way ANOVA.

3.2.3 Characterization of Ndam830 on hippocampal neurons

3.2.3.1 Ndam830 showed little effect on overall NMDAR-gated current but potentiated GluN2A-containing NMDAR-gated current

Direct perfusion of Ndam830 on DIV14 hippocampal neurons did not induce any observable current, indicating Ndam830 by itself does not serve as an agonist and activate NMDARs or other neuronal receptors (Figure 3.3 A&B). Co-application of Ndam830 (10 μM) with NMDA/glycine (10 μM/1 μM) on hippocampal neurons showed little effects on neuronal NMDARs (110.7±7.326% of baseline) (Figure 3.3 A&B), likely due to the canceling effect
between potentiation of GluN2A-gated currents and inhibition of GluN2B gated currents by Ndam830 in HP neurons. Consistent with this, wash-out of Ndam830 produced a significant enhancement of NMDAR-gated (rebound) currents (152.3±20.41% of baseline, P<0.0001). These currents are revealed during wash-out due to Ndam830’s distinct disassociation rates on diheteromeric GluN1/GluN2A and GluN1/GluN2B. The wash-out resulted in a rapid recovery of the inhibited the GluN1/GluN2B-containing NMDARs component, thereby revealing the residual potentiating effects on GluN1/GluN2A-containing NMDARs component (rebound current after washing) (Figure 3.3 A&B). To further confirm these findings, we pre-empted the inhibition of GluN1/GluN2B-containing NMDARs component by Ndam830 through the application of GluN2B-specific antagonist Ro25-6981 (68.6±8.176% of baseline, P<0.0001). As expected, in the presence of Ro25-6981, Ndam830 significantly increased the NMDAR-gated current (103.8±19.07% of baseline, 152.3% of Ro25-6981 alone, P<0.0001) (Figure 3.3 C&D). Lastly, APV (a non-specific NMDAR antagonist) (50 μM) was used to block all NMDAR-mediated current and results confirm that the currents were mediated by NMDARs (5.222±2.186% of baseline, P<0.0001) (Figure 3.3 A-D).
Figure 3.3 Effects of Ndam830 tested on hippocampal neurons.

(A) Representative traces of Ndam830 alone (green), baseline current stimulated by NMDA (10 μM) and glycine (1 μM) (black), NMDA (10 μM) and glycine (1 μM) plus Ndam830 (10 μM) (red), NMDA (10 μM), glycine (1 μM) wash (gray) and APV blockade of all current (blue) on hippocampal neurons. (B) Quantified results of the effects of Ndam830 on hippocampal neurons. Ndam830 alone induced no current change. Ndam830 showed little to no effects on NMDA and glycine induced current (110.7±7.326% of baseline) but showed a rebound current upon washout that was significantly bigger than baseline current amplitude (152.3±20.41% of baseline, n=8, P<0.0001). (C) Representative traces of Ndam830 alone (green), baseline trace stimulated by NMDA (10 μM) and glycine (1 μM) (black), NMDA (10 μM) and glycine (1 μM) plus Ro 25-6981 (0.5 μM) (purple), NMDA (10 μM) and glycine (1 μM) plus Ro 25-6981 (0.5 μM) and Ndam830 (10 μM) (red), NMDA (10 μM) and APV blockade of all current (blue) on hippocampal neurons. (D) Quantified results of the effects of Ndam830 on hippocampal NMDA current gated by GluN2A containing NMDARs. Ndam830 alone induced no current. Ro 25-6981 inhibited the overall NMDAR-gated current to 68.6±8.176% of baseline. Ndam830 significantly enhanced the GluN2A-containing NMDAR-gated current (103.8±19.07% of baseline, n= 11, P<0.0001). (E) Representative traces of concentration-
response of Ndam830 (increasing hue of blue traces) on AMPA (30 μM) (black trace) induced current on neuronal AMPARs. (F) Concentration-response curve of Ndam830 on AMPA (30 μM) induced current on neuronal AMPARs (n=5). (G) Representative traces of concentration-response of Ndam830 (increasing hue of orange traces) on GABA (3 μM) (black trace) induced current on neuronal GABAARs. (H) Concentration-response curve of Ndam830 on GABA (3 μM) induced current on neuronal GABAARs (n=5). ****P< 0.0001, **P< 0.01, *P<0.05, ns=not significant, Tukey’s multiple comparisons after one-way ANOVA.

3.2.3.2 Ndam830 had no effects on neuronal AMPAR-gated currents but slightly inhibited GABAAR-gated currents

Testing Ndam830’s effect on GABAARs and AMPARs would help to determine its specificity for NMDARs and any potential off-target effects on these related receptors. Since the activation of AMPARs accompanies the activation of NMDARs by glutamate during synaptic transmission, it is important to know the compound’s effects on AMPARs. Similarly, GABAARs are prevalent in interneurons relaying messages among various regions. Therefore, it’s also important to observe the compound’s effects on GABAARs. We performed a systematical dose response of Ndam830 on neuronal AMPARs and GABAARs. Ndam830 showed no effects on neuronal AMPARs at all tested concentrations (Figure 3.3 E&F) but inhibited GABAARs dose-dependently with around 20% inhibition at 10 μM. (Figure 3.3 G&H).

3.2.4 Ndam830 exerted neuroprotective effects against NMDA-induced excitotoxicity and hydrogen peroxide (H2O2)-induced oxidative stress in vitro

3.2.4.1 Ndam830 protected cortical neurons from NMDA induced excitotoxicity

Given the activation of synaptic, predominantly GluN2A-containing NMDARs stimulates neuronal survival signaling whereas stimulating extrasynaptic, preferentially GluN2B-
containing, NMDARs triggers cell death signaling pathways, we hypothesized that Ndam830 may exert strong neuroprotective effects against excitotoxic neuronal damages. This prediction was tested in cultured cortical neurons which is a classical model to test NMDA-induced excitotoxicity. NMDAR-mediated excitotoxicity was induced in DIV 14 cortical neurons by treating them with 100 uM NMDA through bath application for 60 mins, which mimics glutamate release and consequently excitotoxicity after a stroke event. Neuroprotective effects of Ndam830 was examined by pre-incubating neurons with increasing concentrations of Ndam830 (2.5, 5, 10 μM) for 60 min; positive control was done by pretreating of neurons with Ro25-6981 (0.5 μM) for 60 min because it has been proven by many studies to be neuroprotective (Hardingham et al., 2002; Liu et al., 2007). After the pretreatment procedure, 100 uM NMDA was added into NMDA plus Ro25-6981 and NMDA plus Ndam830 (2.5, 5, 10 μM) treatment groups. Other control groups included a blank control (only adding equal amounts of DMSO as other groups), Ndam830 alone (2.5, 5, 10 μM), Ro25-6981 alone (0.5 μM), and a triton group (positive LDH assay control). After the 60 min incubation, neurons were washed once with fresh neural basal medium, and then returned to the previously saved conditional medium containing Ndam830 (2.5, 5, 10 μM) and Ro25-6981 (0.5μM). 20-24 hours later, lactate dehydrogenase levels (LDH) was measured. LDH release is a well-recognized marker of loss of plasma membrane integrity, which is a good indicator of necrotic neuronal death. The cell death rate was expressed as a ratio between the absorbance (490nm) of the treated groups and that of the blank control group. 1-hour 100 μM NMDA incubation resulted in a significant increase in cortical neuronal death 24 h after treatment (1.715± 0.3826, P<0.0001). Ndam830 pretreatment significantly protected cortical neurons from NMDA induced insults in a concentration dependent manner with obvious effects observed at the concentrations of 5 μM (1.405±0.191,
P<0.0001) and 10 μM (1.172±0.1039, P<0.0001), although not as effective as the Ro25-6981 (0.5 μM) pretreatment group (1.027±0.08502, P<0.0001) (Figure 3.4 A). We also noticed that 10 μM Ndam830 alone slightly increased the LDH levels (1.169±0.191, P<0.05) but concentrations lower than that did not alter the LDH levels significantly (Figure 3.4 A).

Figure 3.4 Ndam830 protected cortical neurons against NMDA-induced excitotoxicity and hydrogen peroxide (H$_2$O$_2$)-induced oxidative stress.

(A) LDH assay indicated that 1-hour 100 μM NMDA incubation resulted in a significant increase in cortical neuronal death 24 h after treatment (1.715±0.3826). This was rescued by Ro25-6981 (0.5μM) (1.027±0.08502, P<0.0001). Ndam830 2.5 μM (1.537±0.22, ns), 5 μM (1.405±0.191, P<0.0001), 10 μM (1.172±0.1039, P<0.0001) also ameliorated the NMDA-induced excitotoxicity in a concentration dependent manner (n=12). Overnight incubation of 2.5 μM and 5 μM Ndam830 did not cause LDH level alterations but 10 μM Ndam830 slightly elevated LDH release levels (1.169±0.191, P<0.05, n=24). (B) 30-min incubation of 300 μM H$_2$O$_2$ caused significant increase in cortical neuronal death 24 h after treatment (1.709±0.1479, P<0.0001). This was rescued by Ro25-6981 (0.5μM) (1.321±0.09678, P<0.0001). Ndam830 2.5 μM (1.405±0.1548, P<0.0001), 5 μM (1.275±0.1085, P<0.0001), 10 μM (1.186±0.1145, P<0.0001) also significantly ameliorated the H$_2$O$_2$-induced neuronal death in a concentration dependent manner (n=12). Overnight incubation of 2.5 μM and 5 μM Ndam830 did not cause LDH level alterations but 10 μM Ndam830 slightly elevated the LDH release levels (1.169±0.191, P<0.05, n=24). (C) H$_2$O$_2$ caused neuronal death (1.542±0.3472, P<0.0001) was rescued by the treatment of 10 μM Ndam830.
(1.088±0.05726, P<0.0001), which performed significantly better than the 0.1μM Ro 25-6981 (1.342±0.2074, P<0.0001) treatment group (n=12, P<0.0001). ****P< 0.0001, *P<0.05, ns=not significant, multiple comparisons after one-way ANOVA.

3.2.4.2  Ndamp830 showed better protective effects than Ro25-6981 in rescuing H₂O₂ induced oxidative stress

Oxidative stress is known to be present in the ischemic area of the penumbra (Fabian et al., 1995; Fukuyama et al., 1998; Pacher et al., 2007). We suspected that both Ro25-6981 and Ndamp830 would be effective in protecting neurons from this type of neuronal injuries, but Ndamp830 might provide a better protection than Ro25-6981 as it simultaneously promotes synaptic NMDAR cell survival signaling and inhibits extrasynaptic cell death pathways. We tested our prediction using hydrogen peroxide (H₂O₂) to induce neuronal damage. Experimental procedures were the same as described above except that oxidative stress was induced by treating cortical neurons with 300 μM H₂O₂ for 30 min. 30-min incubation of 300 μM H₂O₂ caused significant increase in cortical neuronal death 24 h after treatment (1.709±0.1479, P<0.0001). This was rescued by Ro25-6981 (0.5μM) (1.321±0.09678, P<0.0001). Ndamp830 2.5 μM (1.405±0.1548, P<0.0001), 5 μM (1.275±0.1085, P<0.0001), 10 μM (1.186±0.1145, P<0.0001) also significantly ameliorated the H₂O₂-induced neuronal death in a concentration dependent manner. Moreover, we noticed that 10 μM Ndamp830 (1.186±0.1145) seemed to have better protective effective than 0.5 μM Ro25-6981(1.321±0.09678), although not statistically significant. (Figure 3.4 B). To further investigate this, we reduced the concentration of Ro25-6981 from 0.5 μM to 0.1 μM in order to match its inhibitory effects on diheteromeric GluN1/GluN2B with that of 10 μM Ndamp830 (Ng et al., 2008). As expected, 10 μM Ndamp830
showed significantly better neuroprotective effects compared with 0.1 \mu M Ro 25-6981 treatment (1.342\pm0.2074) against the H2O2 induced insults (P<0.0001) (Figure 3.4 C).

3.2.5 Ndams830 passes blood-brain barrier (BBB) after intravenous injection in rats

The blood-brain barrier (BBB) controls CNS homeostasis to maintain proper neuronal function and also protects the brain from toxins and pathogens (Daneman and Prat, 2015). The BBB penetration of small molecule is crucial for drug efficacy in-vivo because most drugs do not pass the BBB due to their low hydrophobicity (Mikitsh and Chacko, 2014). Therefore, it is very important to know whether a compound passes the BBB and reaches the brain or not. We injected formulated Ndams830 (5mg/kg) through tail vein intravenous injections (I.V.) into rats. Cerebrospinal fluid (CSF) samples were collected via cisterna magna puncture, a procedure that is commonly used to measure drug concentrations in the brain CSF (Duris et al., 2011). The concentrations of compound in CSF were measured by high-performance liquid chromatography- electrochemical detection (HPLC-ECD). After establishing a standard curve via testing samples prepared by spiking Ndams830 into artificial cerebrospinal fluid (aCSF) (Figure 3.5 A&B). Consequently, we tested collected rat CSF samples and measured the concentration of Ndams830 found in these samples. Using the standard curve, we measured the concentration of Ndams830 to be 11.00 \pm 2.677 \mu M in the CSF and demonstrated that the drug passed the BBB in rats after the administration of 5mg/kg Ndams830 via tail vein I.V. (Figure 3.5 C&D).
Figure 3.5 Detection of Ndam830 using HPLC-ECD.

(A) Representative signals of increasing concentrations of Ndam830 (0.25, 0.5, 1, 2.5, 5, 10 μM) in aCSF on HPLC-ECD. (B) Standard curve of increasing concentrations of Ndam830 in aCSF. (C) Representative signal of Ndam830 detected on HPLC-ECD in rat CSF samples collected after intravenous administration of 5mg/kg of formulated Ndam830. (D) Quantifications of estimated Ndam830 concentrations based on standard curve (11.00±2.677 μM, n=4, P<0.01). **P< 0.01, student’s tests.
3.2.6 Ndam830 reduced infarct volume in MCAo models of focal ischemia in rats

3.2.6.1 Pre-operative administration of Ndam830 reduced the infarct volume in a rat MCAo model

Based on the promising data acquired in the in vitro experiments and the satisfactory BBB permeability of Ndam830, we proceeded to determine the effects of Ndam830 (5mg/kg, I.V) in in vivo experiments. We first adopted a model of pre-operative administration strategy by giving Ndam830 (5mg/kg, I.V) one day before and 1 hour prior to the commencement of MCAo surgery on the surgery day. One dose of Ndam830 (5mg/kg, I.V) was also given to the rats daily for 6 consecutive days until they were sacrificed for 2,3,5-Triphenyltetrazolium chloride (TTC) staining (Figure 3.6 A). We found that Ndam830 (22.11±3.184%) was able to significantly protect the ischemic brain region and reduced the infarct volume by ~54% when compared with control groups (50.86±7.584%) (P=0.0155) (Figure 3.6 B&C). This result indicated that pre-operative administration of Ndam830 was neuroprotective against the ischemic insults.
Figure 3.6 Pre-operative administration of Ndam830 reduced the infarct volume in a rat MCAo model.

(A) Timeline of Ndam830 administration and brain slice collection for TTC staining in a rat MCAo model. (B) Sample images of TTC stained-brain coronal sections in either vehicle or Ndam830 (5mg/kg, I.V) treatment groups. (C) Quantifications of infarct volume of control (50.86±7.584%, n=5) and Ndam830 (22.11±3.184%, n=4) groups. *P<0.05, student’s t tests.
3.2.6.2 Post-operative administration of Ndam830 reduced the infarct volume in a rat MCAo model

Knowing that Ndam830 worked in acute MCAo model when given pre-operatively, we then asked the question of whether Ndam830 could exert the same neuroprotective effects even after the onset of stroke. To investigate this, we injected Ndam830 (1 and 3 mg/kg, I.V) 3 hours after the onset of MCAo surgery. To achieve the optimal outcome, one dose of Ndam830 was administered daily for 3 consecutive days before sacrificing the rats for 2,3,5-Triphenyltetrazolium chloride (TTC) staining (Figure 3.7 A). Surprisingly, 1 mg/kg (16.6±3.6%, P=0.0106) and 3 mg/kg (11.5±6.1%, P=0.0063) of Ndam830 administrations both significantly reduced the infarct volume of the ischemic regions in comparison with the control group (28.2±5.2%) (Figure 3.7 B&C), suggesting post-operative administration of Ndam830 also showed a satisfactory neuroprotective effects against ischemia insults, demonstrating the potential for a therapeutic intervention against ischemic stroke.
Figure 3.7 Post-operative administration of Ndam830 reduced the infarct volume in a rat MCAo model. (A) Timeline of Ndam830 administration and brain slice collection for TTC staining in the rat MCAo model. (B) Sample images of TTC stained-brain coronal sections in either vehicle or Ndam830 (1 and 3 mg/kg, I.V) treated groups. (C) Quantifications of infarct volume of control (28.2±4.2%, n=6), Ndam830 (1 mg/kg, I.V) (16.6±3.6%, n=6) and Ndam830 (3 mg/kg, I.V) (11.5±6.1%, n=6) groups. In comparison with the control group, Ndam830 (1 mg/kg, I.V) significantly reduced the infarct volume (P=0.0106) and Ndam830 (3 mg/kg, I.V) also significantly reduced the infarct volume (P=0.0063). **P< 0.01, *P<0.05, Tukey’s multiple comparisons after one-way ANOVA.

3.2.7 Post-stroke treatment with Ndam830 improved neurological function behavioral performance after ischemic insults.

To find out whether Ndam830 can improve the recovery of neuronal function at a behavioral level following transient MCAo, we observed the functional recovery in groups with or without Ndam830 treatments. Post-stroke administration of Ndam830 (1 and 3 mg/kg) were given daily from day 0 to day 7, accompanied and followed by neurobehavioral tests on day 1, day 7, day 14, day 21 and day 28 to determine the effectiveness of Ndam830 in functional rescue (Figure 3.8 A). Three locomotor activity modalities including vertical activity (the total number of beam interruptions that occurred in the vertical sensor), number of vertical movements (number of rat rearing) and vertical movement time (the amount of time, in seconds, the rat spent on rearing) were recorded and analyzed. During the 28-day recovery period after ischemic insults, all three parameters mentioned above were gradually improved. On Day 28, rats in Ndam830 (1 mg/kg) (1505±188, P=0.26748) and Ndam830 (3 mg/kg) (2011±184, P=0.00346) groups interrupted the beams significantly more frequently than the control group (1125±147). Moreover, the rats administered with 3 mg/kg Ndam830 performed significantly better than those treated with 1 mg/kg Ndam830 (P=0.0014). (Figure 3.8 B). Similarly, the vertical
movement time demonstrated that Ndam830 with 1 mg/kg (319±28 s, P=0.039) and Ndam830 with 3 mg/kg (427±33 s, P=0.00474) groups displayed a faster and more pronounced improvement in locomotor activity over the 28 days than the control group (235±41 s). And rats administered with 3 mg/kg Ndam830 spent significantly more time on vertical movements than those treated with 1 mg/kg Ndam830 (P=0.02758). (Figure 3.8 C). Lastly, results of the number of vertical movement test also suggested a more rapid recovery in rats treated with 1 mg/kg Ndam830 (206±21, P=0.04738) and 3 mg/kg Ndam830 (262±24, P=0.00198) at 28 d after stroke onset compared with control group (161±19). Rats administered with 3 mg/kg Ndam830 performed significantly better than those treated with 1 mg/kg Ndam830, suggesting higher dose of Ndam830 is more beneficial in terms of improving neurological function recovery (P=0.0185). (Figure 3.8 D). Overall, these results suggested that Ndam830 not only reduced the infarct volume in a transient stroke model, but also facilitated neurological function recovery resulting from ischemic insults in a dose-dependent manner.
Figure 3.8 Post-stroke administration of Ndam830 accelerated neurological function recovery.

(A) Timeline of post-stroke Ndam830 administration and behavioral tests (symbolized by a rat cartoon). (B) Quantified vertical activities over the 28-day recovery period. On day 28, rats in Ndam830 (1 mg/kg) (1505±188, n=6) and Ndam830 (3 mg/kg) (2011±184, n=6) groups interrupted the beams more frequently than control group (1125±147, n=6). And rats administered with 3 mg/kg Ndam830 performed significantly better than those treated with 1 mg/kg Ndam830. (C) Quantification of vertical movement time over the 28-day recovery period. On day 28, rats in Ndam830 (1 mg/kg) (319±28 s, n=6) and Ndam830 (3 mg/kg) (427±33 s, n=6) groups spent more time on rearing than the control group (235±41 s, n=6). And rats administered with 3 mg/kg Ndam830 spent significantly more time than those treated with 1 mg/kg Ndam830. (D) Quantification of number of vertical movement over the 28-day recovery period. On day 28, rats in Ndam830 (1 mg/kg) (206±21, n=6) and Ndam830 (3 mg/kg) (262±24, n=6) groups reared more frequently than control group (161±19, n=6). And rats administered with 3 mg/kg
Ndam830 performed significantly better than those treated with 1 mg/kg Ndam830. **P< 0.01, *P<0.05.

Bonferroni’s multiple comparisons after two-way ANOVA.

### 3.2.8 Discussion

The heterogeneous composition of NMDARs provides a wide variety of subtypes, which underlies a rich diversity in receptor-mediated cell signaling. Conventional NMDAR antagonists that target the receptors essentially blocked both of the neuronal survival-signaling and death-signaling pathways and inhibit physiological receptor functions, and thus contribute to adverse effects in the CNS (Simon et al., 1984; Fischer et al., 1997; O’Collins et al., 2006). The suspected detrimental effects of the extrasynaptic, GluN2B-containing NMDAR mediated over-activation underlying the pathogenesis of ischemic brain damage has been confirmed along with the previously underappreciated beneficial role of synaptic, largely GluN2A-containing NMDAR in promoting cell survival (Hardingham et al., 2002; Chen et al., 2008). Under physiological conditions, spontaneously released glutamate preferably activate the synaptic NMDARs which are mainly GluN2A-containing that mediates pro-survival signaling pathways (Franchini et al., 2020). However, when stroke happens, excessive release of glutamate in the extracellular space spills to the extrasynaptic sites where GluN2B-containing NMDARs dominate. Those GluN2B-containing NMDARs are then activated by the spilled-over glutamate and lead to the activation of neuronal death complexes (NDC) and neuronal apoptosis (Ge et al., 2020). Our lab previously found a GluN2A selective positive modulator that showed significant neuroprotection in a rat model of stroke with a wider and, clinically relevant therapeutic window than conventional drugs (Axerio-Cilies, 2016). Evidence from above supports both the subcellular ‘location’ and the ‘subtype’ hypotheses of NMDAR-mediated excitotoxicity and further encouraged us to improve
the efficacy and potency of our lead compound Ndam813 that potentiates GluN1/GluN2A and inhibits GluN1/GluN2B simultaneously.

Consistent with previous findings, Ndam830 showed neuroprotective effects in both in vitro and in vivo experiments. The drug protected cortical neurons against neuronal NMDA-induced excitotoxicity and H2O2-induced oxidative stress through its dual allosteric modulating effects of potentiating GluN1/GluN2A and inhibiting GluN1/GluN2B at the same time. It is worth mentioning that potentiating GluN1/GluN2A on top of inhibiting GluN1/GluN2B by Ndam830 exerted better neuroprotective effects than Ro 25-6981 alone against non-NMDA receptor mediated cell death (i.e oxidative stress). Ndam830, either administered pre-operatively or post-operatively, successfully reduced the infarct volume in rat MCAo models. Moreover, post-stroke administration of Ndam830 accelerated the neurological function recovery in rats that that were exposed to ischemic insults. Taken together, our results are in line with the previously proposed subcellular ‘location’ and the ‘subtype’ hypotheses.

As described in the introduction, maintaining the excitation-inhibition (E/I) balance is important for normal functions of the CNS, either dysfunction of GABAARs or over-stimulation of neuronal NMDARs can trigger seizures (Barker-Haliski and White, 2015). The concern of triggering seizures by neuronal over-excitation was discussed in regard of the therapeutic potential of a GluN1/GluN2A selective positive allosteric modulator GNE0723 (Hanson et al., 2020). Compared with the initial lead compound Ndam813, our optimized compound Ndam830 showed better potency in potentiating GluN2A-containing NMDARs. However, given the opposing effects of Ndam830 on GluN2A-containing NMDARs and diheteromeric GluN1/GluN2B, the overall NMDAR-gated currents in neurons were not significantly altered. This potentially could provide Ndam830 with a clinically relevant advantage of maintaining the
excitation-inhibition homeostasis while still stimulating the pro-survival signaling and inhibiting the pro-death signaling.

Last but not least, the fast-on/off kinetic property of Ndam830 is similar to some fast-acting NMDAR channel blockers such as memantine and dextromethorphan (DMX). It was postulated that faster blocking/unblocking kinetics and lower affinity of memantine in comparison with other blockers of its kind, ketamine and PCP were associated with an improved therapeutic index regarding the psychotomimetic effects due to reduced channel blockade during normal synaptic transmission (Chen and Lipton, 2006). This shared kinetic property of Ndam830 with approved NMDAR-related drugs may contribute to the future clinical application, although one limitation for taking this drug to the clinic is its relatively low potency in comparison with most clinically tested drugs that are potent in the nanomolar range.

In summary, this study resulted in the development of a dual allosteric modulator of NMDARs that potentiates GluN1/2A and inhibits GluN1/2B simultaneously, and hence exerts neuroprotective effects against ischemic insults both in vitro and in vivo. This modulator Ndam830 can serve as a prototype drug for a potential novel therapeutic for stroke.
Chapter 4: Characterization of a dual positive allosteric modulator of NMDARs discovered in drug optimization process

4.1 Introduction

During our optimization process of the lead compound Ndam813, we modified multiple functional groups of this molecule in search of compounds with better potency and efficacy. To our surprise, we found a very potent compound Ndam844 that harbored a distinct modulating pattern. Instead of potentiating GluN1/GluN2A and inhibiting GluN1/GluN2B, Ndam844 strongly potentiated both diheteromeric GluN1/GluN2A and GluN1/GluN2B in the initial electrophysiological screening tests.

This intriguing finding reminded us of a previous dual allosteric modulator Npam59 found in our lab. Npam59 was previously demonstrated to potentiate both diheteromeric GluN1/GluN2A and GluN1/GluN2B. It also boosted the dopamine release in the nucleus accumbens (NAc) and affected associated animal behaviors (Li, 2017).

NMDAR hypofunction was reportedly implicated in the pathophysiology of major depressive disorders (MDDs). Moreover, recent study reported that a novel NMDAR positive allosteric modulator (PAM) 32h, increased NMDAR excitability significantly in vitro and induced impressive activity in the forced swimming test, thus showing antidepressant-like activity (Li et al., 2021). In addition to this finding, another drug AGN-241751 was in a clinical trial as an antidepressant. It might also exert rapid antidepressant-like effects via boosting the function of GluN2B-containing NMDARs on the excitatory neurons of mPFC (Pothula et al., 2021). Apart from MDDs, NMDAR hypofunction is also linked with age-related cognitive impairment (Clayton et al., 2002; Magnusson et al., 2010), schizophrenia (Moghaddam and
Javitt, 2012) and autism spectrum disorders (ASD) (Schmeisser et al., 2012), and anti-NMDAR encephalitis (Dalmau et al., 2008; Hughes et al., 2010; Mikasova et al., 2012).

Therefore, we believed that characterization of Ndam844 and understanding the properties of this compound could assist us to prepare it to be a potential therapeutic for hypofunction of NMDARs related schizophrenia, major depressive disorders (MDDs), Alzheimer’s disease (AD), autism spectrum disorders (ASD) and anti-NMDAR encephalitis.

4.2 Results

4.2.1 Characterization of Ndam844 on recombinant NMDARs overexpressed in HEK293

To evaluate the potency and subunit-specificity of Ndam844 on NMDARs, we electrophysiologically characterized the drugs’ effects in HEK293 cells transiently overexpressing recombinant diheteromeric GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, GluN1/GluN2D and triheteromeric GluN1/GluN2A/GluN2B. Whole cell patch recordings were performed at a holding membrane potential of -60mV, and currents were induced by fast perfusion of glutamate (10 μM) and glycine (1 μM) with or without various concentrations of Ndam844. For diheteromeric GluN1/GluN2D, NMDAR-gated current was induced by 100 μM glutamate and 30 μM glycine because 10 μM glutamate and 1 μM glycine could not induce observable peak current during our electrophysiological tests.

We observed that Ndam844 (1 μM) significantly potentiated diheteromeric GluN1/GluN2A (800.2±99.74% of baseline, P< 0.0001), GluN1/GluN2B (509.7±33.57% of baseline, P< 0.0001), GluN1/GluN2C (195.8±16.13% of baseline, P< 0.001), GluN1/GluN2D (533.3±102.3% of baseline, P< 0.01) and triheteromeric GluN1/GluN2A/GluN2B (589.1±73.61% of baseline, P< 0.0001). Ndam844 also potentiated diheteromeric
GluN1/GluN2A (EC$_{50}$=0.0243 μM), GluN1/GluN2B (EC$_{50}$=0.0263 μM), GluN1/GluN2C (EC$_{50}$=0.04125 μM), GluN1/GluN2D (EC$_{50}$=0.01357 μM) and triheteromeric GluN1/GluN2A/GluN2B (EC$_{50}$=0.05298 μM) dose-dependently. The potentiating effects of Ndam844 were relatively smaller on diheteromeric GluN1/GluN2C in comparison with its strong potency on other subtypes (Figure 4.1). The results of Ndam844 tested on recombinant NMDARs expressed on HEK293 cells demonstrated that Ndam844 is a positive allosteric modulator of NMDAR that works on all subtypes of NMDARs including GluN1/GluN2A-D.
Figure 4.1 Effects of Ndam844 tested on different recombinant NMDARs overexpressed in HEK293.

(A) Representative response of baseline current stimulated by glutamate (10 μM) and glycine (1 μM) (black) and glutamate (10 μM) and glycine (1 μM) plus Ndam844 (1 μM) (green) of diheteromeric GluN1/GluN2A. (B) Quantified results of the effects of Ndam844 on diheteromeric GluN1/GluN2A, Ndam844 strongly potentiated glutamate and glycine induced current on diheteromeric GluN1/GluN2A (800.2±99.74% of baseline, n=7). (C) Concentration-response curve of Ndam844 potentiation of glutamate and glycine induced current on diheteromeric GluN1/GluN2A (EC50=0.0243 μM, n=6). (D) Representative response of baseline trace stimulated by glutamate (10 μM) and glycine (1 μM) (black) and glutamate (10 μM) and glycine (1 μM) plus Ndam844 (1 μM) (green) of diheteromeric GluN1/GluN2B. (E) Quantified results of the effects of Ndam844 on diheteromeric GluN1/GluN2B, Ndam844 strongly potentiated glutamate and glycine induced current on diheteromeric GluN1/GluN2B.
(509.7±33.57% of baseline, n=7). (F) Concentration-response curve of Ndam844 potentiation of glutamate and glycine induced current on diheteromeric GluN1/GluN2B (EC₅₀=0.0263 μM, n=6). (G) Representative response of baseline trace stimulated by glutamate (10 μM) and glycine (1 μM) (black) and glutamate (10 μM) and glycine (1 μM) plus Ndam844 (1 μM) (green) of triheteromeric GluN1/GluN2A/GluN2B. (H) Quantified results of the effects of Ndam844 on diheteromeric GluN1/GluN2A/GluN2B, Ndam844 strongly potentiated glutamate and glycine induced current on triheteromeric GluN1/GluN2A/GluN2B (589.1±73.61% of baseline, n=6). (I) Concentration-response curve of Ndam844 potentiation of glutamate and glycine induced current on triheteromeric GluN1/GluN2A/GluN2B (EC₅₀=0.05298 μM, n=6). (J) Representative response of baseline trace stimulated by glutamate (10 μM) and glycine (1 μM) (black) and glutamate (10 μM) and glycine (1 μM) plus Ndam844 (1 μM) (green) of diheteromeric GluN1/GluN2C. (K) Quantified results of the effects of Ndam844 on diheteromeric GluN1/GluN2C, Ndam844 can potentiated glutamate and glycine induced current on diheteromeric GluN1/GluN2C (195.8±16.13% of baseline, n=6). (L) Concentration-response curve of Ndam844 potentiation of glutamate and glycine induced current on diheteromeric GluN1/GluN2C (EC₅₀=0.04125 μM, n=6). (M) Representative response of baseline trace stimulated by glutamate (100 μM) and glycine (30 μM) (black) and glutamate (100 μM) and glycine (30 μM) plus Ndam844 (1 μM) (green) of diheteromeric GluN1/GluN2D. (N) Quantified results of the effects of Ndam844 on diheteromeric GluN1/GluN2D, Ndam844 strongly potentiated glutamate and glycine induced current on diheteromeric GluN1/GluN2D (533.3±102.3% of baseline, n=7). (O) Concentration-response curve of Ndam844 potentiation of glutamate and glycine induced current on diheteromeric GluN1/GluN2D (EC₅₀=0.01357 μM, n=6).

****P< 0.0001, ***P< 0.001, **P< 0.01, student’s t tests.

4.2.2 Characterization of Ndam844 on hippocampal neurons

4.2.2.1 Ndam844 potently potentiated overall, GluN2A-containing and GluN2B-containing NMDAR-gated currents

To further confirm the observation of the potentiating effects of Ndam844 on NMDARs, we consequently tested the drugs’ effects on DIV14 hippocampal neurons. Neuronal NMDAR-gated current was induced by fast perfusing 10 μM NMDA and 1 μM glycine. Ndam844 by itself
did not activate neuronal NMDARs and other receptors but strongly potentiated NMDAR-gated current with Ndam844 (1 μM) was co-applied with NMDA (10 μM) and glycine (1 μM) (392.4±13.97% of baseline, P< 0.0001). We then did a full dose response of Ndam844 on overall neuronal NMDAR currents. The EC₅₀ of Ndam844 on neuronal NMDARs was 0.00949 μM, which is almost 5-fold lower than the EC₅₀ of previously reported NMDAR positive modulator Npam59 (0.044 μM) (Li, 2017). (Figure 4.2 A&C).

Figure 4.2 Effects of Ndam844 tested on neuronal NMDARs in hippocampal neurons.

(A) Representative response of Ndam844 alone (blue), baseline current stimulated by NMDA (10 μM) and glycine (1 μM) (black), NMDA (10 μM) and glycine (1 μM) plus Ndam844 (10 μM) (green) on hippocampal neurons. (B) Quantified results of the effects of Ndam844 on hippocampal neurons. Ndam844 alone induced no current. Ndam844 showed strong potentiating effects on NMDA and glycine induced current (392.4±13.97% of baseline, n=6, P< 0.0001). (C) Concentration-response curve of Ndam844 potentiation of NMDA and glycine induced current

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Moreover, we used Ro25-6981 (0.5 μM) to isolate the GluN2A-containing NMDAR-gated current (70.2±1.798% of baseline) and see how would Ndam844 would respond in the presence of a GluN2B-specific antagonist. Consistent with its strong potentiating effects on overall NMDARs, Ndam844 (1 μM) increased the GluN2A-containing NMDAR-gated current by ~2.5 folds (244.9±14.63% of baseline, P< 0.0001). We also used NVP-AAM077 (0.2 μM) to isolate the GluN2B-containing NMDAR-gated current (18.04±1.143% of baseline) and tested whether Ndam844 could potentiate the GluN2B component. As expected, Ndam844 potentiated the GluN2B-containing NMDAR-gated current by ~7.5 folds (155.29±20.59% of baseline, P< 0.0001). Finally we applied APV (50 μM) to block all currents and confirmed that the currents originated from NMDAR (5.463±1.006% of baseline) (Figure 4.2 D, E, F&G).
4.2.2.2  Ndam844 had no effects on neuronal AMPAR-gated currents but slightly increased GABA\(_A\)R-gated currents

We also investigated whether Ndam844 had any effects on GABA\(_A\)Rs and AMPARs-mediated currents which would help us to profile the drugs specificity towards NMDARs and any potential off-target effects on these related receptors. As we know during synaptic transmission, the activation of AMPARs accompanies the activation of NMDARs by presynaptic release of glutamate, it is important to know Ndam844’s effects on AMPARs. Likewise, GABA\(_A\)Rs are ubiquitous in the CNS, it’s better to know Ndam844’s effects on GABA\(_A\)Rs as well. Neuronal AMPAR-gated currents were induced by fast-perfusing 30 \(\mu M\) AMPA and GABA\(_A\)R-gated currents were induced by 3 \(\mu M\) GABA in the presence of 0.5 \(\mu M\) TTX. We performed a systematical dose response of Ndam844 on neuronal AMPARs and GABA\(_A\)Rs-mediated currents. Ndam844 showed no effect on neuronal AMPARs at all concentrations (Figure 4.3 A&B) but potentiated GABA\(_A\)Rs dose-dependently with a maximal 20% increase at 10 \(\mu M\) (Figure 4.3 C&D). This suggests Ndam844 does not affect neuronal AMPARs and had little effect on GABA\(_A\)Rs in comparison with its strong potentiating effects of NMDARs.
Figure 4.3 Effects of Ndam844 tested on neuronal AMPARs and GABA_ARs.

(A) Representative traces of concentration-response of Ndam844 (increasing hue of blue traces) on AMPA (30 μM) (black trace) induced current on neuronal AMPARs. (B) Concentration-response curve of Ndam844 on AMPA (30 μM) induced current on neuronal AMPARs (n=6). (C). Representative traces of concentration-response of Ndam844 (increasing hue of orange traces) on GABA (3 μM) (black trace) induced current on neuronal GABA_ARs. (D). Concentration-response curve of Ndam844 on GABA (3 μM) induced current on neuronal GABA_ARs (n=6).

4.2.3  Ndam844 did not show toxicity on cortex neurons under basal conditions

Knowing that Ndam844 showed strong potentiating effects on neuronal NMDARs, we wondered whether the large potentiation would affect cell survival in basal condition because there were studies suggesting that stimulating synaptic NMDARs can cause cell death (Zhou et al., 2013a; Chen et al., 2014). In order to investigate this concern, we added increasing
concentrations of Ndam844 into DIV14 cortical neurons and allowed 24-hour incubation before testing the LDH release. We found that Ndam844 only slightly increased the LDH release at 10 μM (1.153±0.01602, P< 0.0001) while Ro25-6981 (0.5 μM) (positive control) and other concentrations of Ndam844 did not significantly cause the elevation of LDH release when compared with control (Figure 4.4). The results suggested that at concentrations that are lower than 1 μM, Ndam844 does not show any toxicity that affect the cell survival.

![Figure 4.4 Toxicity test of Ndam844 on cortical neurons.](image)

Similar to Ro 25-6981(0.5 μM), incubation of increasing concentrations of Ndam844 with cortical neurons overnight did not cause significant LDH release elevation in comparison with control.

### 4.2.4 Future directions and discussion

As a positive allosteric modulator of NMDARs, Ndam844 worked better in comparison with Npam59 because of its lower EC50 and increased efficacy (Li, 2017). The future
experiments may involve testing the pharmacological kinetics of Ndam844 such as the bioavailability after administration and the blood-brain-barrier (BBB) permeability followed by choosing suitable animal models for preclinical tests.

We also need to answer the questions like whether Ndam844 can pass the BBB and whether the concentrations of Ndam844 in the serum or cerebrospinal fluid (CSF) can reach to the therapeutic doses as tested previously in vitro.

After gaining solid evidence for the BBB penetration tests, choosing the right animal models can be done. The most promising disease models would be those implicating hypofunction of NMDARs such as schizophrenia, major depressive disorders (MDDs), Alzheimer’s disease (AD), autism spectrum disorders (ASD) and anti-NMDAR encephalitis.

Schizophrenia is a chronic and disabling mental disorder that is characterized clinically by positive symptoms (delusions, hallucinations, and thought disorder), negative symptoms (social withdrawal, poverty of speech, and anhedonia), and cognitive deficits (attention, working memory, and executive function) (Lewis and Lieberman, 2000). Accumulating evidence indicates that the alterations of excitatory signaling involving the hypofunction of NMDARs is a key contributor to the pathogenesis of schizophrenia (Balu, 2016). NMDAR channel blockers ketamine and phencyclidine (PCP) are dissociative anesthetics that are used to produce the full range of schizophrenia symptoms and cognitive deficits in healthy human and animals (Javitt and Zukin, 1991; Paul et al., 2013). Interestingly, a brain-derived cholesterol metabolite, 24(S)-hydroxycholesterol, was reported as a potent and selective NMDAR positive allosteric modulator (PAM) with a distinct mechanism. The derivatives of 24(S)-hydroxycholesterol, SGE-201 and SGE-301, desirably reversed NMDAR antagonist-induced cognitive and social deficits in the animal model (Paul et al., 2013). Comparing with SGEs, Ndam844 has better potency and
efficacy in terms of potentiating NMDARs, making it a promising candidate drug for treating schizophrenia.

Although NMDA antagonism mediated by ketamine or dextromethorphan shows antidepressant effects (Henter et al., 2018), NMDAR hypofunction is also reportedly implicated in the pathophysiology of major depressive disorders (MDDs). A novel NMDAR-positive allosteric modulator (PAM) 32h, showed antidepressant-like activity via increasing NMDAR excitability in vitro (Li et al., 2021). Another drug AGN-241751 was in a clinical trial as an antidepressant, it might also exert rapid antidepressant-like effects via boosting the function of GluN2B-containing NMDARs on excitatory neurons of mPFC (Pothula et al., 2021). Ndam844 showed comparable potentiating effects on NMDARs as 32h which makes it a promising drug to test in the depression model.

Alzheimer's disease (AD) is characterized by the deposits of insoluble amyloid β-peptide (Aβ) in extracellular aggregated tau protein and also in the intracellular neurofibrillary tangles (Skaper et al., 2017). Mild cognitive impairment in the early stage of AD can be attributed to synaptic dysfunction due to deleterious accumulation of oligomeric Aβ which happens before serious synaptic loss and neurodegeneration (Pozueta et al., 2013; Skaper et al., 2017). Additionally, there is evidence indicating that hypofunction of NMDAR is associated with AD (Ulas and Cotman, 1997; Newcomer et al., 2000; Malinow, 2012). Impaired NMDAR function was observed in the aging brain (Huang et al., 2012) but memory, learning and problem solving ability were significantly improved by the administration of d-serine to healthy old adults, indicating augmenting NMDAR function could be beneficial in regard of cognitive deficits in AD (Avellar et al., 2016). Therefore, the application of Ndam844 should be at the early stage of AD development to improve the cognitive impairment.
Autism spectrum disorders (ASD) is a neurodevelopmental disorder characterized by deficits in social interaction and communication, and repetitive behavior (Schmeisser et al., 2012). Although the causal mechanisms are still not known, several mutations of genes encoding synaptic proteins were identified such as neurexins, neurexins, GKAPs/SAPAPs, DOCK4/Rac1 and ProSAPs/Shanks (Schmeisser et al., 2012; Won et al., 2012; Guo and Peng, 2021). Mutant mice carrying mutations of Shank2 and DOCK4 exhibited ASD-like behaviors including reduced social interaction, reduced social communication by ultrasonic vocalizations, and repetitive jumping and a marked decrease in NMDAR function. Moreover, d-cycloserine and a NMDAR positive allosteric modulator normalized NMDAR function and markedly enhanced social interactions (Schmeisser et al., 2012; Won et al., 2012; Guo and Peng, 2021). Therefore, the hypofunction of NMDARs caused by impaired expression of synaptic protein is a great target for Ndam844 as well.

Anti-NMDAR encephalitis is an auto-immune disease. Patients have severe neurological and psychiatric symptoms such as delusions, hallucinations or mania associated with high titres of auto-antibodies for NMDARs. Those auto-antibodies bind to the N-terminal domain (NTD) of the subunit GluN1 and reduce the surface expression and hence physiological function of the NMDARs (Dalmau et al., 2008; Hughes et al., 2010; Mikasova et al., 2012). Ndam844 may be used as a drug that help to relieve the neurological and psychiatric symptoms associated with the hypofunction of NMDARs in anti-NMDAR encephalitis.

In summary, Ndam844 is a nontoxic positive allosteric modulator that showed desirable potency and efficacy toward GluN1/GluN2A-D but zero to little effects on neuronal AMPARs and GABA<sub>A</sub>Rs. Therefore, Ndam844 can serve as a promising therapeutic compound for a series of neuropsychological disorders associated with impaired function of NMDARs.
Chapter 5: Allosteric modulators rescued distinct functional alterations caused by two pathological de novo variants of the T292 residue of GABRA1 identified in children with epileptic encephalopathy and neurodevelopmental disorders

5.1 Introduction

As a primary mediator of inhibitory synaptic neurotransmission in the central nervous system (CNS), the γ-aminobutyric acid (GABA) type A receptor (GABA\(\alpha\)R) plays a critical role in maintaining the neuronal excitation-inhibition balance (E/I balance) in the CNS. As a result, the dysfunction of the receptor underlies the pathogenesis of many neurological diseases. Accumulating evidence suggests that genetic variants of the GABA\(\alpha\)R may cause conditions including epileptic encephalopathy (EE) (Krampfl et al., 2005; Gallagher et al., 2007; Galanopoulou, 2008; Ding et al., 2010), developmental delay (Galanopoulou, 2008; Braat and Kooy, 2015), Fragile X, Rett Syndrome, and Dravet Syndrome (Duarte et al., 2013; Carvill et al., 2014; He et al., 2014).

Structurally, ionotropic GABA\(\alpha\)Rs are heteropentamers of \(\alpha\) (\(\alpha_1\)-\(\alpha_6\)), \(\beta\) (\(\beta_1\)-\(\beta_3\)), \(\gamma\) (\(\gamma_1\)-\(\gamma_3\)), \(\delta\), \(\varepsilon\), \(\theta\), \(\pi\), or \(\rho\) subunits, with \(\alpha\) and \(\beta\) being the obligatory subunits (Galanopoulou, 2008). Most synaptic GABA\(\alpha\)Rs are assembled from two \(\alpha_1\), two \(\beta_2\), and one \(\gamma_2\) subunits (Pirker et al., 2000). Each subunit contains a large extracellular N-terminal domain, four transmembrane helices, two intracellular loops, and one extracellular C-terminal domain (Figure 5.1 A) (Sieghart and Sperk, 2002; Collingridge et al., 2004; Michels and Moss, 2007). The receptor’s agonist, GABA, binds to the agonist-binding site interfacing the extracellular N-terminal domains of \(\alpha\) and \(\beta\) subunits (Figure 5.1 B). The agonist binding triggers the conformational
opening of the receptor channel, leading to chloride anion influx through the ion pore formed by
the transmembrane segments of all five subunits (Macdonald and Olsen, 1994; Sieghart and
Sperk, 2002; Carvill et al., 2014). Due to the pentameric composition of the GABA\(_A\)R, mutations
of the amino acid residues in many different GABA\(_A\)R subunits have been reported to alter the
function, expression level and subcellular distributions of the receptor (Hernandez et al., 2017;
Butler et al., 2018; Bai et al., 2019; Reyes-Nava et al., 2020).

The \(\alpha1\) subunit of the GABA\(_A\)R, which is encoded by the GABRA1 gene, is ubiquitously
expressed in CNS neurons, suggesting its importance in maintaining the normal function of the
vast majority of native GABA\(_A\)Rs (Jacob et al., 2008). Indeed, increasing numbers of GABRA1
variants have been implicated in causing haploinsufficiency and loss of function of the
GABA\(_A\)Rs, and thereby causally contribute to the pathogenesis of various forms of epilepsy
(Fisher, 2004; Maljevic et al., 2006; Janve et al., 2016; Johannesen et al., 2016; Kodera et al.,
2016), Dravet Syndrome, early onset EEs, and developmental delay (Carvill et al., 2014;
Lachance-Touchette et al., 2014). In nearly all patients with pathogenic GABRA1 variants, the
clinical phenotypes include epilepsies within a spectrum of different severity, ranging from
generalized epilepsies to severe epileptic encephalopathies (Jacob et al., 2008).

It was to our surprise that we have recently identified a novel de novo T292S (C875G)
missense variant of GABRA1 from a pediatric patient with developmental delay without
diagnosed seizure events. Most interestingly, this mutation occurs on the same residue as that of
the previously reported variant T292I (C875T) identified in a pediatric patient with severe
epilepsy (Reyes-Nava et al., 2020). The dramatic difference between the phenotypes of the two
patients and the resistance to the standard care treatments of the patient harboring the T292S
(C875G) mutation prompted us to characterize and compare the pathological impacts of the two
mutations on the function of the GABA\(_A\)R and potentially search for the most suitable and effective pharm-therapeutics. To accomplish our goals, we used surface biotinylation, western blotting, whole-cell and single-channel patch-clamp recordings to characterize the two variants in HEK293 cells overexpressing either the wild-type or mutant rat recombinant GABA\(_A\)Rs containing the \(\alpha_1/\beta_2/\gamma_2\) subunits. We found that the two mutations have drastically different impacts on the receptor function: the \(\alpha_1\text{T}_{292}\text{S}\) variant significantly increased, whereas the \(\alpha_1\text{T}_{292}\text{I}\) variant significantly reduced GABA\(_A\)R function. The opposite functional impacts of these mutations suggest that we would need different therapeutic strategies for treating the patients carrying these mutations. Indeed, through screening several clinically approved drugs that are reportedly acting on GABA\(_A\)Rs, we found that the GABA\(_A\)R allosteric inhibitor thiocolchicoside (TCC) (Fisher, 2004) could reduce the gain of function caused by the T292S mutation, thereby normalizing the receptor function; on the other hand, a combination of GABA\(_A\)R positive modulators, diazepam and verapamil, largely rescued the loss of function caused by the T292I mutation. Our results, particularly the gain of function caused by \(\alpha_1\text{T}_{292}\text{S}\), are in great contrast to the loss of function by all previously reported pathogenic GABRA1 mutations (Reyes-Nava et al., 2020), highlighting the importance of functional characterization and calling for different therapeutic strategies for each individual mutation.

5.2 Results

5.2.1 Phenotypic comparisons of the two patients carrying GABRA1 mutants

Our patient is a 2-year-old boy with severe neurodevelopmental delays, but no diagnosed overt seizure activity despite a few abnormal EEG discharge reported. He also has a visual impairment, feeding difficulties, and significantly below average body weight and organ mass.
No abnormality is found in MRI. Through a whole exome sequencing (WES), we surprisingly identified a novel de novo T292S (C875G) missense variant of GABRA1. The clinical phenotypes, particularly the lack of any observable somatic seizure activity, were drastically different from that of a previously reported patient who carried de novo variant at the same residue T292I (C875T) and had the main clinical features of Lennox-Gastaut syndrome with a light-sensitive myoclonic epilepsy, generalized tonic-clonic seizures and developmental delay (Olivares et al., 2012; Allen et al., 2013; Reyes-Nava et al., 2020). These strikingly different clinical phenotypes may implicate distinct impacts on the receptor functions by these two mutations albeit occurring on the same residue.

5.2.2 Structural analysis and the location of T292 residue in GABRA1

The α1 T292 residue is located in the second transmembrane domain (TM2) of the α1 subunit (Figure 5.1 A) and lines within the inner channel pore (Figure 5.1 B). Parents of both patients have normal alleles, but the patients have heterozygous missense variants of the same site in GABRA1 (NM_000806.5:C.875C>G or 875C>T, NP_000797.2: p.Thr292Ser or Thr292Ile; Figure 5.1 C). Sequence alignment demonstrates that this residue is highly conserved among different species, including Human, Monkey, Rat, Mouse, Bovine, Chicken, Xenopus, and Zebrafish, implying the importance of this residue (Figure 5.1 D); this is in a good agreement with previous studies reporting that modification or pathogenic variant of the channel-lining residues could lead to dramatic alterations of the receptor function, particularly channel gating properties (Xu and Akabas, 1996; Hernandez et al., 2017).
Figure 5.1 Structural analysis and the location of the T292 residue in GABRA1 and depiction of the de novo missense variants c.875C>G (p.Thr292Ser) (green) and c.875C>T (p.Thr292Ile) (red).

(A) Diagram represents the T292 residue located at GABRA1 TM2. (B) Cryo-EM structure of the GABA<sub>A</sub> receptor (PDB ID 6x3u) with highlighted T292 residue (yellow) and the bond agonist GABA (orange). As indicated, T292 residue forms part of the inner channel pore. (C) Depiction of the two de novo missense variants in the patients and unaffected parents. (D) Comparison of the GABRA1 protein from several species indicates that Thr292 (in bold red) and nearby amino acids are evolutionarily conserved. Protein sequences were acquired from Uniprot and Ensembl.

5.2.3 The T292S and T292I variants bidirectionally affect GABA-evoked responses

The drastic differences in clinical phenotypes of the two mutations may imply significantly different impacts on the GABA<sub>A</sub>R function. Since functional impacts of neither variants have previously been fully characterized, we employed whole-cell patch clamp...
recordings of GABA-evoked currents to test whether the variants would affect GABA\_AR functions. The evoked whole-cell currents were measured by fast perfusion (2 s) of GABA with concentrations varied from 0.1 μM to 1 mM at a holding membrane potential of -60mV in HEK293 cells transiently overexpressing wild-type α1/β2/γ2, α1\_T292S/β2/γ2 or α1\_T292I/β2/γ2.

For the wild-type GABA\_ARs, GABA-evoked inward currents were in a dose-dependent manner, with the currents being observable at 1μM and maximized at around 100 μM. In comparison with the wild-type receptor, the GABA sensitivity of α1\_T292S GABA\_ARs were significantly increased (Figure 5.2). The currents gated by the mutated receptor was observable at the concentration of 0.1 μM of GABA and reached maximal at around 10 μM of GABA (Figure 5.2A). When we normalized the currents to the cell’s own maximal response, we found that in comparison with the wild-type GABA\_ARs, T292S GABA\_ARs shifted its dose-response curve to the left (Figure 5.2 C) and lowered its EC50 by more than ten folds (T292S: 0.2895 μM; WT: 3.658 μM) (Figure 5.2 C and E). However, when we normalized the currents of the T292S GABA\_ARs (3287+ 422.5 pA) against the maximum response of WT (3075+526.0 pA), we observed that the maximum response of the T292S GABA\_ARs was not affected (Figure 5.2 B&D). To mimic the patient’s heterozygous expression of the mutant subunit, we also co-transfected both wild-type α1\_T292 and the α1\_T292S mutant in a 1:1 ratio along with the β2 and γ2 subunits in HEK293 cells. We found that this heterozygous expression caused a shift of the GABA-evoked dose response curve in the middle between the wild-type and the homozygous curves (Figure 5.3).

In stark contrast, the expression of the T292I mutant resulted in a significant impairment of GABA\_AR function. It severely reduced the peak current amplitude at each concentration tested (Figure 5.2 A) with a drastically decreased maximum response (1033+286.5 pA, which
was only about 1/3 of the wild-type or T292S GABA\(_R\)s (Figure 5.2 B&D). The reduced maximum response was also associated with a rightward shifted dose response curve and more than 18-fold increased EC50 over the wild-type GABA\(_R\) (T292I: 69.28 \(\mu\)M; WT: 3.658 \(\mu\)M) (Figure 5.2 C-E).

To check whether either of the two variants could affect the ion selectivity of GABA\(_R\), chloride ion channel amplitudes of GABA-evoked peak currents were measured at stepwise membrane potentials ranging from -80 to +80 mV with a 20 mV interval. As shown in Figure 5.2 F and G, we found that neither variant compromised the ion selectivity since the reversal potentials of both variant receptors were similar to that of wild-type receptors, being approximately at 0 mV.
Figure 5.2 GABA-evoked responses of WT (black), α1T292S (red) and α1T292I (blue) of GABA\(\text{A}\)R show bidirectional changes in two de novo mutations at the same T292 residue of GABRA1.

(A) Representative traces of GABA-evoked currents of WT, α1T292S and α1T292I of GABA\(\text{A}\)R. GABA application (2s) is indicated as a bold black line above the traces. (B) Maximum response of α1T292S (n=10) and α1T292I (n=12) of GABA\(\text{A}\)R in comparison with WT (n=8). (C) Dose-response curves of GABA-evoked responses of WT (n=8), α1T292S (n=10) and α1T292I (n=12) of GABA\(\text{A}\)R. The peak current amplitude at each GABA concentration was normalized to the maximum response (1 mM) of each receptor, respectively. (D) Dose-response curves of GABA-evoked responses of WT (n=8), α1T292S (n=10) and α1T292I (n=12) of GABA\(\text{A}\)R. The peak current amplitude at each GABA concentration was normalized to the maximum response (1 mM) of WT. (E) EC50 of GABA-evoked response of WT, α1T292S and α1T292I GABA\(\text{A}\)R. (F) Representative trace of I-V curve. (G) I-V curves for WT (n=5)
and mutants (n=5) GABA_aR-gated currents evoked by 1mM GABA. Statistical differences were determined using student’s t-test (**P<0.001, ns=not significant).

![Image](image.png)

**Figure 5.3** Overexpressing mixed GABRA1 subunits (α1T292 : α1T292S : β2 : γ2=1:1:2:1) in HEK293 cells to mimic heterozygous status in a patient.

(A) Representative trace of GABA-evoked currents of WT (black), α1T292S GABA_A R (red) and mixed expression of α1T292α1T292Sβ2γ2 (green). (B) Dose-response curve of WT (n=10), α1T292S GABA_A R (n=8) and mixed expression of α1T292α1T292Sβ2γ2 (n=8).

5.2.4 Neither T292S nor T292I variants affected GABA_A R total/surface expressions

The marked functional alterations of the two variants in GABA-evoked currents were quite intriguing and prompted us to probe into the mechanisms underlying the bidirectional changes. Functional changes could be due to changes in receptor expression or channel gating properties. Since alteration in the level of GABA_A Rs expression in the cell and/or on the cell membrane has previously been reported, including other previously identified GABA_A R variants (Bradley et al., 2008; Jacob et al., 2008; Vithlani et al., 2011), to be important ways in regulating the functions of the receptors with physiological and pathogenic consequences, we first examined the possibility that these two variants would exert their impacts on the receptor
through affecting the receptor total and/or surface expression levels. We quantified the total receptors expressed in the cell by immunoblotting total cell lysates, and quantified surface receptors expressed on the plasma membrane by specifically immunoblotting biotinylated surface receptors (Figure 5.4) in HEK cells transiently expressing wild-type α1/β2/γ2, α1T292S/β2/γ2 or α1T292I/β2/γ2. To our surprise, unlike other previously identified GABA_AR variants (Butler et al., 2018; Bai et al., 2019; Reyes-Nava et al., 2020), we found that in comparison with the WT receptor, neither variants produced any significant alteration in either the total or surface expression levels of the receptor (Figure 5.4 A-D).

Figure 5.4 T292S and T292I variants did not affect GABA_AR total/surface expressions.

(A) Representative blots of surface biotinylation of WT, α1T292S and α1T292I of GABA_AR and non-transfected HEK293. (B) Quantification of surface α1 subunit expression normalized to Na⁺/K⁺ ATPase (n=9). (C)

Representative blots of the total expression levels from WT, α1T292S and α1T292I of GABA_AR and non-transfected
HEK293 samples. (D) Quantification of the total α1 subunit expression normalized to heat shock protein 90 (HSP90) (n=7). Statistical differences were determined using one-way ANOVA (ns=not significant).

5.2.5 T292S and T292I variants bidirectionally altered GABA$_A$R single channel properties

Since neither T292S nor T292I mutation affected GABA$_A$R expression in the cell and on the plasma membrane as shown above, we next examined the possibility that the functional alterations were resulted from the changes in channel gating properties. We employed cell-attached single channel recordings of the WT and mutant GABA$_A$Rs transiently expressed in HEK cells with subsaturating (3 μM) or saturating (1 mM) concentrations of GABA contained in the recording pipette. As shown in Figure 5.5 A-E, we found that the T292S mutant significantly increased GABA-evoked single-channel activity at subsaturating, but not at the saturating GABA concentrations. When induced by GABA concentration at 3 μM, the T292S variant significantly increased the mean open time (T292S: 31.22+3.532 ms vs WT: 16.58+0.4146 ms), and hence the open probability (T292S: 23.58+4.279% vs WT: 8.528+1.34%) of GABA$_A$R without changing the channel conductance (T292S: 29.16+1.492 pS, WT: 29.51+1.806 pS) and channel open frequency (T292S: 7.982+1.719 s$^{-1}$, WT: 5.172+0.8122 s$^{-1}$) in comparison with the WT GABA$_A$R (Figure4 A-E), being in good agreement with the increased sensitivity to GABA. In contrast, the T292I mutant significantly reduced sensitivity to GABA in comparison with WT receptors as no detectable single-channel event under subsaturating concentration of GABA (Figure 5.5 A-E). When the currents were evoked with GABA at a concentration of 1mM, the T292S GABA$_A$Rs showed no significant alterations of either conductance (T292S: 28.27+2.199 pS vs WT: 24.76 +1.095 pS), mean open time (T292S: 33.77+5.447 ms vs WT: 30.36+4.247
ms), open frequency (T292S: 10.7±2.358 s⁻¹ vs WT: 8.403±2.057 s⁻¹), or open probability (T292S: 30.19±4.249% vs WT: 22.93±5.094%) (Figure 5.5 F-J), being in good agreement with the no change in maximum response observed with the whole-cell current recordings mentioned above (Figure 5.2). As expected, the T292I variant significantly reduced single channel activity evoked by the high concentration of GABA (1 mM). In particular, while no change in the conductance (T292I: 24.71±1.396 pS, WT: 24.76±1.095 pS) could be detected, both channel opening frequency (T292I: 4.806±1.234 s⁻¹, WT: 8.403±2.057 s⁻¹) and open probability (T292S: 7.502±2.224%, WT: 22.93±5.094%) were significantly reduced, leading to reduced mean open time (T292I: 15.41±2.251 ms, WT: 30.36±4.247 ms) when compared with the recordings from the WT counterparts (Figure 5.5 F-J). The results imply that the impaired function of the GABAARs by T292I mutation can be attributed to the decreased open probability and opening frequency of the channel.
Figure 5.5 T292S and T292I variants bidirectionally altered GABA\(_A\)R single channel properties. Cell-attached single channel currents were recorded for WT (black), α1\(_{T292S}\) (red) and α1\(_{T292I}\) (blue) of GABA\(_A\)R under subsaturating and saturating concentrations of GABA.

(A) Representative trace of α1\(_{T292S}\) (n=6), α1\(_{T292I}\) (n=6) and WT (n=6) GABA\(_A\)R under 3 μM GABA. (B) Conductance under 3 μM GABA condition. (C) Mean open time under 3 μM GABA condition. (D) Channel open frequency under 3 μM GABA condition. (E) Open probability under 3 μM GABA condition. (F) Representative trace of α1\(_{T292S}\) (n=6), α1\(_{T292I}\) (n=6) and WT (n=8) GABA\(_A\)R under 1 mM GABA. (G) Conductance under 1 mM GABA condition. (H) Mean open time under 1 mM GABA condition. (I) Channel open frequency under 1 mM GABA condition. (J) Open probability under 1 mM GABA condition. Statistical differences were determined using one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001, ns=not significant).
5.2.6 T292S and T292I variants resulted in different changes of GABA$_A$R-gated tonic and leak currents

We noticed in a recent study by Butler, K.M., et al that a T292K variant of the $\alpha_2$ subunit caused the tonically opening of GABA$_A$Rs (Butler et al., 2018). Since the $\alpha_1$ and $\alpha_2$ subunits are highly homologous, we wondered whether the T292S and T292I variants in the $\alpha_1$ subunit would cause any changes to the tonic/leaking currents of GABA$_A$Rs. The tonic GABA tonic current is the current gated through the GABA$_A$R activated by ambient GABA in the extracellular solution and can be revealed by blocking the GABA$_A$R with competitive antagonist bicuculline (Johnston, 2013). The leak current is the current gated through the channel pore of the non-activated GABAAR and is usually revealed with the receptor channel pore blocker such as picrotoxin (Olsen, 2015). We found that 20 μM bicuculline was already enough to block all tonic current because a higher concentration of it (50 μM) could not further induce more blockade (Figure 5.6 A&B). Bath application of the saturated bicuculline (20 μM) revealed pronounced tonic currents as evident by the upward baseline current drafting in cells expressing T292S GABA$_A$Rs (249+52.6 pA). In great contrast, bicuculline application resulted in only minimal or no tonic currents in cells expressing either WT (13.63+5.641 pA) or T292I (-0.3709+0.8945 pA) receptors (Figure 5.7 A&B). Similarly, bath application of picrotoxin (100 μM) revealed a notable leak current as evident with a small additional increase upward current on top of the bicuculline-induced tonic currents only in cells expressing the T292S GABA$_A$Rs (72.22+16.44 pA), but not in cells expressing WT (1.586+2.496 pA) or T292I (1.913+2.304 pA) GABA$_A$Rs (Figure 5.7 A&C). These results demonstrated that the T292S variant could increase both tonic and leak currents of GABA$_A$Rs while the T292I variant could not affect either currents.
Figure 5.6 Higher concentration bicuculline could not further block tonic current of α1T292S GABAAR.

(A) Representative trace of 20 μM and 50 μM bicuculline blocked tonic current of α1T292S GABAAR. (B) Quantification of tonic current blocked by 20 μM and 50 μM bicuculline of α1T292S (n=7) of GABAAR. Statistical differences were determined using unpaired t-test (ns=not significant).
Figure 5.7 Tonic and leak currents assays on WT, α1T292S and α1T292I of GABAAR show increased tonic and leak currents in T292S mutated receptor.

(A) Representative trace of bicuculline-blocked tonic current and picrotoxin blocked leak current of WT, α1T292S and α1T292I GABAAR. Dotted line indicates the new baseline after tonic current fully blocked by 20 μM bicuculline for the quantification of additional 100 μM picrotoxin blocked leak current. (B) Quantification of tonic current blocked by bicuculline in WT (n=5), α1T292S (n=9) and α1T292I (n=8) of GABAAR. (C) Quantification of leak current blocked by picrotoxin in WT (n=5), α1T292S (n=8) and α1T292I (n=7) of GABAAR. Statistical differences were determined using one-way ANOVA (***,P<0.001, ns=not significant).
5.2.7 Thiocolchicoside restores the function of T292S GABA<sub>A</sub>R

The increased GABA sensitivity of the T292S variant is a surprising result of the present work, suggesting that a gain-of-function mutant of the GABA<sub>A</sub>R is also pathogenic. We reasoned that negative GABA<sub>A</sub>R modulators might be able to normalize the function of mutant receptor to the WT receptor level, thereby having the therapeutic potential to treat patients carrying this gain of function GABA<sub>A</sub>R variant. We examined the effect of three clinically approved negative allosteric GABA<sub>A</sub>R modulators, bemegride (Mistry and Cottrell, 1990), flumazenil (Safavynia et al., 2016) and thiocolchicoside (TCC) (Carta et al., 2006) on a full dose response of whole-cell currents evoked with perfusion of GABA (0.1 μM-1 mM). We found that among the three drugs tested at a concentration of 200 μM, only TCC was able to significantly reduce the increased GABA currents gated through T292S variant (Figure 5.8 A, B, C&D). We then did a dose response of TCC in decreasing the T292S GABA<sub>A</sub>R gated currents evoked by 1 μM GABA in an effort to find an optimal concentration of TCC that was capable of fully restoring the GABA-sensitivity of T292S GABA<sub>A</sub>R (Figure 5.8 A&E and Figure 5.9 A&B). As shown in Figure 5.8 A&E, we found that 1 μM TCC could fully leftwardly shift the dose response curve to the level of WT GABA<sub>A</sub>R.
Figure 5.8 The restoring effects of the negative GABAₐR allosteric modulators on α₁T₂₉₂S mutated GABAₐR.

(A) Representative trace of GABA-evoked currents of WT (black), α₁T₂₉₂S (red) and α₁T₂₉₂S GABAₐR treated by bemegride (purple), flumazenil (blue) and thiocochicoside (TCC) (green). (B) Dose-response curve of α₁T₂₉₂S GABAₐR treated with bemegride (n=5) in comparison with untreated α₁T₂₉₂S (n=8) and WT (n=10) GABAₐRs. (C) Dose-response curve of α₁T₂₉₂S GABAₐR treated by flumazenil (n=6) in comparison with untreated α₁T₂₉₂S (n=8) and WT (n=10) GABAₐRs. (D) Dose-response curve of α₁T₂₉₂S GABAₐR with thiocochicoside (TCC) (n=8) treatment compared with untreated α₁T₂₉₂S (n=8) and WT (n=10) GABAₐRs.
5.2.8 Combination of verapamil and diazepam partially rescues the function of T292I GABAAR

Since the T292I variant’s loss-of-function is primarily a result of reduced channel open probability, we reasoned that its channel abnormality might be restored by some positive GABAAR allosteric modulators, such as diazepam and verapamil that have previously been shown to restore the function of some pathogenic loss of function GABAAR variants via improving channel gating properties (Bai et al., 2019). We first pretreated HEK293 cells transiently expressing $\alpha_1T292I/\beta2/\gamma2$ receptors with verapamil (4 μM) for 24 hours and also acutely applied verapamil during the recordings of the full dose response of the GABA-evoked currents. As shown in Figure 5.10 A&C, verapamil showed slight rescuing effects of the T292I GABAAR. We then tried diazepam (Olsen, 2018) and found that it also only showed partial rescuing effects (Figure 5.10 A&B). Then, we considered the possibility that a combination of both verapamil and diazepam would result in a synergistic rescuing effect. Indeed, it turned out
that acute application of diazepam and verapamil together after chronic treatment of verapamil showed the best effects in shifting the GABA-evoked dose response curve toward its wild-type counterpart (Figure 5.10 A&D), although the shift remained incomplete.

**Figure 5.10 The rescuing effects of diazepam and verapamil on the α1T292I mutated GABA<sub>A</sub>R.**

(A) Representative trace of GABA-evoked currents of WT (black), α1T292I (blue) GABA<sub>A</sub>R and α1T292I with treatment of diazepam (red), verapamil (green) and combination of both (orange). (B) Dose-response curve of α1T292I GABA<sub>A</sub>R with diazepam (n=6) in comparison with untreated α1T292I (n=8) and WT (n=10) GABA<sub>A</sub>Rs. (C) Dose response curve of α1T292I GABA<sub>A</sub>R with verapamil (n=7) treatment in comparison with untreated α1T292I (n=8)
and WT (n=10) GABA$_A$Rs. (D) Dose-response curve of α1T292I GABA$_A$R with combined treatments of diazepam and verapamil (n=8) compared with untreated α1T292I (n=8) and WT (n=10) GABA$_A$Rs.

### 5.2.9 Discussion

In this study, we reported a novel de novo missense variant T292S of the T292 residue of α1 subunit of GABA$_A$R and compared it with a previously found de novo missense variant T292I of the same residue. The patient carrying the T292S variant is featured with developmental delay without observable somatic seizure activity, while the patient with the T292I variant is primarily manifested with severe epilepsy (Allen et al., 2013; Reyes-Nava et al., 2020). Considering the fact that the T292 residue is highly conserved among species and is one of the residues lining the channel pore, functional characterization of these variants identified from patients may improve our understanding of the underlying pathophysiology. Our functional analysis performed in HEK293 cells showed that the T292S and T292I variants of the GABA$_A$R α1 subunit conferred opposing changes in GABA agonist sensitivity and potency. The T292S variant induced leftward shift of the GABA dose-response curve and lowered the EC50 of GABA without altering the maximum response, suggesting that T292S is causing gain-of-function of the GABA$_A$R. On the other hand, the T292I variant caused a rightward shift of the GABA dose-response curve, increased the EC50 of GABA and reduced maximum response, indicating that T292S is causing loss-of-function of the GABA$_A$R. To our knowledge, this is the first study that performs detailed functional characterizations of the T292S and T292I variants and, importantly, the first to report that the same subunit residue of GABA$_A$R replaced by different amino acids would result in opposite impacts on the receptor functions.
There are several potential reasons that can explain these functional changes, which were examined in details in our experiments (Butler et al., 2018). First, the genetic mutations observed in the GABARA1 variants may theoretically alter the receptor expression, assembly, and trafficking that lead to the change of the surface/total receptor numbers (Bai et al., 2019). However, we found that neither T292S nor T292I variants caused changes in receptor numbers, as evidenced by the results of immunoblotting and surface biotinylation assays.

Second, single amino acid mutations may induce structural changes in the GABA agonist-binding pocket and result in increased or decreased GABA binding affinity (Ceulemans et al., 2010; Janve et al., 2016; Steudle et al., 2020). However, as the T292 residue is located at the inner channel pore, which is far away from the GABA binding region, the chance that the mutation causes allosteric change in the structure of the GABA agonist-binding pocket is low.

Third, since this residue lies in the middle of the channel pore, its mutations may produce conformational changes that lead to altered GABA sensitivity in inducing channel gating (Fisher, 2004; Janve et al., 2016). Supporting this conjecture, our single channel recording data showed significant changes of channel gating properties in both variants. The T292S variant increased single-channel open time and open probability under subsaturating (3μM) and saturated (1mM) GABA concentrations, suggesting the alteration of GABAAR function by increasing the sensitivity of GABA to induce channel opening. In contrast, the T292I variant showed significant decrease in the GABA’s ability to keep the channel opened as evident by the lack of opening activity at sub saturating GABA, and significantly reduced channel open time and open probability at saturating GABA stimulation (1mM). Taken together, these results strongly suggest that the residue T292 in the α1 subunit has a critical role in determining the channel open threshold by its agonists. The idea that the opposing functional alterations of these two mutations
primarily resulted in changing GABA’s sensitivity in gating the receptor channel is further supported by the results of our tonic and leak currents assay. We found that the T292S (but not T92I) GABA<sub>A</sub>R showed increased tonic currents and leak currents. We postulated that the T292S variant not only decreases the threshold of GABA induced channel opening, but also keep the channel at some degree of open status even in the absence of agonist binding; whereas the T292I variant turns the channel into a more closed status by decreasing the ability of GABA to cause it open. Given that the T292 residue is located at the TM2 segment and forms part of the channel pore, it should not be surprising to see such changes in channel gating properties. In particular, the T292I variant involves a threonine to isoleucine mutation, which may result in a significant change in side chain length and polarity that are not in favor of channel opening. Similarly, as the T292S variant involves a more subtle threonine to serine mutation, which results in less alteration in side chains in terms of their length and polarity, and such relative subtle changes may in turn favor GABA to cause the channel open. Mechanistically, T292S and T292I may differentially alters a conserved pore-peripheral salt bridge that couples with GABA<sub>A</sub>R channel opening and gating in similar way as described in the recent work on the archetypal muscle nicotinic acetylcholine receptor (AChR) by Steven Sines (Strikwerda and Sine, 2021). Taken together, the ability of both T292S and T292I to dramatically and oppositely change the channel open probability demonstrates that α<sub>1</sub>T<sub>292</sub> is a critical residue for controlling GABA<sub>A</sub>R channel opening and gating.

So far, there are more than 25 reported GABRA1 mutations that are related to pediatric encephalopathy and most of these characterized mutations show a negative impact on GABA<sub>A</sub>R function (Bradley et al., 2008; Ding et al., 2010; Liu et al., 2018; Hernandez et al., 2019). Only an A332V variant in GABRA1 located in the TM3 channel pore was recently reported to
enhance the receptor function in addition to our T292S variant (Steudle et al., 2020). In the present work, we report for the first time that the T292S variant, unlike most previously reported pathogenic GABA$\_A$R variants, is a gain of function variant. Furthermore, the T292I variant, which occurs at the same residue with a different amino acid substitution as T292S, is a loss of function variant. Following functional profiling of the pathological mechanisms of the T292S and T292I mutations, we also attempted to identify potential therapeutic options for more effective and personalized treatments. Guided by the detailed functional phenotypes of the two variants, we performed a quick screening of some clinically approved drugs that directly or indirectly act on GABA$\_A$Rs, and thereby reducing the functional abnormalities of certain mutant variants of GABA$\_A$Rs. Thiocolchicoside (TCC) is used in the clinic as a muscle relaxant, but it is also an allosteric GABA$\_A$R inhibitor that shows potent antagonistic effects against GABA$\_A$R (Carta et al., 2006). At 1 $\mu$M concentration, TCC could fully restore the increased GABA-evoked dose response of the T292S variant to WT level, with superior effects over other GABA$\_A$R negative allosteric modulators, bemegride and flumazenil. Our study, along with others, suggests that these drugs can be a potential therapeutic option for gain of function GABA$\_A$R variants (Butler et al., 2018; Steudle et al., 2020).

In contrast, we found that the loss of function variant T292I GABA$\_A$R-gated currents could be partially restored by a combination of two positive GABA$\_A$R modulating drugs, diazepam and verapamil. Chronic treatment of verapamil was shown to enhance channel gating with elongated open time and increased open probability of R214C GABA$\_A$R, suggesting diazepam and verapamil may work synergistically to ultimately improve the function of the T292I variant GABA$\_A$Rs. Our studies indicate that these mutations have opposite impacts on the function of GABA$\_A$Rs although occurring at the same residue and thereby require different
functional and pharmacological strategies to restore the function of the receptor to the level of their wild-type counterpart. Thus, our findings suggest that it is of paramount importance to perform functional and pharmacological analysis after exome sequencing to determine the pathological mechanisms. This would aid in searching for the appropriate therapeutic options for patients carrying a de nova mutation of principle neurotransmitter receptors, such as the GABA\(_A\)Rs.

In conclusion, our functional analyses of the two de novo variants, T292S and T292I, of the same GABRA1 T292 residue from two patients with distinct clinical phenotypes have revealed their gain of function and loss of function impacts on the GABA\(_A\)R, respectively. The study not only provides evidence for the pathogenic contributions of the two variants to the patients’ pathology but also indicate the crucial role of the T292 residue in controlling channel gating. There are around 30% of children with refractory epilepsy that do not respond to conventional drug treatments due to unknown functional alterations or unidentified causes (Geffrey et al., 2015; Sills and Rogawski, 2020). Moreover, traditional anti-seizure drugs show little to no effect toward rescuing deficits caused by specific mutations in GABA\(_A\)R subunits (Greenfield, 2013; Lösch et al., 2020), and in certain case can even exacerbate the symptoms (Absalom et al., 2020; Billakota et al., 2020). The pharmacological characterizations in our study provided differential therapeutic suggestions for managing these two patients and endorse the importance of precision medicine for pediatric channelopathies.
Chapter 6: Conclusion

6.1 Overview

N-methyl-D-aspartate (NMDA) receptor and type A γ-aminobutyric acid receptor (GABA\(_A\)R) participate in various neuronal activities in the mammalian central nervous system (CNS) (Pugh and Raman, 2006; Tatti et al., 2017), their crucial roles make them ideal targets for developing therapeutic drugs for neurological and psychiatric disorders implicating the dysfunctions of these receptors.

Ischemic stroke causes sudden neurologic deficits and subsequent neurodegeneration due to oxygen deprivation results from blood clot wherein NMDAR-mediated excitotoxicity plays a major role (Simon et al., 1984; Rothman and Olney, 1995; Lee et al., 1999). During an cerebral ischemic event, a suddenly discontinued supply of blood and oxygen leads to impaired mitochondrial energy metabolism and depolarization of neuronal cells, resulting in excessive release and accumulation of extracellular glutamate, which overactivates NMDARs and triggers a massive influx of calcium into the intracellular space (Kristián and Siesjö, 1998), lactate level elevation (Rossi et al., 2007), acidosis and oxidative stress with the formation of reactive oxidative species (ROS) (Su and Wang, 2020). NMDARs are heterotetramers. Despite the common and obligatory GluN1 subunits, the diverse combinations of GluN2 subunits give rise to a bunch of diheteromers and triheteromers which largely determine NMDAR’s biophysical and pharmacological properties, subcellular localizations and intracellular signaling cascades (Paoletti et al., 2013; Zhou et al., 2013a). Two prevalent theories about the role of NMDARs in the pathogenesis of ischemic stroke are the ‘NMDAR subtype hypothesis’ and ‘NMDAR location hypothesis’. The ‘NMDAR subtype hypothesis’ states that activating GluN2A-containing NMDAR promotes neuronal survival, whereas activating GluN2B-containing
NMDARs induces neuronal death (Lai et al., 2011). This hypothesis is largely supported by more recent evidence accumulated using in-vitro and in-vivo models of stroke and fuels the development of subtype specific drugs or drugs that can modulate their activities (modulators) for treating stroke (Sattler et al., 1999b; Aarts et al., 2002a). The ʻNMDAR location hypothesis (Liu et al., 2007; Hardingham and Bading, 2010) implies that activating synaptic NMDARs which are predominantly GluN2A-containing NMDARs is pro-survival while activating extrasynaptic NMDARs, which are predominantly GluN2B-containing NMDARs is pro-death. However, the two hypotheses have also been challenged by several other studies. There are groups reporting that synaptic NMDARs mediate neuronal death and extrasynaptic NMDARs mediate neuronal death (Papouin et al., 2012). Additionally, activating synaptic and extrasynaptic NMDARs alone is reported to be pro-survival while activating both induces cell death (Stark and Bazan, 2011; Zhou et al., 2013b; Zhou et al., 2013a; Chen et al., 2014). Nevertheless, therapies that modulate the subpopulations of NMDARs by location are shown to be effective in treating ischemic injury in preclinical studies. In light of the large amount of evidence, through virtual screening and in silico drug design, a lead compound 813 was found to function as an NMDAR dual allosteric modulator (Ndam) that potentiates GluN2A-containing NMDARs and at the same time inhibits GluN2B-containing NMDARs with very low potency (Axerio-Cilies, 2016). In this thesis I successfully further optimized the lead compound into a new allosteric modulator Ndam830 with largely improved potency and efficacy and Ndam830 showed neuroprotective effects against cytotoxic injuries in vitro and in vivo.

In contrast to the overactivation of NMDARs happening in the process of ischemic stroke, hypofunction of NDMARs is associated with a series of psychoneurological disorders such as schizophrenia, major depressive disorders (MDDs), Alzheimer’s disease (AD), autism
spectrum disorders (ASD) and anti-NMDAR encephalitis. There is accumulating evidence indicating that the alterations of excitatory signaling involving the hypofunction of NMDARs are key contributors to the pathogenesis of schizophrenia (Balu, 2016). NMDAR channel blockers ketamine and phencyclidine (PCP) are dissociative anesthetics that are used to produce the full range of schizophrenia symptoms and cognitive deficits in healthy humans and animals (Javitt and Zukin, 1991; Paul et al., 2013). Although NMDA antagonism mediated by ketamine or dextromethorphan showed anti-depressant effects (Henter et al., 2018), NMDAR hypofunction was also reportedly implicated in the pathophysiology of major depressive disorders (MDDs). Hypofunction of NMDAR is reportedly associated with AD (Ulas and Cotman, 1997; Newcomer et al., 2000; Malinow, 2012) and impaired NMDAR function was observed in the aging brain (Huang et al., 2012). Autism spectrum disorders (ASD) is a neurodevelopmental disorder characterized by deficits in social interaction and communication, and repetitive behavior (Schmeisser et al., 2012). Several mutations of genes encoding synaptic proteins were identified such as neuroligins, neurexins, GKAPs/SAPAPs, DOCK4/ Rac1 and ProSAPs/Shanks (Schmeisser et al., 2012; Won et al., 2012; Guo and Peng, 2021). Anti-NMDAR encephalitis is an auto-immune disease with high titres of auto-antibodies for NMDARs. Those auto-antibodies bind to the N-terminal domain (NTD) of subunit GluN1 and reduce the surface expression and hence physiological function of the NMDARs (Dalmau et al., 2008; Hughes et al., 2010; Mikasova et al., 2012). In the process of drug development of Ndams, I also found a nontoxic positive allosteric modulator Ndams844 that strongly potentiated all GluN1/GluN2A-D subtypes with desirable selectivity against neuronal AMPARs and GABAARs. Ndams844 harbors great therapeutic potential in rescuing the NDMAR hypofunction related neuropsychological disorders as discussed above.
Apart from NMDARs, dysfunction of GABA\(_A\)Rs is implicated with various neurological conditions as well. Accumulating evidence suggests that genetic variants of the GABA\(_A\)R may cause conditions including epileptic encephalopathy (EE) (Krampfl et al., 2005; Gallagher et al., 2007; Galanopoulou, 2008; Ding et al., 2010), developmental delay (Galanopoulou, 2008; Braat and Kooy, 2015), Fragile X, Rett Syndrome, and Dravet Syndrome (Duarte et al., 2013; Carvill et al., 2014; He et al., 2014).

The \(\alpha_1\) subunit of the GABA\(_A\)R, which is encoded by the GABRA1 gene, is ubiquitously expressed in CNS neurons, suggesting its importance in maintaining the normal function of the vast majority of native GABA\(_A\)Rs (Jacob et al., 2008). Indeed, increasing numbers of GABRA1 variants have been implicated in causing haploinsufficiency and loss of function of the GABA\(_A\)Rs, and thereby causally contribute to the pathogenesis of various forms of epilepsy (Fisher, 2004; Maljevic et al., 2006; Janve et al., 2016; Johannesen et al., 2016; Kodera et al., 2016), Dravet Syndrome, early onset EEs, and developmental delay (Carvill et al., 2014; Lachance-Touchette et al., 2014). In nearly all of the patients with pathogenic GABRA1 variants, the clinical phenotypes include epilepsies within a spectrum of different severity, ranging from generalized epilepsies to severe epileptic encephalopathies (Jacob et al., 2008).

We recently identified a novel de novo T292S (C875G) missense variant of GABRA1 from a pediatric patient with developmental delay but without diagnosed seizure events. This mutation occurs on the same residue as that of the previously reported variant T292I (C875T) identified in a pediatric patient with severe epilepsy (Reyes-Nava et al., 2020). The dramatic difference between the phenotypes of the two patients and the resistance to the standard care treatments of the patient harboring the T292S (C875G) mutation prompted us to characterize and compare the pathological impacts of the two mutations on the functions of the GABA\(_A\)R and
potentially search for the most suitable and effective pharm-therapeutics. We found that the two mutations have drastically different impacts on the receptor function: the $\alpha_1T292S$ variant significantly increased (gain-of-function), whereas the $\alpha_1T292I$ variant significantly reduced (loss-of-function) $\text{GABA}_A\text{R}$ function. The opposite functional impacts of these mutations suggest that we would need different therapeutic strategies for treating the patients carrying these mutations. Through screening several clinically approved drugs that are reportedly acting on $\text{GABA}_A\text{Rs}$, we found that a negative allosteric modulator (NAM) of $\text{GABA}_A\text{R}$ thiocolchicoside (TCC) (Fisher, 2004) can reduce the gain of function caused by the T292S mutation, thereby normalizing the receptor function; on the other hand, a combination of $\text{GABA}_A\text{R}$ positive allosteric modulators (PAMs), diazepam and verapamil, largely rescued the loss of function caused by the T292I mutation. Our results, particularly the gain of function caused by $\alpha_1T292S$, are in great contrast to the loss of function by all previously reported pathogenic $\text{GABRA}_1$ mutations (Reyes-Nava et al., 2020), highlighting the importance of functional characterization of and calling for different therapeutic strategies for each individual mutation. This study is also a good example of precision medicine that tailors allosteric modulators of $\text{GABA}_A\text{R}$ based on detailed functional characterization of $\text{GABA}_A\text{R}$ variants.

6.2 Lead compound Ndam813 optimization and development

6.2.1 Optimizing Ndam813 with low potency and efficacy into Ndam830 with improved potency and efficacy

We used a computer-assisted in-silico screening assay to screen commercial chemical libraries to select chemicals that are analogs of Ndam813. Following this, we tested these initially selected chemicals electrophysiologically on HEK293 cells expressing recombinant
NMDARs to determine their effects on diheteromeric GluN1/GluN2A and GluN1/GluN2B. The effects of tested compounds on NMDARs were fed back to the structure-activity relationships (SAR) analysis in combination with simulated compound dockings using structure models of NMDARs (PDB: 6IRA and 5IOU) to guide further optimizations and syntheses of the lead compounds.

Compounds were tested at concentrations of their optimal solubility on HEK293 cells expressing recombinant diheteromeric GluN1/GluN2A and GluN1/GluN2B. These structure-activity relationships (SAR) analysis together with the in-vitro functional screenings led to the discoveries of Ndam830 (potentiates GluN1/GluN2A but inhibit GluN1/GluN2B).

When compared with the old lead compound Ndam813, Ndam830 showed better potency in potentiating diheteromeric GluN1/GluN2A and triheteromeric GluN1/GluN2A/GluN2B and inhibiting diheteromeric GluN1/GluN2B at 10-fold lower concentration (improved efficacy) (Figure 3.1 and Table 3.1).

6.2.2 Ndam830 showed differential unbinding kinetics on recombinant diheteromeric GluN1/GluN2A, triheteromeric GluN1/GluN2A/GluN2B and diheteromeric GluN1/GluN2B

During the testing of Ndam830 on recombinant NMDARs expressed on HEK293 cells, we noticed the dissociation rates of Ndam830 bound diheteromeric GluN1/GluN2A and triheteromeric GluN1/GluN2A/GluN2B may differ a lot from that of diheteromeric GluN1/GluN2B. The reason why we made such an assumption was that when we adopted a 40-sec wash out strategy the effect mediated by Ndam830 on diheteromeric GluN1/GluN2A and triheteromeric GluN1/GluN2A/GluN2B were lingering (longer-lasting effects) (Figure 3.2, A,
B, F&G) while the effect on diheteromeric GluN1/GluN2B went back to baseline (Figure 3.2, C&D). Consistent with what we observed in tests on recombinant NMDARs expressed on HEK293 cells, co-application of Ndam830 (10 μM) with NMDA/glycine (10 μM/1 μM) on hippocampal neurons showed little effects on neuronal NMDARs (Figure 3.3 A&B), likely due to the canceling of each other between potentiation of GluN2A-gated currents and inhibition of GluN2B gated currents by Ndam830 in these neurons. Wash-out of Ndam830 produced an enhancement of NMDAR-gated (rebound) currents. Due to Ndam830’s distinct disassociation rates on diheteromeric GluN1/2A, triheteromeric GluN1/GluN2A/GluN2B and diheteromeric GluN1/2B, washing out resulted in a rapid recovery of the inhibited GluN1/2B component, thereby revealing the residual potentiating effects on GluN2A component (rebound current after washing) (Figure 3.3 A).

The fast-on and fast-off kinetic properties of Ndam830 on diheteromeric GluN1/GluN2B mimic that of clinically approved drugs, some fast-acting NMDAR channel blockers such as memantine and dextromethorphan (DMX). It was postulated that faster blocking/unblocking kinetics and lower affinity of memantine in comparison with other blockers of its kind, ketamine and PCP were associated with an improved therapeutic index regarding psychomimetic effects due to reduced channel block during normal synaptic transmission (Chen and Lipton, 2006). This shared kinetic property of Ndam830 with approved NMDAR-related drugs may contribute to future clinical application.

There are many studies reported that a large portion of native NMDARs are in triheteromeric GluN1/GluN2A/GluN2B form and account for >50% of the total NMDARs in the hippocampus and cortex in the adult rodent brain (Sheng et al., 1994; Chazot and Stephenson,
1997; Luo et al., 1997; Al-Hallaq et al., 2007; Rauner and Köhr, 2011; Tovar et al., 2013). Most recent advances in this field were the functional and pharmacological study of selectively expressed recombinant triheteromeric GluN1/GluN2A/GluN2B (Hansen et al., 2014) and the resolution of the structure of triheteromeric GluN1/GluN2A/GluN2B receptor by single-particle cryogenic electron microscopy (cryo-EM) (Lu and Du, 2017). Selective expression of triheteromeric GluN1/GluN2A/GluN2B was achieved either by engineering the C-tails of GluN2A and GluN2B to only enable triheteromers’ surface expression or by combining the GluN2A subunit that was generated from GluN2B by mutating a dozen of residues of GluN2B. Interestingly, the pharmacological properties of triheteromeric GluN1/GluN2A/GluN2B are reportedly to be dominated by its GluN2A subunit (Hansen et al., 2014). Our experiments on HEK cells supported this as Ndam830 acted as a potentiator on triheteromeric GluN1/GluN2A/GluN2B similarly to its action on diheteromeric GluN1/GluN2A. However, the overall effects of Ndam830 on hippocampal neurons were very little due to canceling of each other between potentiation of GluN2A-gated currents and inhibition of GluN2B gated currents by Ndam830 in these neurons. In addition, there was a rebound current that revealed current mediated by GluN2B-containing NMDARs upon washout. This observation made us wonder whether triheteromeric GluN1/GluN2A/GluN2B really account for >50% of the total NMDARs because Ndam830 wound have shown stronger overall potentiating effects similar to what observed on recombinant NMDARs. Our results, along with another report (Al-Hallaq et al., 2007), suggested the percentage of triheteromeric GluN1/GluN2A/GluN2B accounted for in native NMDARs might not be as high as previously expected.
6.3 Ndam830 as a novel neuroprotectant that passed the BBB

In the in vitro experiments, when pre-treat cortical neurons with Ndam830, it protected from NMDA induced excitotoxicity and showed even better protection than traditional preclinical neuroprotectant Ro 25-6981 in H$_2$O$_2$ induced oxidative stress. NMDA induced excitotoxicity was achieved by the incubation of 100 μM NMDA for 1 hour, and oxidative stress insults were done by incubating the cortical neurons with 300 μM H$_2$O$_2$ for 30 mins. Ndam830 concentration-dependently exerted its protective effects against either excitotoxicity or oxidative stress. However, overnight incubation of 10 μM Ndam830 alone slightly increased the LDH release, suggesting its potential toxicity for cultured cortical neurons under basal condition.

After confirming the neuroprotective effects of Ndam830 in vitro, we asked the question of whether it would pass the blood brain barrier (BBB) which was important for the future in vivo experiments. We injected formulated Ndamp830 (5mg/kg) through tail vein intravenous injections (I.V) into rats and collected the cerebrospinal fluid (CSF) samples via cisterna magna puncture. The concentrations of the compound CSF were measured by high performance liquid chromatography electrochemical detection (HPLC-ECD). After establishing a standard curve via testing samples prepared by spiking Ndamp830 into the artificial cerebrospinal fluid (aCSF). We went on to test CSF samples collected from rats, and then Ndamp830 was detected. Based on the standard curve, we made a rough calculation showing that Ndamp830 passed the BBB and had reached a concentration of 11.00 ± 2.677 μM in the collected CSF 30 min after IV injection of 5 mg/kg. This is an ideal amount of Ndamp830 found in the CSF as the therapeutic dose for drug in-vitro was at the similar uM range.

The desirable neuroprotective effects from in vitro experiments and good bioavailability of Ndamp830 encouraged to try this compound on an in-vivo MCAo model, a classic ischemic
stroke model. We first adopted a mode of pre-operative administration strategy by giving Ndam830 (5mg/kg, I.V) one day before and 1 hour prior to the commencement of MCAo surgery on surgery day. One dose of Ndam830 (5mg/kg, I.V) was also given to rats daily for consecutive 6 days after surgery until rats were sacrificed for 2,3,5-Triphenyltetrazolium chloride (TTC) staining. We found that Ndam830 was able to significantly protect the ischemic brain region and reduced the infarct volume. Knowing that Ndam830 worked in the acute MCAo model when given pre-operatively, we then asked the question of whether Ndam830 could exert the same neuroprotective effects at even lower concentrations if it is given post-stroke. To investigate this, we injected Ndam830 (1 and 3 mg/kg, I.V.) 3 hours after the onset of MCAo surgery and sacrificed the rats for 2,3,5-Triphenyltetrazolium chloride (TTC) staining 3 days later. Surprisingly, 1 mg/kg and 3 mg/kg Ndam830 groups both significantly reduced the infarct volume of the ischemic regions in comparison with the control group, suggesting Ndam830’s neuroprotective effects. On top of this, when Ndam830 was given post-operatively for 7 days after the first bolus and then tested periodically for the rats’ neurological function recovery, it significantly accelerated the recovery of locomotor behavioral performance in comparison with the rats in the control group.

These promising results from in vivo experiments demonstrated that Ndam830 could serve as drug prototype that could be used to treat ischemic stroke. Moreover, since Ndam830 also protected the ischemic brain regions when given pre-operatively, the application of Ndam830 might be expanded to scheduled surgery for brain aneurysm. Since the neurosurgical interventions for large or complex brain aneurysm sometimes involve clipping the artery that the aneurysm is formed on, pre-operative administration of Ndam830 could potentially protect the
affected brain region as the surgery goes on. However, the real effects of the drug on aneurysm surgery still warrant further investigation.

Ndam830 is the first potent compound of its kind in terms of the mechanism of potentiating GluN1/GluN2A and simultaneously inhibiting GluN1/GluN2B. It showed good neuroprotective effects against NMDAR-mediated excitotoxicity and non-NMDAR mediated oxidative stress, which are both involved in the pathogenesis of Alzheimer’s disease. It was proposed that activating GluN2B-containing NMDARs mediated amyloid-β (Aβ)-induced alterations in synaptic plasticity and synapse loss, thus enhancing the Aβ- and tau-induced excitotoxicity (Paoletti et al., 2013). Plus, the most recent study demonstrated that a GluN2A selective modulator, GNE-0723 improved the cognitive functions in both Dravet syndrome and Alzheimer’s disease model (Hanson et al., 2020). Considering Ndam830’s pharmacological properties on NMDARs, it may be able to improve cognitive deficits, protect neurons from Aβ- and tau-induced excitotoxicity and preserve synaptic plasticity and synapse loss.

6.4 **Ndam844 as a nontoxic and selective positive allosteric modulator for all NMDARs**

During our optimization process of the lead compound Ndam813, we modified multiple functional groups of this molecule in search of compounds with better potency and efficacy. To our surprise, we found a very potent compound Ndam844 that harbored a distinct modulating pattern. Instead of potentiating GluN1/GluN2A and inhibiting GluN1/GluN2B, Ndam844 strongly potentiated both diheteromeric GluN1/GluN2A and GluN1/GluN2B in the initial electrophysiological tests.

Subsequent systemic evaluation on HEK293 cells overexpressing recombinant diheteromeric GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, GluN1/GluN2D and triheteromeric GluN1/GluN2A/GluN2B. We observed that Ndam844 potentiated diheteromeric
GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, GluN1/GluN2D and triheteromeric GluN1/GluN2A/GluN2B in a dose-dependent manner. The potentiating effects of Ndam844 were relatively smaller on diheteromeric GluN1/GluN2C in comparison with its strong potency on other subtypes. The results of Ndam844 tested on recombinant NMDARs expressed on HEK293 cells demonstrated that Ndam844 is a pan-potentiator of NMDAR that worked on all subtypes of NMDARs including GluN1/GluN2A-D.

Consistently, Ndam844 strongly potentiated overall, GluN2A-containing and GluN2B-containing NMDAR-gated currents. When tested on neuronal AMPARs and GABA\(_A\)Rs, Ndam844 showed decent selectivity towards NMDARs since it shows minimal effects on neuronal AMPARs and GABA\(_A\)Rs.

Intriguingly, when we incubated a series of concentrations of Ndam844 alone overnight with DIV14 cortical neurons, Ndam844 below saturating concentration did not cause any elevation of LDH release at all. This indicated that Ndam844 is not toxic to cultured cortical neurons at the basal condition, suggesting good tolerance for future preclinical experiments.

The potential application of Ndam844 in treating NMDAR hypofunction related diseases has already been detailed discussed in the future direction and discussion part in Chapter 4. Briefly, Ndam844 as a nontoxic and selective positive allosteric modulator for all NMDARs can be tested in future on diseases models involving schizophrenia, major depressive disorders (MDDs), Alzheimer’s disease (AD), autism spectrum disorders (ASD) and anti-NMDAR encephalitis.
6.5 Precision medicine of repurposing allosteric modulators for rare variants of GABA\(_{\alpha1}\)R

In Chapter 5 we reported a novel de novo missense variant T292S of the T292 residue of GABRA1 and compared it with a previously found de novo missense variant T292I of the same residue. The patient carrying the T292S variant is featured with developmental delay without observable somatic seizure activity; while the patient with the T292I variant is primarily manifested with severe epilepsy (Allen et al., 2013; Reyes-Nava et al., 2020). Our functional analysis performed in HEK293 cells showed that the T292S and T292I variants of the GABA\(_{\alpha1}\)R \(\alpha1\) subunit conferred opposing changes in GABA agonist sensitivity and potency. The T292S variant induced leftward shift of the GABA dose-response curve and lowered the EC\(_{50}\) of GABA without altering the maximum response. On the other hand, the T292I variant caused a rightward shift of the GABA dose-response curve, increased the EC\(_{50}\) of GABA and reduced maximum response. However, neither T292S nor T292I variants caused alterations in total and surface receptor numbers as shown by the results of immunoblotting and surface biotinylation assays.

Since this residue lies in the middle of the channel pore, its mutations may produce conformational changes that lead to altered GABA sensitivity in inducing channel gating (Fisher, 2004; Janve et al., 2016). Indeed, our single channel recording data revealed significant changes of channel gating properties in both variants. The T292S variant increased single-channel open time and open probability under subsaturating (3\(\mu\)M) and saturated (1mM) GABA concentrations, indicating the alteration of GABA\(_{\alpha1}\)R function by increasing the sensitivity of GABA to induce channel opening. On the other hand, the T292I variant showed a significant decrease in the GABA’s ability to keep the channel open due to the decreased opening activity at sub-saturating GABA, and significantly reduced channel open time and open probability at
saturating GABA stimulation (1mM). These results strongly indicate that the residue T292 in the α1 subunit plays a critical role in dictating the channel open threshold by its agonists. This idea of opposing functional alterations of these two mutations primarily resulted in changing GABA’s sensitivity in gating the receptor channel is also supported by the results of our tonic and leak currents assay in which we found that the T292S (but not T92I) GABAAR showed increased tonic currents and leak currents.

Up to date, more than 25 GABRA1 mutations are reported to be involved in pediatric encephalopathy, and most of these characterized mutations show negative impact on GABAAR function (Bradley et al., 2008; Ding et al., 2010; Liu et al., 2018; Hernandez et al., 2019). Except an A332V variant in GABRA1 that is located in the TM3 channel pore was recently reported to enhance the receptor function in addition to our T292S variant (Steudle et al., 2020). The T292S variant, unlike most previously reported pathogenic GABAAR variants, is a gain of function variant. Interestingly, the T292I variant that occurs at the same residue with a different amino acid substation as T292S, is a loss of function variant. After functional characterizations of the pathological mechanisms of the T292S and T292I mutations, we also tried to find potential therapeutics for more effective and personalized treatments. Based on the detailed functional phenotypes of the two variants, we did a quick screening of some clinically approved drugs that directly or indirectly act on GABAARs, and thereby reducing the functional abnormalities of certain mutant variants of GABAARs. Thiocolchicoside (TCC) is used in the clinic as a classic muscle relaxant, but it is also an allosteric GABAAR inhibitor that shows potent antagonistic effects against GABAAR (Carta et al., 2006). In our experiments, 1 μM TCC could fully restore the increased GABA-evoked dose response of the T292S variant to WT level, with superior effects over other GABAAR negative allosteric modulators, bemegride and flumazenil. Our
study, along with others, suggest that these drugs can be a potential therapeutic option for the gain of function GABA\textsubscript{A}R variants (Butler et al., 2018; Steudle et al., 2020).

In contrast, we found that the loss of function variant T292I GABA\textsubscript{A}R-gated currents could be partially restored by a combination of two positive GABA\textsubscript{A}R modulating drugs, diazepam and verapamil. Chronic treatment of verapamil was shown to enhance channel gating with elongated open time and increased open probability of R214C GABA\textsubscript{A}R, suggesting diazepam and verapamil may work synergistically to improve the loss of function caused by T292I variant GABA\textsubscript{A}Rs. Our studies demonstrated that these mutations have opposite impacts on the function of GABA\textsubscript{A}Rs although occurring at the same residue and thereby require different functional and pharmacological strategies to restore their function to the level of wild-type. Therefore, it is of paramount importance to perform functional and pharmacological analysis after exome sequencing to determine the pathological mechanisms precisely. This would help precision medicine via searching for the appropriate therapeutic options for patients carrying de novo mutations of GABA\textsubscript{A}Rs.

There are around 30\% of children with refractory epilepsy that do not respond to conventional drug treatments due to unknown functional alterations or unidentified causes (Geffrey et al., 2015; Sills and Rogawski, 2020). Traditional anti-seizure drugs show little to no effect toward rescuing deficits caused by specific mutations in GABA\textsubscript{A}R subunits (Greenfield, 2013; Löcher et al., 2020), and in certain cases can even exacerbate the symptoms (Absalom et al., 2020; Billakota et al., 2020). The pharmacological characterizations in our study have provided differential therapeutic advice for managing these two patients and endorsed the importance of precision medicine for treating rare variants of GABA\textsubscript{A}Rs.
However, there are some limitations of these allosteric modulators in future applications. As for Ndam830, at its optimal working concentration of 10 μM, Ndam830 inhibits the GABA\textsubscript{A}R by around 20% which raises the possibility of disturbing the E/I balance and altering the excitability of the network in the brain. Moreover, when compared with most clinically tested drugs that are potent in the nanomolar range, Ndam830 has relatively low potency. But, the solution to this could be the reformulation of Ndam830 with nanoparticles which harbors the characteristics of low toxicity, high loading capacity, excellent biocompatibility, appropriate colloidal stability, ability to target specific tissues, protection of agents from degradation and fast clearance during circulation (Bahreyni et al., 2023). These features would to some extent compensate the relatively low potency of Ndam830. The TCC is a negative allosteric modulator of GABA\textsubscript{A}Rs could be used to rescue the rare variants that causes gain-of-function of the receptor. But, the dosing of TCC that can be applied on real patients carrying rare variants may differ very much from the dose used as a muscle relaxant. Therefore, treating gain-of-function of GABA\textsubscript{A}Rs may necessitate a CFS pharmacokinetic study to find out the optimal dose.

Taken together, the allosteric modulators Ndam830 and Ndam844 tailored for NMDAR-related disorders or TCC, diazepam and verapamil tailored for rare variants of GABA\textsubscript{A}Rs have shown great potential for future clinical therapeutic applications.
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Appendix: NMR and mass spectroscopy of synthesized Ndcm830.