FUNCTIONAL CHARACTERIZATION OF A NOVEL NMDA RECEPTOR POSITIVE ALLOSTERIC MODULATOR

by

Victor Li

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Abstract

The NMDA receptor is a glutamatergic ionotropic receptor key in mediating neuronal plasticity across virtually all synaptic circuits in the brain. An increasing list of neurological disorders have implicated NMDA receptor hypofunction as an integral part of pathogenesis, necessitating the production of NMDA receptor potentiators as therapeutics. To date, most of these attempts have used increased co-agonism at the glycine binding site of NMDA receptors, but this strategy has been plagued by low specificity and efficacy. Specific allosteric modulation of NMDA receptors is an ideal solution, but until recently, no known drugs were capable of doing so. Building off previous work in our lab that discovered a novel family of compounds capable of modulating NMDA receptor activity through its apical N-terminal domain, we identified and characterized a drug candidate, Npam59, predicted to potentiate both GluN2A- and 2B-containing NMDA receptors. Npam59 was shown to potentiate NMDA currents mediated by both subtypes with EC50 in the low-micromolar range. Npam59 also potentiated d-amphetamine-induced dopamine release in the ventral striatum in an NMDA receptor-dependent manner, but had no observable effect when administered alone. Finally, Npam59 potentiated d-amphetamine-induced hyperlocomotion in Sprague-Dawley rats. These results demonstrate that Npam59 can potentiate the function of NMDA receptors, including both GluN2A- and 2B-containing ones, suggesting its potential as a research tool and drug candidate for further development.

Npam59 is the first known NMDA receptor allosteric potentiator with specificity for both GluN2A and GluN2B. Its characterization provides the foundation for therapeutic development and novel insights into the interaction of dopamine-glutamate signaling in the ventral striatum.

Lay Summary

The NMDA receptor is a type of ion-conducting glutamate receptor with characteristics that allow them to serve crucial functions in adaptability of the synapse. An increasing list of neurological disorders, such as schizophrenia, have had NMDA receptor hypofunction identified as a major contributing factor. Rescuing NMDA receptor hypofunction would theoretically act as a viable therapeutic strategy for these disorders, but existing drugs have problems with target specificity. This thesis characterizes a novel drug, Npam59, which is capable of potentiating NMDA receptors at multiple levels of brain function. It was demonstrated that Npam59 could allosterically potentiate NMDA receptors with consequences seen in neurochemistry and behavior. This work provides the foundation for future development of Npam59 as a potential therapeutic for NMDA hypofunction disorders.

Preface

All figures were reprinted with permission. Citations are contained in figure legends. A version of Chapters 3-5 will be assembled into a manuscript for submission.

This thesis is the product of a collaborative effort. My study design and overall direction were developed under the supervision of Dr. Yu Tian Wang (YTW) and Dr. Anthony Phillips (AP). Electrophysiological experiments were performed by Yang Ge, Serena Boccella, Dr. Peter Axerio-Cilies (PAC) and me. Screening data on Npam candidate compounds were taken from previous work by PAC. I conducted all data analysis and figure construction.

In the late stage of my thesis research, due to development of life-threatening anaphylaxis towards rats over the course of my Ph.D., I refrained from direct contact with animals for most experiments contained in this thesis. I designed the *in vivo* experiments in consultation with AP and YTW, prepared drug treatments, and oversaw the experiments as they were carried out. Cannulation surgeries for micro-dialysis experiments were performed by Giada Vacca (GV) and Haiyan Zou (HZ). Animal handling and injection of treatments were conducted by HZ in micro-dialysis and locomotor studies. High performance liquid chromatography measurements of micro-dialysis samples or cerebrospinal fluid and serum was conducted by GV, PAC, and Soyon Ahn (SA). Collection of rat cerebrospinal fluid was conducted by Camille Potey. All data analysis and figure construction for *in vivo* experiments were performed by me in consultation with SA. The figure in Chapter 6 was kindly illustrated by Jennifer Ji.

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 A13-0230.

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AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
NMDA	N-methyl-D-aspartate
GABA	γ-Aminobutyric acid
CNS	Central nervous system
BBB	Blood-brain barrier
APV	(*2R*)-amino-5-phosphonovaleric acid
TTX	Tetrodotoxin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DA	Dopamine
Glu	Glutamate
DOPAC	3,4-Dihydroxyphenylacetic acid
HVA	Homovanillic acid
5-HIAA	5-Hydroxyindoleacetic acid
LTP	Long-term potentiation
LTD	Long-term depression
NTD	N-terminal domain
LBD	Ligand-binding domain
Npam	NMDA positive allosteric modulator
HEK293	Human embryonic kidney 293 cells
DMSO	Dimethyl sulfoxide
CREB	cAMP response element binding protein

pCREB	Phosphorylated cAMP response element binding protein
IF	Ifenprodil
NVP	NVP-AAM077 PEAQX tetrasodium hydrate
MK-801	Dizocilpine, also (5S,0R)-(+)-5-Methyl-0,-dihydro-5H-dibenzoa,d-cyclohepten-5,0-imine maleate
CSF	Cerebrospinal fluid
aCSF	artificial cerebrospinal fluid
EC50	Concentration that gives half-maximal response; Effective concentration
i.p.	Intraperitoneal
i.v.	Intravenous
NAc	Nucleus accumbens
VTA	Ventral tegmental area
PFC	Prefrontal cortex
MSN	Medium spiny neuron
Amph	amphetamine
d-Amph	dextroamphetamine
NO	Nitric oxide
nNOS	Neural nitrous oxide synthase
Apaf-1	Apoptotic protease activating factor 1
EndoG	Endonuclease G
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
SSRI	Selective serotonin reuptake inhibitor
SNRI	Serotonin-norepinephrine reuptake inhibitor

TCA	Tricyclic antidepressant
MAOI	Monoamine oxidase inhibitor
PCP	Phencyclidine
MAO	Monoamine oxidase
TH	Tyrosine hydroxylase

Acknowledgements

I am very grateful to have had an inspiring and supportive circle of friends and colleagues who have supported me along this journey. Foremost of these are my supervisors Dr. Yu Tian Wang and Dr. Tony Phillips, who have been invaluable for their advice, insights, and direction. Their unwavering encouragement and support have helped me immeasurably through my (many) setbacks. I thank Dr. Lynn Raymond and Dr. Weihong Song for their insights and support as excellent members of my supervisory committee.

I'd like to thank Dr. Peter Axerio-Cilies, of course, for discovering the compounds which led to the conception of my thesis. He has also been a fantastic collaborator who is infinitely patient and helpful in guiding me through the ropes and lending a helping hand. I also thank Yang Ge for his help in gathering some of the electrophysiology data in this thesis and Serena Boccella for providing the early groundwork in the discovery and characterization of Npam59. I am grateful to Dr. Soyon Ahn for her guiding expertise in micro-dialysis and being a knowledgeable and approachable mentor. I also thank Giada Vacca, Haiyan Zou, and Gemma Floresco for being patient, helpful, and willing when I was restricted from animal work for health reasons. I thank Dr. Jie Lu, Dr. Lidong Liu, Dr. Camille Potey, Dr. Steven Connor, and Yuping Li for help inside and outside of the lab with all matter of miscellaneous things.

Lastly, I thank my parents for being my shelter and support over the last five years and Jennifer Ji, for her unwavering support

Dedication

To my parents, who have guided and believed in me every step of the way.

Chapter 1: Introduction

1.1 Overall aims

N-methyl D-aspartate (NMDA) receptors are ionotropic glutamatergic receptors key in regulating normal brain functions. These receptors have critical roles in mediating synaptic plasticity, learning, and memory, and are implicated in a number of neurological disorders (Morris, 1989; Aroniadou and Teyler, 1991; Olney et al., 1999; Berman et al., 2000; Newcomer et al., 2000; Koutsilieri and Riederer, 2007). Previous work in our lab has characterized a family of NMDA receptor positive allosteric modulator (Npam) compounds which resulted in the development of a prototype drug which can increase the function of the GluN2A-containing subtype NMDA receptor (Axerio-Cilies, 2016). This earlier work also described a separate compound that appeared capable of potentiating GluN2A-containing and GluN2B-containing NMDA receptors and the primary aim of the present thesis is to examine using both *in vitro* and *in vivo* techniques whether this compound does indeed act as an Npam at *both* the GluN2A and GluN2B subunits of the NMDA receptor. Starting from the most basic, cellular level, we will progress to neurochemistry in select regions of the intact brain, and finally conclude by studying the effect of the compound on behavior, proving the effectiveness of our drug and provide indications into how it might best be further applied. Dual potentiation would in theory offer new possibilities for treating disorders involving hypo-functioning NMDA receptors such as schizophrenia and Alzheimer's disease, or for application as a cognitive enhancer.

1.2 Overview of NMDA receptor structure and function

NMDA receptors are formed from four heteromeric subunits, including two obligatory GluN1 subunits, and two of either GluN2 or GluN3 subunits. Each GluN1 subunit has a binding

site for glycine (Bonhaus et al., 1987), and each GluN2 subunit contains a binding site for glutamate (Laube et al., 1997; Anson et al., 1998). For GluN2-containing NMDA receptors, activation requires occupancy of both glycine and glutamate binding sites, which results in influx of calcium and sodium, and efflux of potassium, depolarizing the cell (Voglis and Tavernarakis, 2006).

The GluN2 subunit family consists of GluN2A, GluN2B, GluN2C, and GluN2D, with each conferring a distinct expression and electrophysiological profile to the complete receptor complex (Seeburg, 1993; Bliss and Schoepfer, 2004). Compared to other ionotropic receptors, NMDA receptors act over much longer timespans. Other glutamatergic ionotropic receptors, such as the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor, conduct a current with a rise time of 0.2-0.4ms and inactivate after 2ms, whereas NMDA receptors show a rise time of 10-50ms and inactivate after longer intervals—50-500ms (Wyllie et al., 2013). A comparison of the GluN2 subtypes confirms that GluN2A-containing receptors inactivate the quickest, at 100ms following a 1ms stimulation by agonist. GluN2B and GluN2C inactivate slower, at 250ms, and GluN2D receptors are slowest, at 4000ms (Blanke and VanDongen, 2009; Wyllie et al., 2013). The long duration of NMDA receptor currents allows them to temporally integrate excitatory inputs, and thereby play a role in detection of synchronous firing (Dudman et al., 2007; Paoletti et al., 2013). There is also differential expression for these receptor subunits. Whereas GluN1 subunits are expressed globally in all NMDA receptors throughout the central nervous system (Monyer et al., 1994), GluN2 subunits have different regional specificity. GluN2A and GluN2B subunits are widely expressed in cortical and hippocampal neurons (Ewald and Cline, 2009) as well as in the striatum (Fantin et al., 2007), while GluN2C and GluN2D are

expressed highly in the cerebellum, striatum, and midbrain (Akazawa et al., 1994; Monyer et al., 1994; Watanabe et al., 1994).

A key unique feature of NMDA receptors is the additional presence of a physiological voltage-dependent blockade by magnesium ions in the central pore, which inhibits the channel activity regardless of the absence or presence of an agonist (Sacaan and Johnson, 1991; Calabresi et al., 1992; Voglis and Tavernarakis, 2006). During sufficient membrane depolarization, magnesium ions are electrostatically repelled from the channel complex, unblocking the NMDA receptors and allowing them to become activated by glutamate. In this manner, NMDA receptors can detect coincidence between depolarization and glutamatergic stimulation, and convey that such an event has occurred by conducting intracellular calcium entry. This consequence of NMDA receptor activation is the critical phenomenon that enables neuronal plasticity (Aroniadou and Teyler, 1991; Debanne, 1996; Shipton and Paulsen, 2014).

GluN3 subunits do not contain a glutamate binding site, nor are they sensitive to magnesium blockade (Henson et al., 2010). They are activated by glycine alone to mediate inward cationic currents that are calcium impermeable and unresponsive to canonical NMDA receptor antagonists (Henson et al., 2010). While difficult to study given the absence of GluN3-specific antagonists, it is thought that they may play a more developmental role, or provide a possible additional inhibitory component in tri-heteromeric NMDA receptors (Pachernegg et al., 2012; Kehoe et al., 2013). Due to their dramatically different function compared to GluN2-containing receptors, we will not examine these receptors further in the present work.

The function of NMDA receptors is also dependent on its subcellular localization. NMDA receptors are found in both the synaptic and extra-synaptic membranes in the post-

synaptic neuron with a segregation pattern that is subtype-specific. GluN2A-containing receptors preferentially localize to the synapse, and GluN2B-containing receptors localize primarily to extra-synaptic space (Perez-Otano and Ehlers, 2005; Lai et al., 2014), but with imperfect segregation allowing for a slightly heterogenous population of NMDA receptors found in both locales (Petralia et al., 2005; Thomas et al., 2006). The spatial to subtype correlation in NMDA receptors is purported to allow differential responding between contained glutamate release in the synapse and spill-over glutamate signaling outside the synapse. Synaptic GluN2A-containing receptors initiate calcium-dependent signaling that result in long-term potentiation (LTP) while activation of GluN2B-containing receptors inside or outside the synapse mediates formation of long-term depression (LTD) (Sakimura et al., 1995; Hrabetova et al., 2000; Liu et al., 2004; Massey et al., 2004; Berberich et al., 2007; Zhao and Constantine-Paton, 2007; Gardoni et al., 2009), although it is increasingly recognized that GluN2B-containing receptors may also mediate LTP (Berberich et al., 2005; Miwa et al., 2008). Additionally, GluN2A-containing receptors have been shown to promote cell survival (Lujan et al., 2012; Hu et al., 2016), whereas GluN2B-containing receptors have been shown to participate in processes linked to cell death (Martel et al., 2009; Lujan et al., 2012; Vizi et al., 2013), especially in the context of excitotoxicity, as occurs during stroke, seizures, or similar pathologies of excessive excitation (Lai et al., 2014; Li and Wang, 2016).

1.3 Downstream signaling cascades mediated by NMDA receptors

NMDA receptor activation results in the immediate influx of a high local concentration of calcium, with kinetics determined by the subtype of the receptor's GluN2 subunit, as described above. Additionally, the c-terminal region of GluN2A and GluN2B subunits are binding partners to various synaptic and non-synaptic proteins, which gives specificity between

the stimulation received, the receptor subtype activated, and the resulting intracellular signal, as is detailed below.

1.3.1 Activation of GluN2A-containing receptors

GluN2A-containing NMDA receptors are primarily found at the synaptic cleft alongside ionotropic glutamatergic αAMPA receptors, the primary mediator of excitatory signaling in the brain. Once magnesium blockade is removed by a concurrent depolarization, glutamatergic stimulation allows calcium entry into the cell, activating numerous intracellular targets. Briefly, following high frequency stimulation, calcium transients from synaptic GluN2A-containing receptors initiate several calcium-sensitive cascades, of which calmodulin kinase II (CaMKII) is of key importance (Malenka and Nicoll, 1999). These pathways result in increased membrane insertion of AMPA receptors by phosphorylation via modulation of protein phosphatase 1 (PP1) and direct phosphorylation by activated CaMKII (Malenka and Nicoll, 1999). The changes are upheld via protein synthesis beyond the first few hours, and eventually local cytoskeletal remodeling causes the dendritic spine to expand as well (Kauer and Malenka, 2007). Together, these processes create a long-lasting increase in sensitivity from a given synaptic input, potentiating the synapse's future contributions to the firing probability of the post-synaptic neuron in a phenomenon known as LTP (Lu et al., 2001). Functionally, LTP is thought to be crucial in almost all learning and plasticity processes in the brain.

Beyond synaptic plasticity, GluN2A-containing receptors also have an important role in promoting cell survival. The calcium transient from receptor activation, through pathways involving CaMKII, Ras, and protein kinase A, also eventually leads to the phosphorylation and activation of cAMP response-element binding protein (CREB) (Liu et al., 2007; Hardingham,

2009). CREB and its binding partner CREB binding protein (CBP), are key mediator of neuron survival signaling and another important downstream target of GluN2A activation (Liu et al., 2007; Hardingham, 2009). (Figure 1.1)

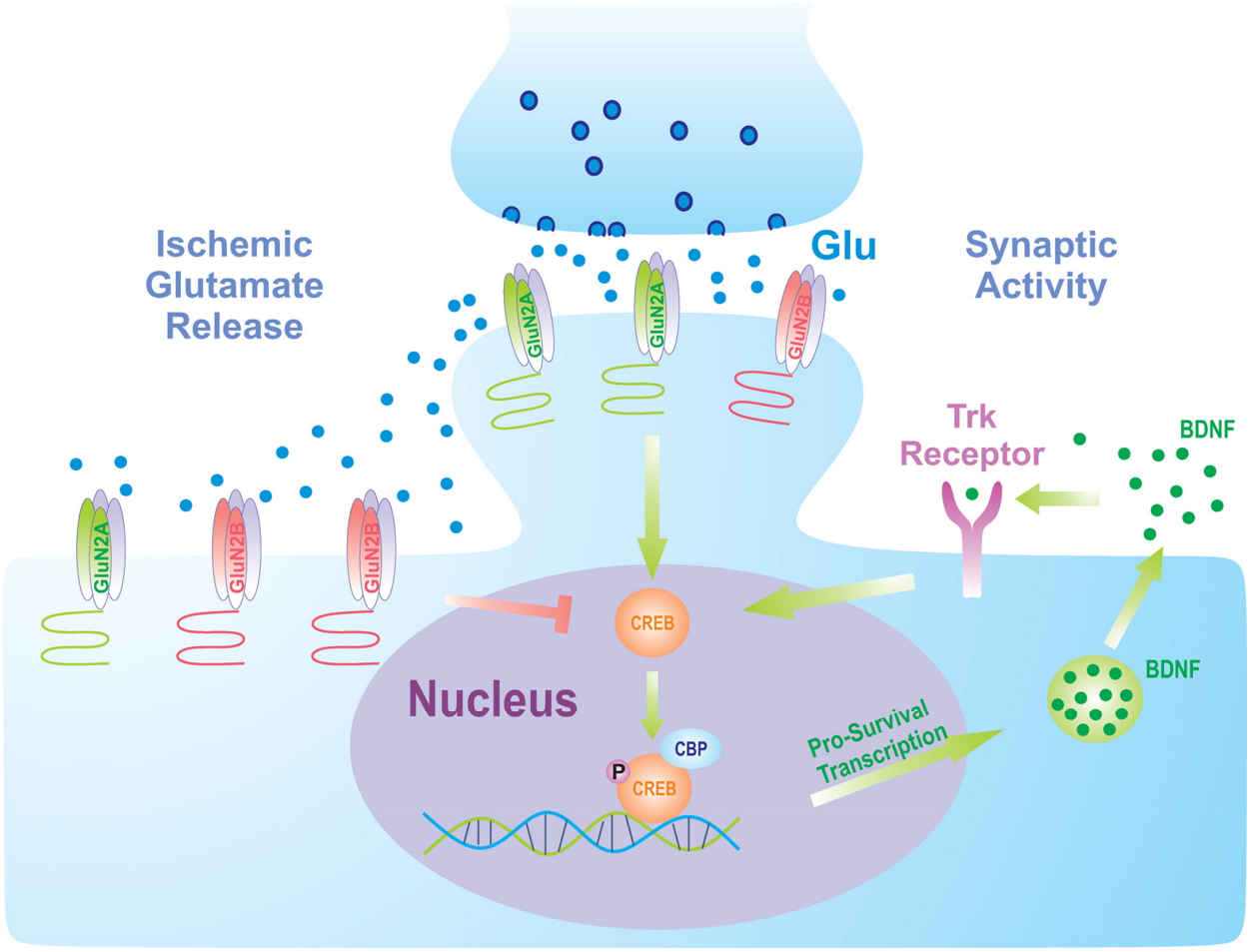

Figure 1.1 Illustration of cell survival pathways following GluN2A activation.

GluN2A-containing NMDA receptors mediate signaling resulting in activation of CREB and survival genes under its control. This pathway can be inhibited following extra-synaptic

activation of GluN2B-containing receptors in excitotoxicity. (Reproduced from Lai et al., 2014 with permission)

1.3.2 Activation of GluN2B-containing receptors

GluN2B-containing receptors are primarily extra-synaptic in localization, but are also present on postsynaptic membranes. Low frequency stimulation of glutamate produces LTD (Lu et al., 2001; Liu et al., 2004), a phenomenon diametrically opposed to LTP. Whereas LTP involves increasing dendritic spine size and increased AMPA receptor membrane insertion, LTD causes the reverse, reducing dendritic spine size and AMPA receptor surface expression. The opposing mechanism is thought to be caused by lower calcium fluxes than those that enable LTP, which act through calcineurin to dephosphorylate AMPA receptors and cause their endocytosis through clathrin-dependent mechanisms (Kauer and Malenka, 2007). Additionally, specificity of GluN2B subunits for LTD may be due to differential effects on the Ras/ERK signaling pathway. Whereas GluN2A-containing receptors promote activation of Ras via calcium influx, GluN2B-containing receptors block the activation of Ras through the action of synaptic Ras/Rap GTPase activating protein, which selectively associates with the c terminal tails of GluN2B and not GluN2A (Kim et al., 2005). Behaviorally, LTD is responsible for memory consolidation (Ge et al., 2010) and behavioral flexibility (Nicholls et al., 2008; Dong et al., 2013), at least for spatial memory and navigation.

Physiological release of glutamate is highly localized in the synapse, which is tightly controlled by uptake via EAAT1 and EAAT2 on nearby glial cell processes to limit excitotoxic

signaling at normal conditions (Herman and Jahr, 2007; Zhou and Danbolt, 2013). As GluN2B-containing receptors are localized mainly to the extra-synaptic regions, it is thought that these non-synaptic receptors initiate cell death signaling only during pathological events such as excitotoxicity, and play a minimal, or at least nonlethal role in normal function (Lai et al., 2014). During ischemia or hypoxic stress, status epilepticus, or physical trauma, glutamate release can far exceed the system's limits for containment and thereby can activate extra-synaptic NMDA receptors. Should the stressor be insufficient to cause immediate cell death through necrotic mechanisms (Nicholls, 2009), large-scale activation of GluN2B receptors could still result in excessive calcium influx and precipitation of delayed death pathways. Initially, calcium entry depolarizes the mitochondria, inhibiting ATP production and contributing to cellular energy failure (Nicholls, 2009; Cheng et al., 2012). Cytochrome c is also released, initiating caspase-dependent death via formation of the apoptosome with apoptotic protease activating factor 1 (Apaf-1) and subsequent activation of caspase 9 (Tait and Green, 2013). Other caspase-independent process may also be involved, including activation of Endonuclease G by BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) following mitochondrial leakage (Zhang et al., 2007), or overproduction of nitric oxide (NO) from neural nitric oxide synthase (nNOS) coupled to GluN2B c-tail via PSD95 (Figure 1.2) (Aarts et al., 2002).

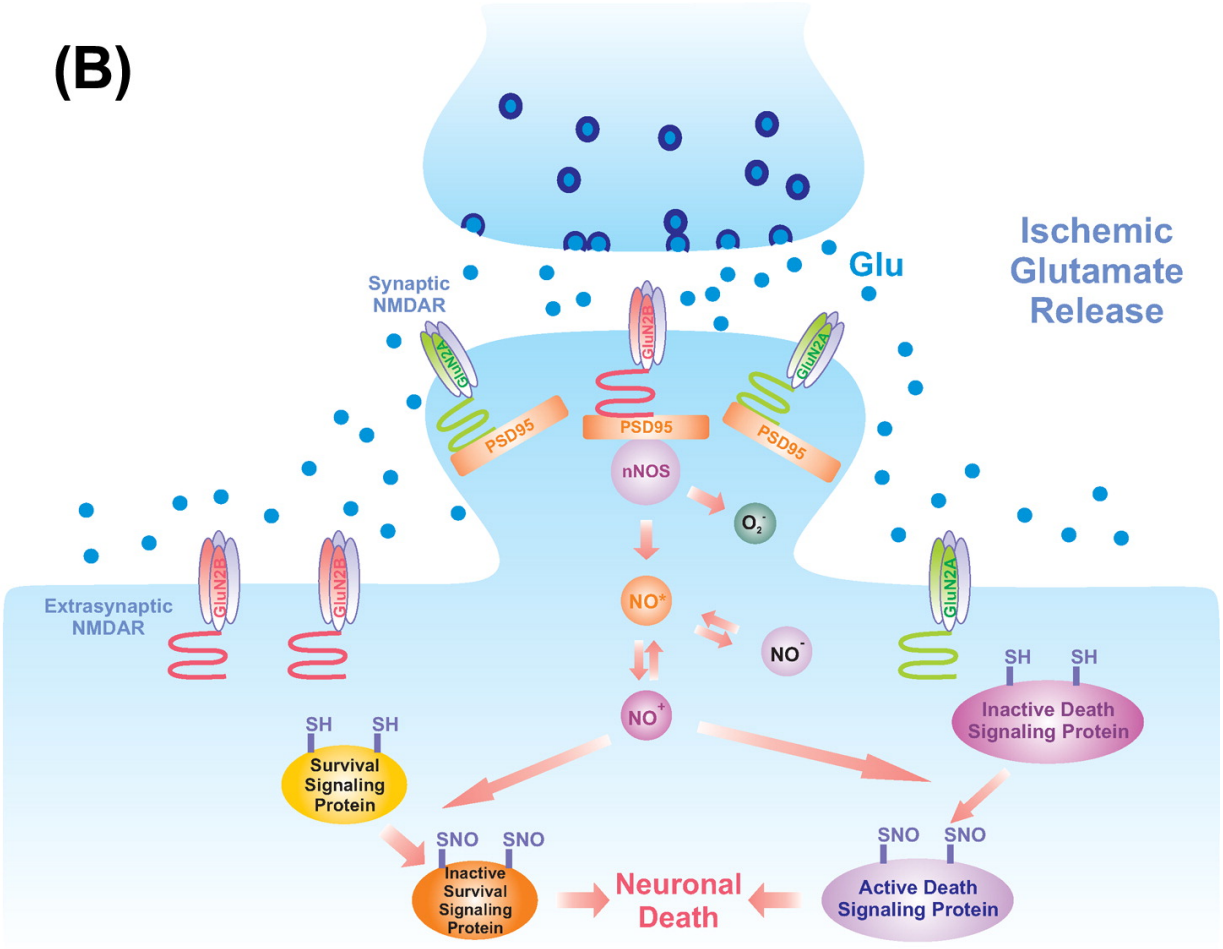

Figure 1.2 Illustration of cell death pathways mediated by nNOS.

In excitotoxic conditions, nNOS complexed to GluN2B receptors is activated by calcium to produce a range of reactive oxygen species including NO, causing cell death via intracellular damage. (Reproduced from Lai et al., 2014 with permission)

1.4 NMDA receptors in diseases

NMDA receptors have been implicated in the neurodegeneration of different diseases, including Alzheimer's disease, Huntington's disease, and Parkinson's disease (Parsons and Raymond, 2014). Their dysfunction is also contributory to other brain disorders, such as in depression, schizophrenia, and ADHD. As such, the NMDA receptor is a popular target in disease modeling and in the development of new therapeutic strategies.

1.5 NMDA hyperfunction disorders and NMDA receptor antagonism as a therapeutic strategy

1.5.1 Excitotoxicity

Numerous diseases have been linked to excitotoxicity through overactivation of GluN2B-containing NMDA receptors as a driving mechanism. Ischemic stroke (Tai et al., 2001; Montoliu et al., 2002; Planells-Cases et al., 2002), epilepsy (Smolders et al., 1997; Pelletier et al., 1999; Dominguez et al., 2006), Huntington's disease (Beal, 1994; Taylor-Robinson et al., 1994; Zeron et al., 2002), Parkinson's disease (Beal, 1998; Rodriguez et al., 1998), late stage Alzheimer's disease (Harkany et al., 2000; Molinuevo et al., 2005) all have strong evidence implicating excitotoxicity as a significant contributor to cell death. Strategies to block excitotoxicity include targeting the NMDA receptor directly with global, pan-NMDA channel blockers such as Selfotel and dizocilpine (MK-801) in stroke, which have narrow therapeutic indices (Wood, 2005; Lai et al., 2014), and glycine binding site antagonists such as licostinel and gavestinel, which looked promising until their clinical trials failed due to low efficacy (Woodward et al., 1995; Bordi et al., 1997). While NMDA receptor antagonism was general failure in stroke, blockade of NMDA receptors showed benefit in chronic degenerative diseases. Memantine, a noncompetitive NMDA

receptor antagonist, showed benefits in Alzheimer's disease (Kornhuber et al., 1994; Danysz and Parsons, 2003; Lipton, 2004) and has seen widespread clinical use since its introduction in the early 2000s. Its use prophylactically has also shown some promise in an animal model of stroke (Trotman et al., 2015), although it has yet to be shown if memantine prophylaxis is effective in stroke in humans.

More successful approaches were made by moving away from antagonism at the NMDA receptor. For example, a peptide-based approach using the Tat-NR2B9c peptide demonstrated substantial neuroprotection by preventing the coupling of nNOS to the GluN2B C-terminal tail, disrupting the endogenous death signaling cascade that results from calcium-activated overproduction of NO (Aarts et al., 2002) (Figure 1.2).

1.5.2 Major depressive disorder

Major depressive disorder, or simply depression, is a mood disorder characterized by low mood, decreased interest in most activities, anhedonia, low energy, poor concentration, and suicidal ideation (APA, 2013). While also classically understood as a disorder of serotonin and norepinephrine hypofunction, increasing evidence is implicating NMDA receptors either as a key player in pathophysiology, or at least as a viable therapeutic target due to observations that sub-anesthetic doses of ketamine have immediate and long-acting effects as an antidepressant (Berman et al., 2000)

First-line treatments for depression include selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), which work via blockade of serotonin and/or norepinephrine reuptake. Other drugs include tricyclic antidepressants (TCAs), the prototypical antidepressant with similar mechanism to SSRIs, monoamine oxidase inhibitors

(MAOIs), which decrease metabolism of all monoamine transmitters (dopamine, serotonin, norepinephrine), and more recently and experimentally, cannabinoids. A limitation for each of these treatments is a delay of several weeks between the initiation of treatment and an improvement in symptoms, sometimes leading to an increased burst of energy and motivation without mood improvement which is thought to be just sufficient for patients with major depression to commit suicide, although data surrounding this is still controversial (Bridge et al., 2007; Sharma et al., 2016). Recent research efforts have turned to the NMDA receptor to find treatments with no lag in effect, following the discovery that a single treatment with ketamine has immediate and sustained antidepressant effects (Berman et al., 2000) which are mediated by NMDA receptor antagonism (Maeng et al., 2008). Furthermore, blockade of NMDA receptors by MK-801 or Ro25-6981 also had antidepressant effects, but these were not sustained as long as those of ketamine (Maeng et al., 2008). While new evidence has identified some metabolites of ketamine as responsible for the long duration of its antidepressant effect (Zanos et al., 2016), it is still recognized that antagonism of NMDA receptors is important to initiate changes such as synaptic spine density and AMPA receptor expression that anticorrelate depressive symptoms (Aleksandrova et al., 2017).

1.6 NMDA receptor hypofunction disorders

1.6.1 Schizophrenia

NMDA hypofunction was first proposed as a mechanism behind the neurochemical changes observed in schizophrenia in the 1990s (Carlsson and Carlsson, 1990; Javitt and Zukin, 1991). Classically, schizophrenia has been described as a pathology of the dopamine signaling systems. The link of schizophrenia to dopamine was made following observations that anti-

dopaminergic drugs were anti-psychotic (Carlsson, 1978), and that antipsychotics increased the metabolites of dopamine when administered *in vivo* (Carlsson and Lindqvist, 1963). Additionally, it was noted that amphetamine was able to greatly increase mesolimbic dopamine efflux, and that amphetamine-induced psychosis included features of auditory hallucinations and persecutory delusions which are similar to the positive symptoms of schizophrenia (Angrist and Gershon, 1970; Griffith, 1970; Snyder, 1972). These, and other corroborating findings supported the hypothesis that hyperdopaminergia was at the core of the disease pathogenesis, and drove efforts into the design of antipsychotics such as haloperidol and other typical antipsychotic drugs, which act as D2 dopamine receptor antagonists. However, this original hypothesis did not account for the dimension of negative symptoms or cognitive symptoms in schizophrenia, nor did it address why the contemporary antipsychotics were ineffective at treating negative symptoms, such as blunted emotion, apathy, and poor social function, or the cognitive impairments (Crow, 1980). By the 1990's, more evidence had arisen, which had started to point to regional pathologies of dopamine, rather than a global dopamine excess (Davis et al., 1991). It was proposed that the negative symptoms were due to a hypofrontality, or diminished dopamine function in the frontal cortical regions to explain negative symptoms, and a coexisting hyperdopaminergia specifically in the mesolimbic pathway (Davis et al., 1991).

Subsequently, Arvid Carlsson and colleagues advanced the hypothesis that glutamate function was also compromised in schizophrenia and suggested that NMDA may play a key role in modulating dopamine function in different brain regions (Carlsson and Carlsson, 1990; Carlsson et al., 2004). Further work with NMDA receptor antagonists such as phencyclidine (PCP) (Javitt and Zukin, 1991) or ketamine (Lahti et al., 2001) showed that they could cause symptoms mimicking both the positive and negative symptoms of schizophrenia in healthy

volunteers, and exacerbate symptoms in schizophrenics. Importantly, positive modulation of NMDA receptor function using glycine or glycine reuptake inhibitors was therapeutic (Choi et al., 2013). Several genes linked to schizophrenia also had effects on NMDA receptor function (Coyle, 2006; Kehrer et al., 2008). The model identified NMDA receptors on γ-aminobutyric acid (GABA)ergic interneurons (Lisman et al., 2008), which through neural circuits, could have a bidirectional influence on dopaminergic tone.

Briefly, dopamine neurons in the midbrain project to various targets throughout the brain, and are themselves under control from inputs from numerous regions, including excitatory inputs from cortical pyramidal neurons. The cortical inputs are in turn modulated in large part by GABAergic interneurons expressing NMDA receptors (Lisman et al., 2008; Schwartz et al., 2012). Blockade of these receptors, or their hypofunction in a disease state could reduce inhibition in the cortex, resulting in greater glutamatergic stimulation into the midbrain. Increased activity in these projections which may terminate directly on dopaminergic neurons could lead to greater dopamine release (Figure 1.3); alternatively, modulation of inhibitory interneurons in the midbrain could provide a separate mechanism of changing dopamine release in other regions of the forebrain (Figure 1.4). The net effect of these changes could include a potentiation of dopamine release in the mesolimbic pathway to the ventral striatum, causing positive symptoms, and a deficit of dopamine release in the mesocortical pathway to the prefrontal cortex, causing negative and cognitive symptoms (Schwartz et al., 2012).

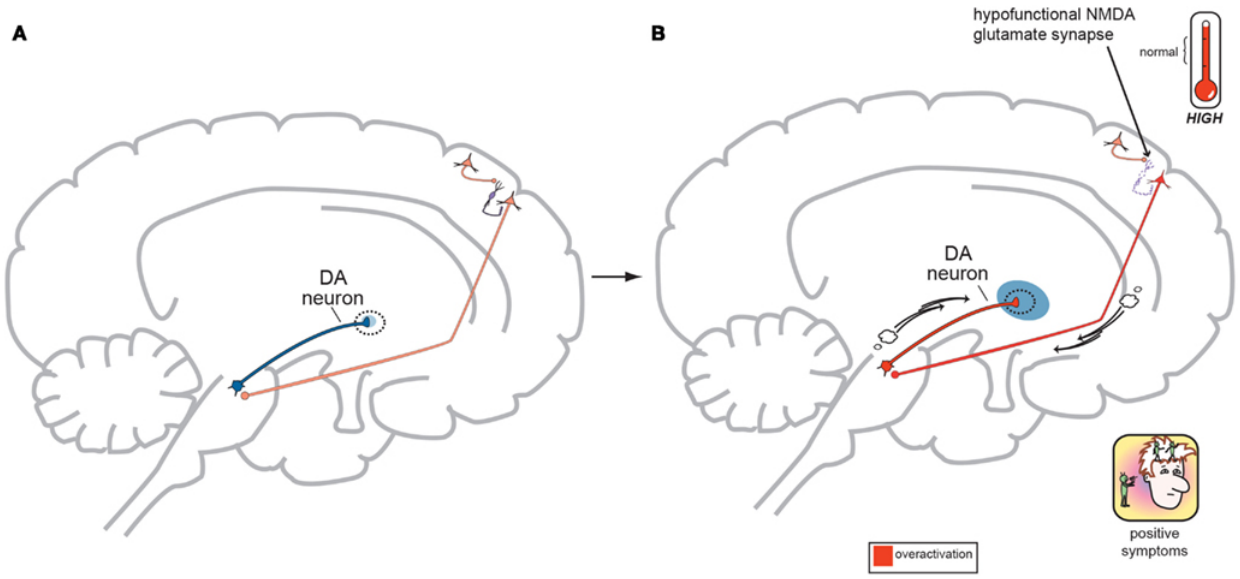

Figure 1.3 NMDA receptor hypofunction leads to increased dopamine release in the striatum.

NMDA receptor hypofunction on cortical GABAergic neurons through a polysynaptic pathway cause a reduction in dopamine release to the prefrontal cortex. This is thought to be the underpinning of schizophrenia cognitive and negative symptoms. (Stahl, 2008)

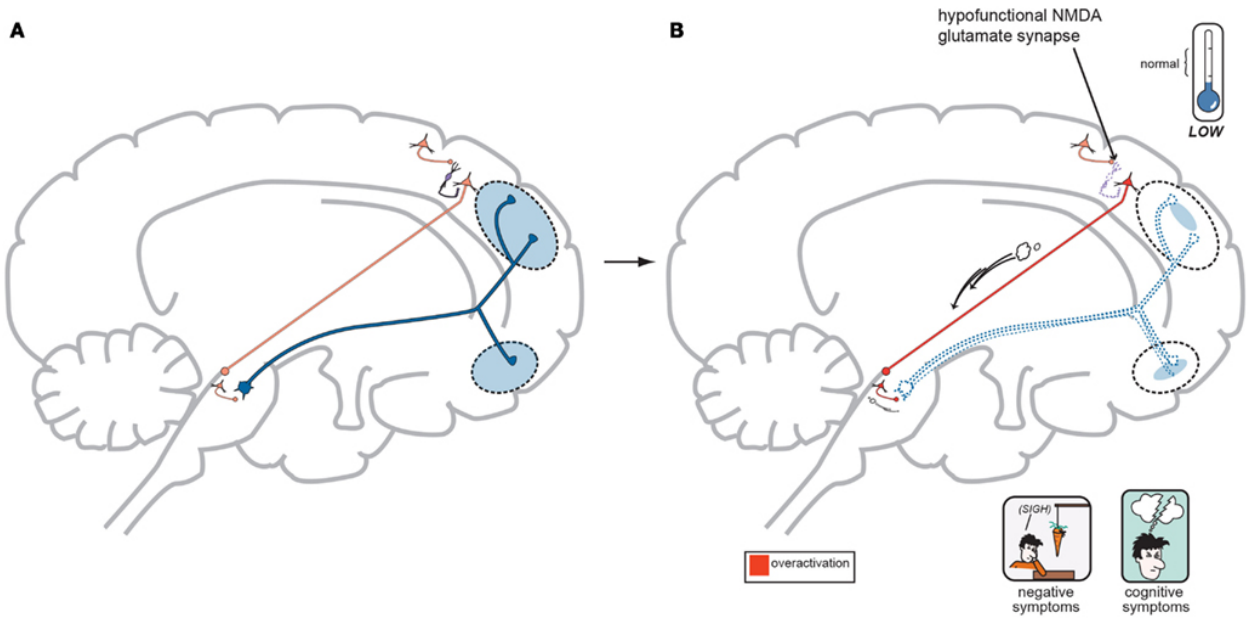

Figure 1.4 NMDA receptor hypofunction leads to reduction of dopamine release in the prefrontal cortex.

NMDA receptor hypofunction on cortical GABAergic neurons through a polysynaptic pathway cause a reduction in dopamine release to the prefrontal cortex. This is thought to be the underpinning of schizophrenia cognitive and negative symptoms. (Stahl, 2008)

1.6.2 Major depressive disorder

While the prevailing hypothesis is that NMDA antagonism rescues depression, the story is complicated by reports which suggest allosteric potentiation of NMDA receptors has an antidepressant effect. Recent clinical trials demonstrated greater benefit from sarcosine, a glycine type 1 transporter (GLYT-1) blocker that potentiates NMDA receptors via increasing glycine co-activation, compared to citalopram over a 6-week period on depressed patients (Huang et al.,

2013). This finding corroborates observations that NMDA receptor potentiation by D-serine and GLYT-1 antagonists have anxiolytic and antidepressant effects in animal models (Depoortere et al., 2005; Malkesman et al., 2012) through an mTOR-mediated pathway that increases surface expression of AMPA receptors (Chen et al., 2015; Chen et al., 2017). *In vivo*, NMDA receptor activation can also activate mTOR (Gong et al., 2006), consistent with the observation that increasing NMDA signaling can rescue depression. Ketamine also activates mTOR signaling as a crucial component of its antidepressant mechanism (Welberg, 2010; Hashimoto, 2011; Akinfiresoye and Tizabi, 2013; Yang et al., 2013; Zhou et al., 2014), perhaps by an NO-dependent mechanism secondary to NMDA receptor antagonism (Harraz et al., 2016). It is unclear how these findings might be reconciled, but it indicates that additional study is necessary to test if depression may also be responsive to NMDA receptor potentiation.

1.6.3 Attention deficit hyperactivity disorder

Attention deficit hyperactivity disorder (ADHD) is a disorder commonly diagnosed in childhood characterized by motor hyperactivity, inattention, and impulsivity (APA, 2013). It can affect student performance and development in school as well as interfere with the patient's interpersonal relationships with both caregivers and peers. The neurochemistry of ADHD is currently understood to involve dopamine dysregulation, which was discovered accidentally by Charles Bradley in the 1900s (Erin M. Miller, 2013). Using amphetamines to treat headaches of children following pneumoencephalography, he found that this treatment improved school performance and social interactions. Amphetamine became a treatment for ADHD in the 1950s, but it was only after subsequent preclinical studies, wherein amphetamine attenuated the hyperlocomotor response in a dopamine depletion model of ADHD, that dopamine was implicated in the disorder (Shaywitz et al., 1976). More recently, several dopamine-related genes

have been associated with ADHD, including genes for the dopamine receptors, as well as genes for dopamine synthesis, metabolism, and reuptake (Comings et al., 2000). Other genes related to serotonin, norepinephrine, and GABA signaling and metabolism have also been associated with ADHD, suggesting that the disease has a complicated etiology involving numerous neurotransmitter systems (Comings et al., 2000). At the anatomical level, brain imaging studies found that ADHD was linked to lower cortical and striatal neuronal activity as well as to the involvement of brain-wide circuits (Schneider et al., 2006; Wallis et al., 2008; Cortese and Castellanos, 2012).

Similar to schizophrenia, hypodopaminergia in the prefrontal cortex has been implicated in the inattentive and hyperactive behaviors of ADHD. Likewise, the involvement of NMDA receptors is related in a similar way. Increasingly, NMDA receptor hypofunction is becoming implicated in ADHD (Chang et al., 2014). The spontaneously hypertensive rat (SHR), a strain based off the Wistar-Kyoto rats bred for hypertension, is a widely-used model for ADHD. Experiments with cortical slices of SHRs observed that application of NMDA failed to elicit a calcium response on par with a wild-type response (Lehohla et al., 2004), indicating some form of dysfunction of NMDA receptors that may be involved in the attention and cognitive deficits characteristic of this animal model.

However, the picture is complicated by other studies suggesting a hyperfunction of NMDA receptors. Following the discovery that the commonly prescribed ADHD drug atomoxetine was an NMDA receptor antagonist, a few studies were conducted investigating the effect of memantine, an NMDA receptor antagonist, on ADHD. Memantine improved scores in both pediatric (Findling et al., 2007) and adult populations (Biederman et al., 2017) with ADHD.

Further evidence for this phenomenon is otherwise scant, and further assessment is required to determine how NMDA receptor modulation may impact ADHD symptoms.

1.6.4 Alzheimer's disease

Alzheimer's disease is characterized by the dual pathology of beta-amyloid plaques and neurofibrillary tangles within the brain parenchyma with progressive grey matter loss (Perl and Brody, 1980; Glenner and Wong, 1984b, a; Glenner et al., 1984; Sumpter et al., 1986; Kowall, 1994; Kalaria, 1997), especially in the hippocampus (Sudo et al., 2005). At onset of disease, a patient's ability to form short-term memories becomes weaker, and as the disease progresses, memories are lost, usually in a retrograde temporal fashion. Cognitive functions are also impaired, and patients slowly lose autonomy and ability to function. Canonical treatments for Alzheimer's disease exist as neuromodulators of two systems: acetylcholinesterase inhibitors for mild to moderate dementia (Calabria et al., 2009), as increased acetylcholine seemed to boost memory function, and NMDA receptor antagonists for late-stage disease to delay excitotoxic damage that is thought to drive late-stage grey matter loss (Kertesz, 2003).

Several lines of investigation point to a potential link between NMDA receptor hypofunction and Alzheimer's disease (Ulas and Cotman, 1997; Newcomer et al., 2000; Cisse et al., 2011; Huang et al., 2012; Malinow, 2012). Furthermore, NMDA function is impaired in the aging brain (Huang et al., 2012), which may be improved by treatment with D-serine (Avellar et al., 2016). In this model, extra-synaptic NMDA receptor hyperfunction is attributed to beta amyloid oligomer toxicity and widely accepted means of cell death in AD over the long term (Birnbaum et al., 2015; Zhang et al., 2016; Rammes et al., 2017). However, before the substantial neuronal loss characteristic of late-stage Alzheimer's disease, NMDA hypofunction

in the cortex and hippocampus may explain the symptoms of mild cognitive impairment and mild dementia (Newcomer et al., 2000). Additionally, there is evidence that NMDA hypofunction can similarly lead to cell death in a process parallel to beta-amyloid excitotoxicity (Newcomer et al., 2000).

Timing the use of NMDA potentiators in Alzheimer's disease could be challenging, as it would be important to rescue the putative NMDA receptor hypofunction without exacerbating NMDA receptor-mediated excitotoxicity. Accordingly, potential therapies based on NMDA receptor potentiation may need to be used in early stages to prevent hypofunction, thereby delaying or precluding development of the disease towards late-stage excitotoxicity-mediated neuronal loss.

1.7 Potentiation of NMDA receptors as a therapeutic strategy

A great deal of research has been directed towards the potentiation of NMDA receptor function as a therapeutic strategy. Until very recently, no specific, direct positive modulators of NMDA receptors existed, so previous efforts have been focused on modulation of the glycine binding site as a means to increase NMDA receptor sensitivity to glutamate.

1.7.1 Glycine and glycine mimetics as NMDA receptor potentiators

As NMDA receptor activation requires both glycine and glutamate, increasing either co-agonist should make the receptor more sensitive to activation by the other, as long as saturation is not reached. Indeed, it was found that *in vivo* levels of glycine do not reach a high enough level to saturate NMDA receptors, offering a possibility for modulation of NMDA receptor activity by glycine (Monaghan and Jane, 2009; Collingridge et al., 2013). However, due to glycine's poor blood-brain barrier (BBB) penetration (Pollay, 1976), this molecule is a poor drug

choice, as extremely high doses must be administered continuously to reach effective concentrations. In addition to glycine, two other glycine-mimetic drugs are commonly used as potentiators of the NMDA receptor: D-serine and cycloserine (Tsai et al., 1998; Goff et al., 1999; Tsai et al., 1999). D-serine is a popular glycine mimetic with much better BBB penetration, but its use carries concerns about nephrotoxicity (Ganote et al., 1974). Cycloserine is another analogue already FDA approved for use against drug-resistant tuberculosis, but is paradoxically contraindicated in patients with psychosis, and in some cases, can cause psychosis (Sharma et al., 2014; Holla et al., 2015). Unfortunately, in addition to these caveats, glycine site modulation remains suboptimal, as glycine is itself an inhibitory neurotransmitter, especially in the spinal cord and brainstem, as well as being present diffusely in the prosencephalon (Todd et al., 1996; Avila et al., 2013). Given the poor specificity compounded with modest efficacy in clinical trials as described immediately below, and numerous caveats to the safety and practicality of these drugs, their widespread clinical use for neuropsychiatric disorders has been delayed.

Nevertheless, more recently glycine-based strategies have gained interest as new therapies for NMDA hypofunction disorders, including schizophrenia. These clinical studies are supported by with numerous preclinical studies demonstrating benefit from glycine site agonism of NMDA receptors in animal models (Monahan et al., 1989; Andersen and Pouzet, 2004; Gaisler-Salomon et al., 2008; Kanahara et al., 2008; Bado et al., 2011). Human trials have examined the effects of glycine, glycine mimetics, and glycine reuptake inhibitors with mixed success. One meta-analysis of 26 studies found a small improvement in overall psychiatric symptoms for NMDA potentiation via glycine agonism, and a moderate improvement for negative symptoms. However, there was no improvement on scores for general cognition, memory, or spatial reasoning, or speed of processing (Choi et al., 2013). Notably, the largest

single randomized control trial, CONSIST, found no benefit from either cycloserine or glycine in the treatment of negative symptoms or cognitive impairments across 157 enrolled patients (Buchanan et al., 2007). In contrast, another open-label study has shown that cycloserine is effective at improving negative and cognitive symptoms when given at high doses (Kantrowitz et al., 2010), suggesting that insufficient potency of these drugs may be responsible for the inconsistent outcomes.

Glycine reuptake inhibition by drugs such as sarcosine extends and allows the buildup of glycine at the synapse, providing an alternative approach to glycine and glycine mimetics for potentiating NMDA receptors. Sarcosine has better BBB penetration, better tolerability, and a larger effect size in negative and total symptoms compared to glycine and glycine mimetics in a meta-analysis of 29 trials (Singh and Singh, 2011). However, the same caveats exist regarding off-target effects within glycine signaling systems of the brain and spinal cord.

Overall, there are indications that glycine-based potentiation of NMDA receptors has mild effects on the negative and cognitive symptoms of schizophrenia. However, modest effects of these compounds in clinical trials might be attributable to a weak pharmacological effect limited by both bioavailability and therapeutic windows. Clearly, there is a need for alternative classes of drugs for the treatment of NMDA hypofunction disorders.

1.8 Positive allosteric modulation of NMDA receptors

Allosteric modulation refers to the indirect influence of a highly selective molecule acting at a site distinct from the direct agonist binding site to change the affinity of a receptor for its orthosteric, or primary agonist. Generally, this is accomplished when a separate ligand binds to an allosteric site that triggers conformational changes in the agonist binding sites. Importantly,

the allosteric modulator is not capable of activating the receptor in the absence of the agonist. In drug development, allosteric modulators have strong advantages for applications involving ionotropic receptor potentiation or inhibition. In contrast to orthosteric agonists or antagonists which can override endogenous signaling, or partial agonists which serve to "buffer" receptor activity away from extremes by acting either as a weak agonist or antagonist depending on the concentration of a full agonist, an allosteric modulator only tunes the receptor's sensitivity for a selective receptor ligand. This allows amplification or attenuation of natural, endogenous signaling only, theoretically with no effect at inactive synapses (Wenthur et al., 2014).

One illustrative example to the advantages of allosteric modulation can be seen with benzodiazepines. These drugs are anxiolytics which positively modulate $GABA_A$ receptors to cause sedation, but avoid the lethal depressive effects exhibited by direct agonists (Mohler et al., 2002; Conn et al., 2009). An additional advantage of allosteric modulation is receptor selectivity. While use of NMDA may allow selective activation of NMDA receptors over AMPA or kainate receptors, it is much more difficult to design agonists capable of only activating GluN1/GluN2A receptors over GluN1/GluN2B receptors. A well-designed allosteric modulator could selectively potentiate only one type or even one or more subtypes of glutamate receptor, as was accomplished recently with the characterization of an GluN2A-specific allosteric modulator by our lab (Axerio-Cilies, 2016). As a therapeutic strategy for NMDA receptor hypofunction, non-glycine site allosteric modulation may be the ideal strategy to rescue NMDA hypofunction without off-target effects on glycine signaling.

A general concern for potentiation of NMDA receptors in experimental therapeutic approaches is that it might lead to exacerbated neuronal death by aggravating cell death signaling pathways mediated by GluN2B-containing receptors. Use of an allosteric modulator is near

mandatory in this case to minimize pro-death effects. By only demonstrating an effect in the presence of highly localized synaptic glutamate, an Npam selectively potentiates synaptic NMDA receptors over extra-synaptic receptors, minimizing aggravating effects on cell death pathways. Just as benzodiazepines can have therapeutic, depressive effects without causing widespread, lethal depression of neuronal activity, Npams may rescue NMDA hypofunction without inducing neurotoxicity.

1.8.1 Compounds with positive allosteric effects on NMDA receptors

Positive NMDA receptor allosteric modulation outside of glycine-based strategies has been an elusive goal. The first class of compounds discovered to have positive allosteric effects on NMDA currents were the polyamines (Rock and Macdonald, 1995). Spermine, one such polyamine, was found to potentiate NMDA currents when applied in neuron cultures (Benveniste and Mayer, 1993), but this effect was highly variable, and presented also as strong inhibition, depending on the neuron being recorded. Later, it was realized that differing NMDA receptor subtype expression was responsible, as polyamines can conditionally potentiate GluN2B-containing NMDA receptors. Specifically, polyamines have three effects at the NMDA receptor. First, they cause a voltage-dependent inhibition, much like Mg^{2+}, by occupying and occluding the channel pore (Rock and MacDonald, 1992; Benveniste and Mayer, 1993). Second, they increase the receptor's affinity for glycine (McGurk et al., 1990), likely by binding to sites normally bound by Ca^{2+} or Mg^{2+} (Paoletti et al., 1995; Wang and MacDonald, 1995). Third, they potentiate currents by blocking the tonic inhibition provided by protons from GluN2B-containing NMDA receptors only (Williams, 1994; Zhang et al., 1994; Traynelis et al., 1995). While the

degree of potentiation by polyamines can be dramatic (Benveniste and Mayer, 1993), it is not possible to divorce the three effects, limiting the usefulness of these compounds. Furthermore, due to preferential potentiation of GluN2B-containing receptors over GluN2A-containing receptors, potentiation of cell death pathways remains a concern.

Pregnenolone sulfate, a neurosteroid, is another known potentiator of NMDA receptors with selective potentiation of GluN2A- and GluN2B-containing receptors and inhibition on GluN2C- and GluN2D-containing receptors (Wu et al., 1991; Irwin et al., 1992; Malayev et al., 2002; Horak et al., 2006; Adamusova et al., 2013). However, it is also an important negative modulator of $GABA_A$ receptors (Majewska and Schwartz, 1987; Mienville and Vicini, 1989; Nilsson et al., 1998; Eisenman et al., 2003), and lacks the specificity needed to be a useful NMDA allosteric modulator.

Other than the research described above with these two classes of compounds and their numerous flaws, prior to 2016, there have been no published reports focused on Npams. One of our lab's long-standing objectives has been to develop a series of Npams, ideally with varying subtype specificity. Initial experiments used computer-aided drug discovery (CADD) to screen a database of compounds for candidates. A few million compounds were screened against a computer model of the NMDA receptor derived from X-ray crystallography work (Axerio-Cilies, 2016). A potential binding pocket predicted to have allosteric effects on the glutamate binding pocket was identified, and candidate compounds were tested for binding affinity within the pocket accounting for steric effects, polarity, and attractive forces between residues and functional groups. A shortlist of a few thousand compounds was then refined manually down to roughly 60 compounds. Of these, Npam02 and Npam43 were found to be a GluN2A-containing

NMDA receptor specific positive allosteric modulator and was characterized extensively as a possible neuroprotective drug (Axerio-Cilies, 2016).

At the same time, an independent group, also recognizing the value in an NMDA receptor allosteric modulator, published the discovery of a GNE family of drugs in 2016. These drugs act with GluN2A specificity by binding in a subunit interface within the ligand binding domain (Hackos et al., 2016; Volgraf et al., 2016). While the effects of the GNE family of compounds is comparable, but slightly weaker to that of Npam43, this group did not target the same binding site and has not yet had demonstration of any *in vivo* or behavioral effects. Furthermore, no dual potentiator of GluN2A- and GluN2B-containing receptors, or even a GluN2B-specific allosteric modulator, has been characterized, leaving ample opportunity for further development of Npams.

1.8.2 NMDA receptor crystal structure, and the search for NMDA receptor positive allosteric modulators

Briefly, the NMDA receptor can be divided into 3 extracellular regions or layers: a transmembrane domain rich in alpha helices that contains the receptor pore, a ligand binding domain immediately above that which contain clamshell-like structures for glycine and glutamate binding, and a globular N-terminal domain (NTD) above the ligand binding domain (Figure 1.5). The receptor is rotationally symmetric, with GluN1 and GluN2 subunits arranged in a 2x2 checkerboard pattern when viewed from above. The crystal structure used for drug screening was obtained from previous work done to characterize the ligand-binding domains of the GluN1/GluN2A-containing NMDA receptor (Furukawa et al., 2005). Above the ligand-binding domain is the NTD, which has not yet been crystalized for GluN1/GluN2A. However, the structure of this portion of the receptor has been defined for GluN1/GluN2B (Karakas et al.,

2009), and the entire GluN1/GluN2B receptor of Xenopus laevis had been crystalized to reveal a rotationally symmetric, checkerboard arrangement of subunits (Lee et al., 2014). Additionally, it was demonstrated that connections between the NTD and the ligand binding domain is permissive for allosteric influences towards the ligand binding sites (Lee et al., 2014). This region was the site of interest for our lab's efforts towards discovering a novel allosteric modulator of NMDA receptors.

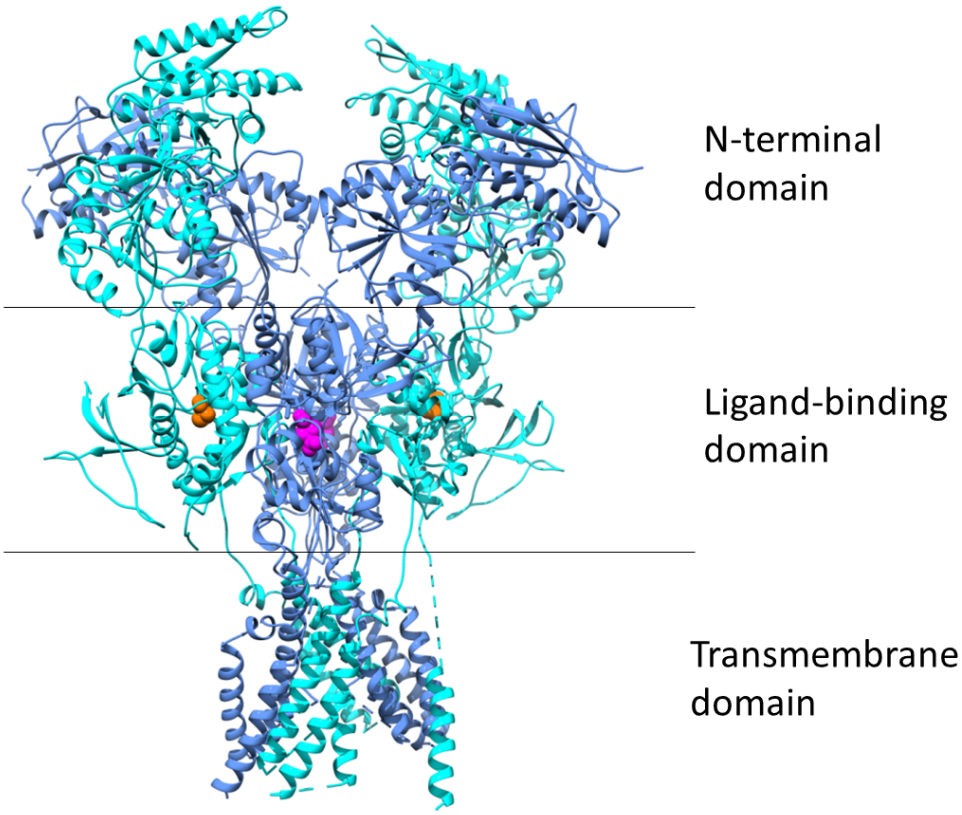

Figure 1.5 Structural overview of the GluN1/GluN2 NMDA receptor.

The NMDA receptor consists of GluN1 subunits (teal) and GluN2 subunits (indigo). Binding sites for glycine (orange) and glutamate (magenta) exist in clamshell-like structures in the

ligand-binding domain of the GluN1 and GluN2 subunits respectively. Changes in conformation of the NTD can be transmitted down to the ligand-binding domain, offering opportunities for allosteric modulation. (Axerio-Cilies, 2016)

There were three main considerations when searching for an ideal binding site that may allow allosteric regulation of the NMDA receptor. First, it was known that the conformational changes in the NTD could in turn induce conformational changes in the ligand binding domain (Lee et al., 2014), and that in other proteins which share a clamshell-like structure of binding sites, adjacent NTDs were capable of modulating those as well (Axerio-Cilies et al., 2011; Zoraghi et al., 2011; Axerio-Cilies et al., 2012). Second, targeting the interface between subunits promised to be a higher yield strategy than otherwise (Lack et al., 2011), and the involvement of the GluN2 subunit allows the potential for subtype specificity. Third, targeting a region nearby or in the ligand binding domain, while likely to produce working candidates, suffers from the drawback of being susceptible to the concentration of glycine and glutamate compared to allosteric modulation by a more distal location (Axerio-Cilies, 2016). As a result, the NTD was chosen as the highest yield target for further development.

1.8.3 The success of Npam02 and Npam43

Npam02 was the first developed drug candidate produced from drug screening, which showed potentiation of GluN2A-containing, but not GluN2B-containing receptors. In neuron culture, Npam02 potentiated NMDA currents by up to 200%, which could be abolished by the application of an GluN2A-specific antagonist such as NVP (Axerio-Cilies, 2016). Npam43 did

not potentiate isolated GluN2B currents (Axerio-Cilies, 2016). Furthermore, Npam02 was shown to potentiate induction of LTP in hippocampal slices (Axerio-Cilies, 2016).

Selective potentiation of GluN2A-containing NMDA receptors has the obvious application towards affecting the balance of neuron survival versus death. When Npam43 was tested *in vitro* with excitotoxicity studies, it upregulated pCREB and protected against NMDA- and hydrogen peroxide-induced cell death (Axerio-Cilies, 2016). *In vivo*, it was demonstrated that Npam43 was protective in a middle cerebral artery occlusion model of ischemic stroke (Axerio-Cilies, 2016).

1.9 Applications for allosteric modulation of both GluN2A- and GluN2B-containing NMDA receptors

Whereas selective potentiation of GluN2A-containing receptors is useful for neuroprotective applications (Axerio-Cilies, 2016), it has less clear applications in non-excitotoxic circumstances. In less extreme conditions, such as in normal synaptic function, GluN2A-containing and GluN2B-containing receptors have equally important roles in the acquisition and consolidation of new memories (Bannerman et al., 2008; von Engelhardt et al., 2008; Ge et al., 2010). GluN2A-containing receptors mediate LTP (Liu et al., 2004), as described above, and are critical for the induction of LTP and acquisition of learning (Morris et al., 1986; Morris, 1989; Lynch, 2004; Bannerman et al., 2008), and GluN2B-containing receptors are crucial for induction of LTD, which is crucial for the consolidation of memory (Ge et al., 2010; Dong et al., 2012), and behavioral flexibility and reversal learning (Kumar et al., 2015). Both GluN2B-containing receptors and GluN2A-containing receptors have been implicated in NMDA hypofunction pathology (Li and He, 2007; Weickert et al., 2013; Dawson et al., 2015). Given

that both GluN2A signaling and GluN2B signaling appear to play opposing and complementary functions in plasticity, it follows that potentiation of both receptors subtypes may be more beneficial than potentiation of either one alone, especially in cognitive processes. For this reason, the continued search for a dual GluN2A and GluN2B Npam holds exciting possibilities for applications as a therapeutic or cognitive enhancer.

Here, it is also important to note the possible existence of tri-heteromeric NMDA receptors *in vivo* (Sheng et al., 1994; Gray et al., 2011; Rauner and Kohr, 2011; Delaney et al., 2013; Tovar et al., 2013; Hansen et al., 2014), which consist of two GluN1 subunits and one each of GluN2A and GluN2B subunits, although little is understood about the functional role of these mixed receptors (Paoletti et al., 2013). However, given that there are two binding sites within the N-terminal domain of NMDA receptors for our Npam compounds which show subtype specificity for the GluN2 subunit, we could imagine that a 2A-specific modulator would only target one of those sites, and have a much-reduced efficacy on tri-heteromeric receptors, while a 2A- and 2B-specific modulator may not lose efficacy on these tri-heteromeric receptors due to binding to both binding sites. Considering that these GluN1/GluN2A/GluN2B receptors may consist of 15% to >50% of the total NMDA receptor population (Paoletti et al., 2013), a dual modulator may prove to be more useful than GluN2A-specific Npams such as both Npam43 and the GNE series of drugs.

1.10 Hypothesis and specific aims

Given the numerous significant roles that NMDA receptors play in normal cognition and the involvement of NMDA receptor hypofunction in various neurological and psychiatric pathologies, we predict that drugs which enhance NMDA receptors may have great potential in

therapeutic applications. Building upon our past success with Npam43 in using a combination of *in silico* screening and functional characterization in both HEK cells expressing recombinant NMDA receptors and in neurons expressing native NMDARs, I hypothesize that the same strategies would also lead to the discovery and characterization of a new dual potentiator of GluN2A- and GluN2B-containing NMDA receptors within the same Npam family. The thesis is designed to test this hypothesis by realizing the following specific aims using a combination of molecular, electrophysiological, neurochemical and behavioral techniques:

1. Characterize the efficacy and specificity of NMDAR positive allosteric modulators (Npam) in HEK cells expressing recombinant human NMDARs
2. Characterize the efficacy and specificity of the Npam in the potentiation of native NMDARs in cultured hippocampal neurons
3. Determine the effects of the Npam on NMDA receptors in the intact brain *in vivo*
4. Determine the consequences on behavior by application of the Npam

Each of these aims will be presented in the following chapters chronicling the discovery and multilevel characterization of a novel Npam.

Chapter 2: Materials and Methods

2.1 Primary neuron culture

Pregnant Sprague-Dawley mothers were sacrificed 18 or 19 days post-fertilization via a lethal 3.25mL intraperitoneal injection of 25% urethane solution. Sterile surgical procedures in a class II biosafety cabinet were used after reaching surgical plane in the mothers to recover the embryonic brains. The uterus was visualized and removed through an abdominal incision and placed in a 10cm culture dish containing ice cold dissection buffer (ingredients and prep protocol). The uterus was then cut open and rat embryos were transferred to another ice-cold 10cm culture dish containing dissection buffer. Each embryonic brain was then carefully removed and dissected under microscope to separate out the hippocampi. The dissected portions were sorted and collected into two 10cm culture dishes where the cells were separated by incubation in 2-3mLs of 37°C 0.25% trypsin-EDTA (provider) in DMEM containing 10% FBS for 30 minutes. An additional 8-10mL of warm DMEM with 10% FBS was added to each plate to halt digestion, and the total contents were transferred to individual 15mL falcon tubes. The tubes were set upright for 2-3 minutes to allow cells to settle to the bottom and the supernatant was discarded. The cells were washed twice with 10mL of fresh DMEM containing 10% FBS and centrifuged before the DMEM media was replaced with neurobasal growth medium. Cell density was determined by a hemocytometer and neurons were plated with a target density of $2.5x10^5$ cells/well on poly-D-lysine coated glass cover slips in a 24 well plate for both cortical and hippocampal neurons. Roughly two thirds of the culture medium were drawn off and replaced with fresh neurobasal medium every four days to maintain cellular health.

2.1.1 Dissection buffer:

- Neurobasal Media (Invitrogen)
- 2% B-27 supplement (Invitrogen)
- 0.5mM GlutaMAX supplement (Invitrogen)

2.1.2 Neurobasal growth medium:

- 489.75mL Neurobasal media (Invitrogen)
- 2% B-27 supplement (Invitrogen)
- 0.5mM GlutaMAX supplement (Invitrogen)
- 25uM glutamic acid (Sigma)

2.2 Human embryonic kidney 293 (HEK293) cell line maintenance

HEK293 cells were cultured in 10 cm culture dishes and Dulbecco's modified eagle medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) in an incubator at 37°C and 5% CO_2. Culture medium was replaced every 2-3 days as needed and cell health and confluency were monitored daily.

The HEK293 cells were passaged at 80-90% confluency. The medium was discarded, and 2-3mLs of 37°C 0.25% trypsin-EDTA (Invitrogen) was added for 10 minutes to detach cells from the dish. 3mL of DMEM was added to neutralize the trypsin, and the cell suspension was centrifugated at 1000rpm for 5 minutes to pellet the cells. The supernatant was discarded, and 7mL of fresh DMEM with 10% FBS was added to re-suspend the pellet. 1mL of the cell suspension was added to 9mLs of growth medium in a new 10cm culture dish for maintenance of

the cell line, and 0.5mLs of the suspension was added to each well of a 6 well plate for transfection experiments the following day, as required.

2.3 HEK293 transfections

Lipofectamine 2000 (Invitrogen, #15338-500) was used to transfect plasmid DNA into HEK293 cells as per manufacturer instructions. pcDNA3-CMV vectors expressing GluN1, GluN2A, and GluN2B rat recombinant subunits were co-transfected to produce cells expressing GluN1/GluN2A or GluN1/GluN2B. A Green fluorescent protein (GFP) encoded on a pcDNA3-GFP plasmid was also transfected for visual report.

2.4 Electrophysiology:

Whole cell patch-clamp recordings were performed using an Axopatch 200B or 1D patch-clamp amplifier (Molecular Devices). Whole-cell currents were recorded under voltage clamp mode at a holding potential of -60 mV, and signals were filtered at 2 kHz and digitized at 10 kHz (Digidata 1322A). Glass micropipettes were drawn to 3-5 MΩ and filled with intracellular solution, which is described below.

Hippocampal neurons on glass cover slips were harvested and cultured for 11-14 days *in vitro* as described in Section 2.1. Before recording, a cover slip was transferred to a 3cm petri dish containing magnesium-free artificial cerebrospinal fluid (aCSF) solution for transport to the recording apparatus. The cover slip was then placed in the recording chamber and superfused with constantly flowing extracellular solution, described below. One neuron per cover slip was selected, and micromanipulators were used to position the stimulus port over the cell and the glass electrode onto the cell membrane. Once a >1GΩ seal between the cell and the electrode was achieved, light suction accompanied by 20-40mV shocks was used to break through the

membrane and achieve whole cell recording. All experiments were performed at room temperature.

Induced currents were evoked by application of NMDA, GABA, or AMPA through perfusion fast-step (Warner Instruments) delivered through two-barrel glass capillaries. Recordings from at least six neurons were performed for all test conditions. Dose-response data were fitted by the equation "Y= Top/(1+10^((LogEC50-X)))", where LogEC50 is the log concentration of agonist that produces a half-maximal response and Top is the response at maximally effective concentration relative to the maximal response of glutamate. CNQX (10μM) and TTX (0.5μM) were applied to block non-NMDA ionotropic glutamate receptors and voltage-gated sodium channels, respectively. Bicuculline methobromide (10μM) was added to block $GABA_A$ receptors. Glycine (1μM) was also added as co-agonist for NMDA receptor activation in all ECS solutions, unless otherwise indicated.

2.4.1 Intracellular solution

CsCl 140mM, HEPES 10mM, Mg-ATP 4mM, QX-314 5mM, pH 7.20, 290-295 mOsm. BAPTA (10 mM) was added in the intracellular solution (otherwise specified). Solution was aliquoted and stored at -20°C before use.

2.4.2 Extracellular solution

NaCl 140mM, KCl 5.4mM, HEPES 10mM, CaCl2 1.3mM, pH 7.4, 305-315 mOsm. pH was adjusted and glucose 20mM was added to the above stock on the day of use. Note solution is magnesium-free.

2.5 Chemicals

N-methyl-D-aspartate (NMDA), D-2-amino-5-phosphonovaleric acid (APV), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Torcis (Ellisville, Missouri, US). Glutamate, gamma-Aminobutyric acid (GABA) was purchased from Sigma-Aldrich. Bicuculline methobromide was purchased from Alexis Biochemicals.

2.6 Drug injections

Drugs were administered with 25 gauge needles intraperitoneally (i.p.) unless otherwise indicated. Npam59 formulation was injected with low dead-space syringes (BD). Volumes injected were standardized at 330μL/kg for Npam59 and 1mL/kg for d-amphetamine at all doses tested.

2.6.1 Npam59 formulation

Npam59 was formulated in two preparations depending on application. For electrophysiology and reverse micro-dialysis applications, Npam59 was dissolved in dimethyl sulfoxide (DMSO) at 10mM and serially diluted with aCSF to obtain the indicated working concentrations. For animal injections, Npam59 was dissolved fresh daily in dimethyl formamide (DMF) at a concentration of 1mg/10μL, then emulsified by a ratio of 1:10 into corn oil (Mazola) to create a final concentration of 9.091mg/mL for use at 3mg/kg. When preparing lower doses of Npam59, the vehicle volume and ratio was kept constant while the mass of Npam59 dissolved in DMF was reduced.

2.6.2 d-Amphetamine formulation

d-Amphetamine was prepared at a stock concentration of 10mg/mL in sterile 0.9% saline and was diluted to working concentrations with additional 0.9% saline. d-Amphetamine solutions were stored at 4°C in 5mL glass vials sealed in parafilm for up to 3 months.

2.7 Animal subjects

Male Sprague Dawley rats (Charles River, Quebec) were procured at 200-250g body weight and kept on a 12-hour reverse light cycle (lights off 7am-7pm), with all experiments carried out during the dark phase. Following arrival, animals were double housed and acclimatized to the colony room over 7 days with unlimited access to rat chow and acidified tap water on a 12-hour reverse light cycle (lights off 7am-7pm). Animals received handling daily during this time. After these 7 days, animals were used for experiments and continued to have unlimited access to food and water unless specified. All animal husbandry, experiments, handling, and surgery were conducted in accordance to the Canadian Council on Animal Care and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003). Ethics of the studies were approved by the Committee on Animal Care, University of British Columbia.

2.8 Micro-dialysis

Prior to surgery, rats were anesthetized with isoflurane and bilateral stainless steel micro-dialysis guide cannulas (19 gauge x 15 mm) were implanted in the nucleus accumbens (from bregma +1.7 mm anterior and ±1.1 mm medial; from dura -1.0 mm ventral). Micro-dialysis probes were fabricated in house and inserted the day prior to experiments. Rats remained in the testing chamber overnight (14-16 hr) and aCSF was perfused continuously through the probes at

1 µl/min. Once baseline levels were stable, the drug treatment phase of each experiment was initiated. Micro-dialysis samples were collected every 10 min and analyzed immediately using high-pressure liquid chromatography (HPLC) with electrochemical detection via two identical systems.

The aCSF consisted of a 10.0 mM sodium phosphate buffer with 147.0 mM NaCl, 3.0 mM KCl, 1.0 mM $MgCl_2$ and 1.2 mM $CaCl_2$ (pH 7.4).

2.9 CSF and serum collection

Rats were injected i.p. or intravenously through the tail vein with Npam59, then anesthetized with i.p. overdose urethane 0.5h, 1h, 2h, 4h, and 8h after injection. Immediately when animals entered surgical plane, the head was fully flexed and the nape of the neck was dissected down to the dura of the cisterna magna. CSF was collected by piercing the dura with glass micropipettes. Animals were then euthanized by decapitation and blood was collected and centrifuged to yield a serum sample. CSF and serum samples were analyzed on HPLC to determine the concentration of Npam59.

2.10 High performance liquid chromatography

Micro-dialysate samples and animal body fluid samples were separated using reverse-phase HPLC and analyzed with electrochemical detection on two separate setups. For micro-dialysis, the HPLC setup consisted of:

- Antec Leyden LC100 HPLC pump (Netherlands)
- Scientific Systems Inc. pulse damper (State College, PA)
- Rheodyne 9125i manual injector (20µL injection loop, Rohnert Park, CA)

- Tosoh Bioscience Super ODS TSK column (2µm particle, 2mm x 10cm, Montgomeryville, PA)
- Antec Leyden Intro electrochemical detector with a VT-03 flow cell ($V_{applied}$=+650mV, Netherlands)

The mobile phase (70mM sodium acetate buffer, 40mg/L EDTA, 6mg/L sodium dodecyl sulfate (variable), pH 4.0, 10% methanol) flowed through the system at 0.17mL/min. Acquisition and analysis of chromatographic data were accomplished with EZChrome Elite software (Scientific Software, Pleasanton, CA). For analysis of rat CSF and serum samples, the HPLC setup consisted of:

- ESA 582 pump (Bedford, MA)
- Scientific Systems Inc. pulse damper (State College, PA)
- Rheodyne 9125i manual injector (20µL injection loop, Rohnert Park, CA)
- Tosoh Bioscience Super ODS TSK column (2µm particle, 2mm x 10cm, Montgomeryville, PA)
- Antec Leyden Intro electrochemical detector with a VT-03 flow cell ($V_{applied}$=+800mV, Netherlands)

The mobile phase (20mM phosphate buffer, acetonitrile (80: 20, v/v) mixture, pH 7.0) was flowed through the system at 0.1mL/min. Acquisition and analysis of chromatographic data were accomplished with EZChrome Elite software (Scientific Software, Pleasanton, CA).

2.11 Locomotor activity

Eight black plexiglass boxes measuring 41.25cm * 41.25cm * 41.25cm containing biofresh Natural Cellulose Comfort bedding (biofresh, Ferndale WA) were used as arenas. Red LED lights placed above the boxes provided lighting and the room was otherwise held in darkness. Rats were placed in the arenas on two consecutive days for 30 minutes each prior to the test day for habituation. On the third day 20 min prior to placement in the arena, animals were injected i.p. with Npam59 or vehicle. After 20min, each animal was injected with d-amphetamine and immediately placed into the arena for locomotor tracking. Locomotor activity within each arena was tracked by two overhead video cameras and scored with Ethovision XT (Noldus), which was set to track 2 hr of locomotor activity upon detection of movement within each arena zone. Following testing completion, tracking errors were manually corrected using the stored video recording.

2.12 Statistics

Analyses for dose response curve fitting were conducted in GraphPad Prism 6. All other data were analyzed in Sigmaplot 13.0. All group effects were first assessed by analysis of variance testing (ANOVA) and Sidak-Holms method was used for all post-hoc tests except where stated otherwise. Significance in all analysis was set at $\alpha = 0.05$. All data in the text and figures are presented as the mean ± standard error of the mean unless indicated otherwise.

Chapter 3: Identification and characterization of Npam59 as a potent and specific dual potentiator of GluN1/GluN2A and GluN1/GluN2B receptors

3.1 Introduction

Based on previous work by our lab, we have determined that there is a binding site in the NMDA receptor NTD between the GluN1 and GluN2 subunits which can allosterically modulate the conformation of the ligand binding domain of the receptor. It has been shown that it was possible to selectively potentiate the GluN1/GluN2A heteromeric receptor *in vitro* and *in vivo* through this site, with effects on plasticity and neuroprotection following stroke (Axerio-Cilies, 2016).

Given reasons discussed in Chapter 1, there are numerous advantages to targeting the GluN2A- and GluN2B-containing NMDA receptors. Our first step in the development of such an Npam was to determine if one of the previously obtained Npams has the properties of dual potentiator for GluN2A-containing or GluN2B-containing NMDARs. We initially focused on Npam43 as our starting point based on preliminary results from previous study in our lab (Axerio-Cilies, 2016)). From previous screening of numerous Npam compounds, it was clear the backbone of two benzene rings linked by a hydrazine group was the critical shape capable of binding to the NTD allosteric site. On this backbone, the R2 benzene ring was relatively deeper into the pocket, and functional groups attached to this ring conferred steric effects that can result in specificity for GluN2A vs GluN2B. For example, addition of a methyl group in the ortho position caused Npam02 to also cause inhibition upon GluN2B-containing receptors, in the absence of any other changes (Axerio-Cilies, 2016). On the R1 benzene ring, functional groups were responsible for the potentiating or inhibitory effects of the compound through interactions

with key residues within the binding pocket. The difference between Npam02 and Npam43 is the addition of two chlorines in the meta and ortho positions, and replacement of the methoxy group with an ethoxy group on the R1 ring (Figure 3.1), which increased its potentiation dramatically (Axerio-Cilies, 2016) on GluN2A-containing receptors. From these trends, we hypothesized that removal of steric hindrances on the R2 ring of Npam43, while preserving the R2 ring functional groups would allow potentiation of both GluN2A- and GluN2B-containing receptors.

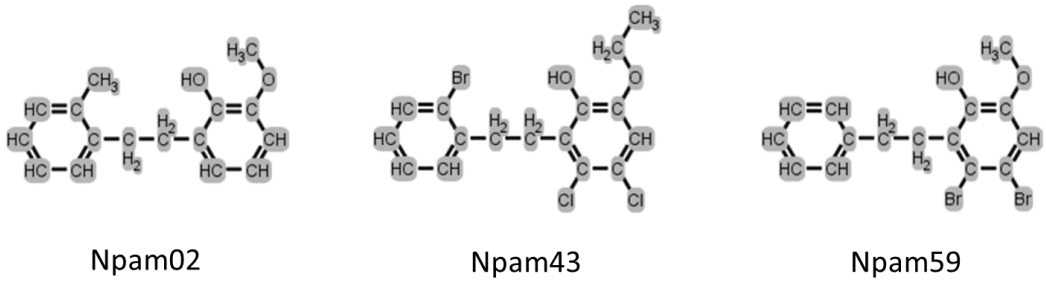

Figure 3.1 Molecular structures of Npam02, Npam43, and Npam59.

Npam02 was the initial lead compound developed as an allosteric modulator acting within the NTD of the NMDA receptor. It was found to potentiate GluN2A-containing receptors but inhibit GluN2B-receptors. Npam43 was an extension upon Npam02 that introduced greater steric hindrance at the R2 ring (left side in the depicted orientation) for greater selectivity towards GluN2A, and increased functional groups for a greater potentiating effect. Npam59 is another extension upon Npam43, wherein the functional group characteristics on the R1 ring are retained, but the steric bromine is removed entirely. (Axerio-Cilies, 2016)

We first started our search for such a compound within the shortlist of other screened compounds that Axerio-Cilies had found to show NMDA receptor potentiation of unknown specificity (Axerio-Cilies, 2016). One compound, Npam59, was very similar to our hypothesized dual potentiator (Figure 3.1), with no steric hindrances on R2, and functionally similar groups on R1, replacing chlorines for bromines (which are both halogens), and the ethoxy group for a methoxy group, which differ only slightly in size and polarity. During the initial compound screen conducted on cortical neurons, Npam59 also showed very strong potentiation of NMDA receptors with unknown specificity (Axerio-Cilies, 2016), which may indicate activity on more than one subtype of receptor. An additional practical consideration existed that favored study of Npam59 over Npam43 with a removed bromine: Npam59 was available commercially for purchase, but the modified Npam43 was not. For these reasons, Npam59 was selected as the best candidate moving forward to discovery and characterize a dual GluN2A and GluN2B positive allosteric modulator.

We briefly returned to our computer model of the NMDA receptor to confirm the validity of our reasoning by placing Npam59 and Npam43 within the allosteric binding pocket using computer modeling of the receptor structure. As was predicted, Npam43 was unable to bind to GluN1/GluN2B due to the extra bromine, while Npam59 was not limited in this way (Figure 3.2). There was indication that Npam59 binds to both GluN1/GluN2B and GluN1/GluN2A, which led us to perform the following electrophysiological experiments to fully verify and characterize these effects.

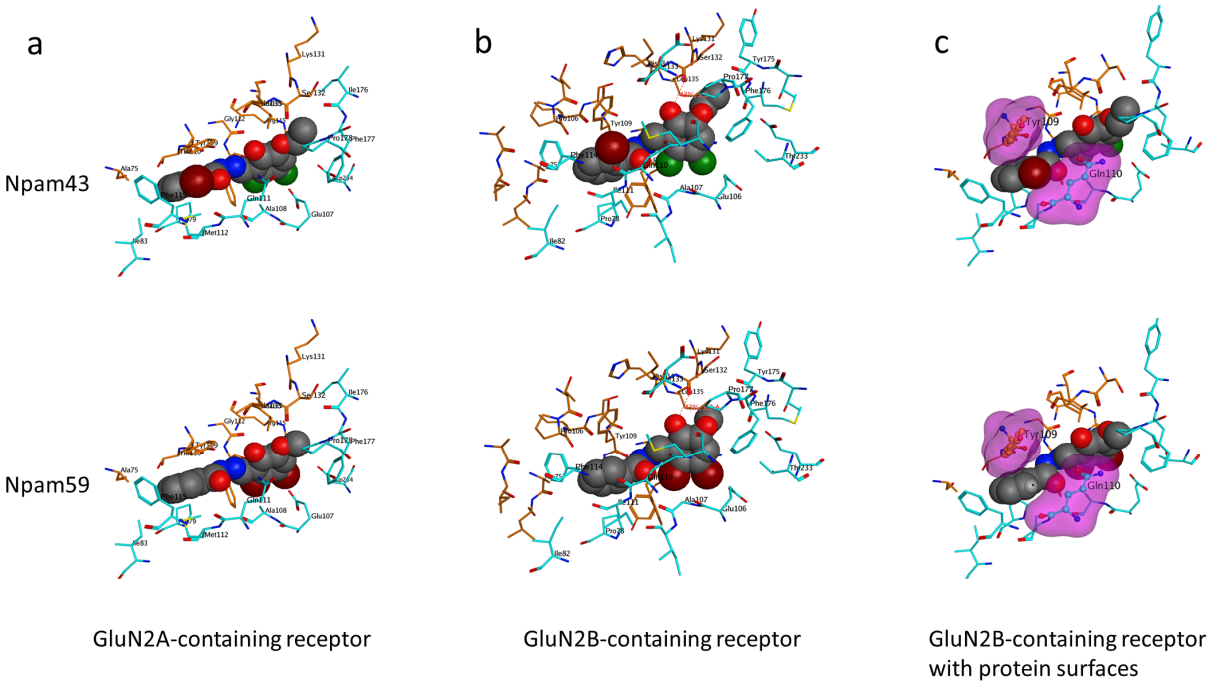

Figure 3.2 Comparison of Npam43 and Npam59 in the allosteric site located in the N-terminal domain of the NMDA receptor.

a) Within the GluN2A receptor, there are no steric hindrances or unfavorable interactions which prevent binding of either Npam compound in the interface between GluN1 and GluN2A. Orange residues belong to GluN1, and teal residues belong to GluN2. b) The bromine group on Npam43 overlaps volume occupied by Gln110, whereas Npam59 does not have such steric interference. c) Magenta volumes represent the space occupied by Gln110 and Tyr109, in the GluN2B allosteric site.

3.2 Results

3.2.1 Npam59 potentiates NMDA receptors containing only GluN1/GluN2A or only GluN1/GluN2B in transiently transfected HEK293 cells in a dose dependent manner

To evaluate the ability and subunit-specificity of Npam59 to potentiate NMDAR function, we electrophysiologically characterized its effects in HEK293 cells transiently co-expressing either human recombinant GluN1 and GluN2A or GluN1 and GluN2B NMDARs. Whole cell patch recordings were conducted under voltage-clamp configuration at a holding membrane potential of -60mV, and currents were induced by fast perfusion of glutamate (10μM) and glycine (1μM) with or without various concentrations of Npam59. The percent potentiation was determined by dividing total current with Npam59 by total current with glutamate alone and subtracting 100%. Data were analyzed with a one-way ANOVA. Results obtained from HEK cells expressing GluN1/2A showed that Npam significantly potentiated NMDA currents in a dose-dependent manner (Fig. 3.3a and c; $F(4,22) = 44.372$, $p<0.001$). Post-hoc analysis revealed significance at doses of 0.1μM through 10μM caused significant potentiation of GluN2A-mediated currents (p values as indicated in Figure), with a maximal potentiation of 505%. Data were also fitted to a 3 parameter Hill's equation, which provided an EC50 of 1.1± 0.40μM (Figure 3.3d).

Similarly, in HEK293 cells expressing GluN1/2B receptors, NPam59 exhibited a significant potentiation of NMDA currents (Fig. 3.3b and c; $F(5,34) = 164.066$, $p<0.001$). Post-hoc analysis revealed significance at doses of 0.1μM through 10μM caused significant potentiation of GluN2B-mediated currents ($p<0.001$ for each of these doses), with a maximal

potentiation of 188%. Data were also fitted to a 3 parameter Hill's equation, which provided an EC50 of 0.12 ± 0.023μM (Figure 3.3d).

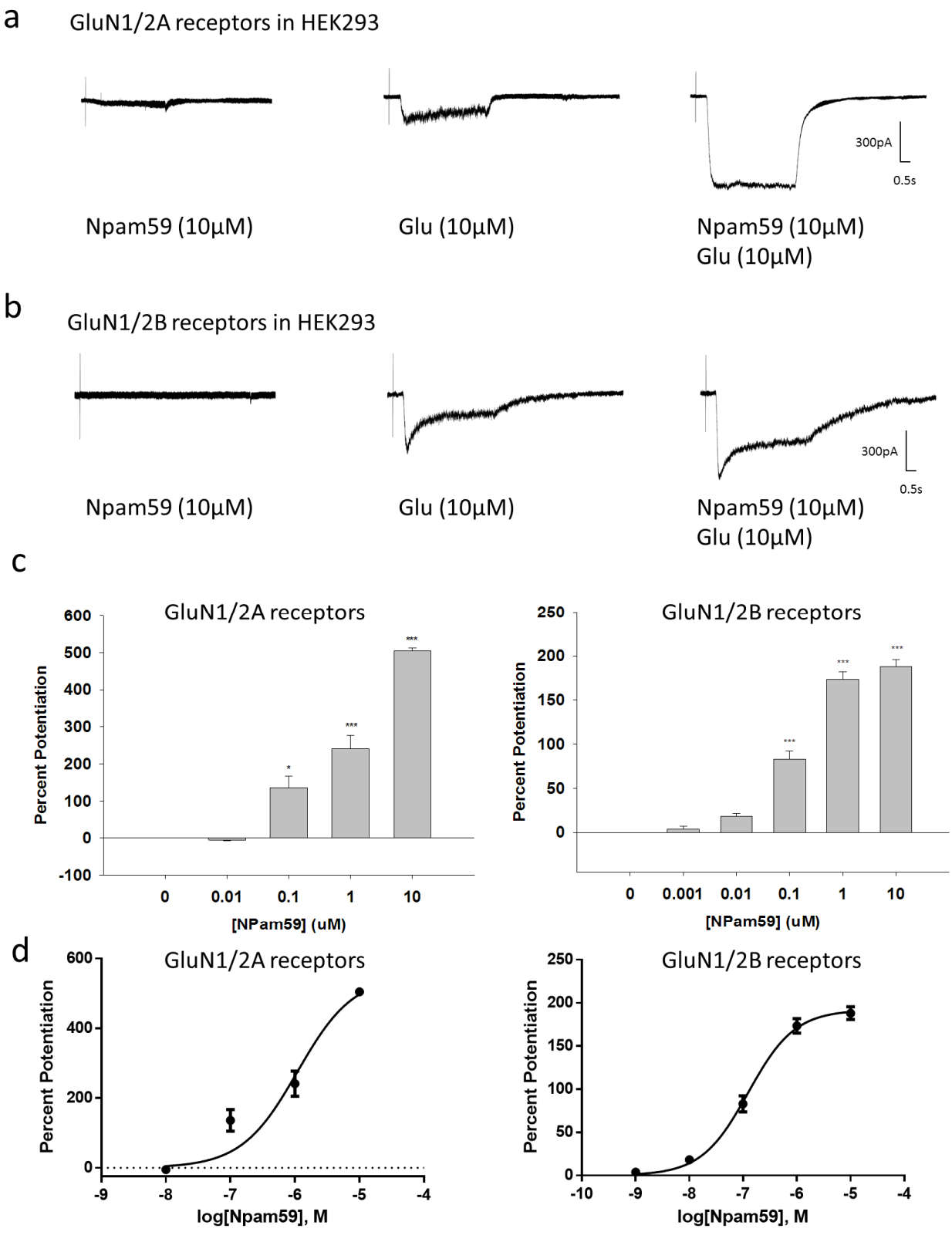

Figure 3.3 Npam59 potentiates NMDA currents in transiently transfected HEK293 cells.

(a) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 had no effect alone, but potentiated glutamate-induced currents in GluN1/GluN2A receptors. (b) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 had no effect alone, but potentiated glutamate-induced currents in GluN1/GluN2B receptors. (c) Quantification of the potentiation by different doses of Npam59 on 10μM of glutamate. GluN1/GluN2A: n=3-7 neurons per dose. One-way ANOVA, $F(4,22)=44.372$, $p<0.001$. GluN1/GluN2B: n=5-8 neurons per treatment. One-way ANOVA, $F(5,34)=164.066$, $p<0.001$. (d) 3-parameter fit of the quantified data from (c). EC50 for GluN1/GluN2A was 1.1 ± 0.40 μM, and EC50 for GluN1/GluN2B was 0.12 ± 0.023μM. Data expressed as mean ± SEM. *$p<0.05$, ***$p<0.001$ relative to control (0M Npam59).

3.2.2 Npam59 potentiates GluN2A- and GluN2B-containing receptors in hippocampal neuron cultures

Potentiation of GluN1/GluN2A and GluN1/GluN2B receptors in HEK293 cells may be due to the artificial nature of the transfected cell system. It is important to verify these findings in the more natural setting of the wild-type neuron. Whole-cell patch-clamp recordings of NMDA (10μM)-induced currents were made in hippocampal neurons maintained in primary cultures. First, total potentiation of NMDA receptors was measured without GluN2A- or GluN2B-specific blockers. Data was analyzed on a one-way ANOVA, which found a significant effect of Npam59 dose ($F(6,27)=171.347$, $p<0.001$). Post-hoc analysis revealed that doses 0.01μM and above caused significant potentiation ($p<0.001$ for each dose), with a maximal potentiation of 247%

and an EC50 of 0.044 ± 0.010 µM. We observe that in hippocampal neurons, the maximal response can be reached at a minimum of ~1µM Npam59, which was the dose chosen for the following investigations (Figure 3.4).

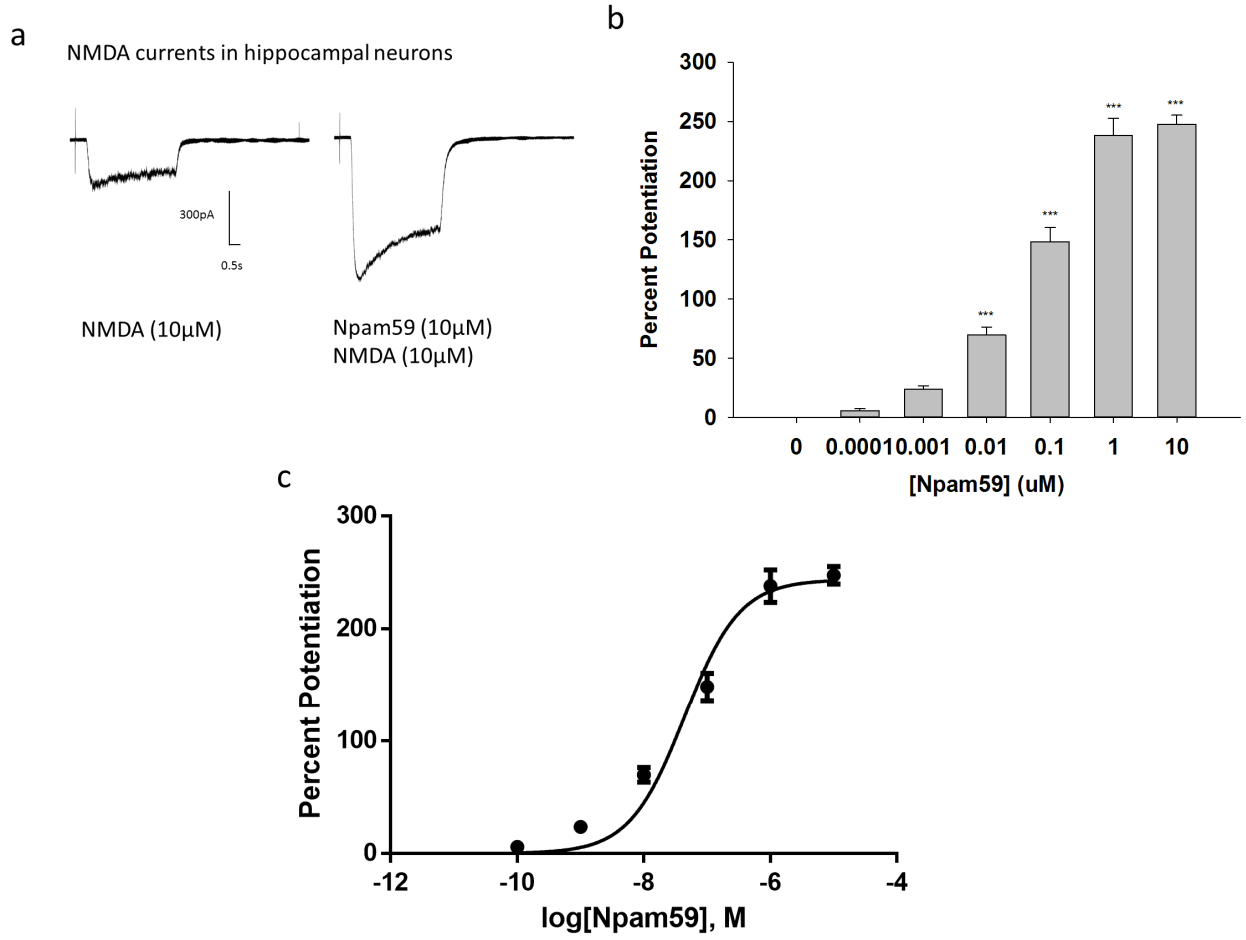

Figure 3.4 Npam59 potentiates NMDA currents in hippocampal neurons.

(a) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 potentiated NMDA-induced currents in neurons without pharmacological blockade of any NMDA receptor subtypes. (b) Quantification of the potentiation by different doses of Npam59 on 10µM of NMDA. n=4 neurons per treatment. One-way ANOVA, F(6,27)=171.347,

$p<0.001$. Data expressed as mean ± SEM. ***$p<0.001$ relative to Control (0M Npam59). (c) 3-parameter fit of the quantified data from (b). EC50 was 0.044 ± 0.010 μM. All data expressed as mean ± SEM.

To assess specificity for GluN2A-containing and GluN2B-containing receptors, we then recorded NMDA currents in the presence or absence of the GluN2A-specific blocker NVP-AAM077 (NVP 0.2μM) or the GluN2B-specific blocker ifenprodil (IF 3μM) and the effect of Npam59 (1μM) on these pharmacologically isolated NMDA currents were evaluated. At the end of each experiments, Non-subunit specific NMDAR blocker APV was applied to verify that no other non-NMDA receptor-mediated current component contributed to these currents.

For neurons treated with IF, a one-way ANOVA was conducted across treatments, which showed significance ($F(4,40) = 229.944$, $p<0.001$). Post-hoc analysis revealed that all pairwise comparisons versus control were significant ($p<0.001$ for all) except for control vs. IF+Npam59 ($p=0.257$). Additionally, there was a significant difference between IF and IF+Npam59 ($p<0.001$). We determined that IF blockade of GluN2B-containing receptors removed roughly 55% of the control baseline current, and that Npam59 was able to potentiate IF-treated neurons from 45% to 110%, representing a ~240% potentiation specific for GluN2A. This current could be blocked completely by application of APV, verifying that it is not acting through non-NMDA receptors (Figure 3.5).

To assess GluN2B-specific currents, we applied NVP instead of IF. Evoked NMDA currents were measured under the same combinations of blocker x Npam59 treatments, and a

one-way ANOVA was conducted on the total currents, which showed a significant effect of treatment ($F(4,41) = 138.539$, $p<0.001$). Post-hoc analysis revealed that all pairwise comparisons were significant vs control ($p<0.001$ for all, except control vs NVP+Npam59 $p=0.007$). Furthermore, NVP versus NVP+Npam59 was significant ($p<0.001$). We concluded that blockade of GluN2A-containing receptors by NVP removed roughly 45% of the control baseline current, and that Npam59 potentiated NVP-treated neurons from 55% to 125%, representing a ~230% potentiation specific for GluN2B. This current could be blocked completely by application of APV (50μM), verifying that it is not acting through non-NMDA receptors (Figure 3.5).

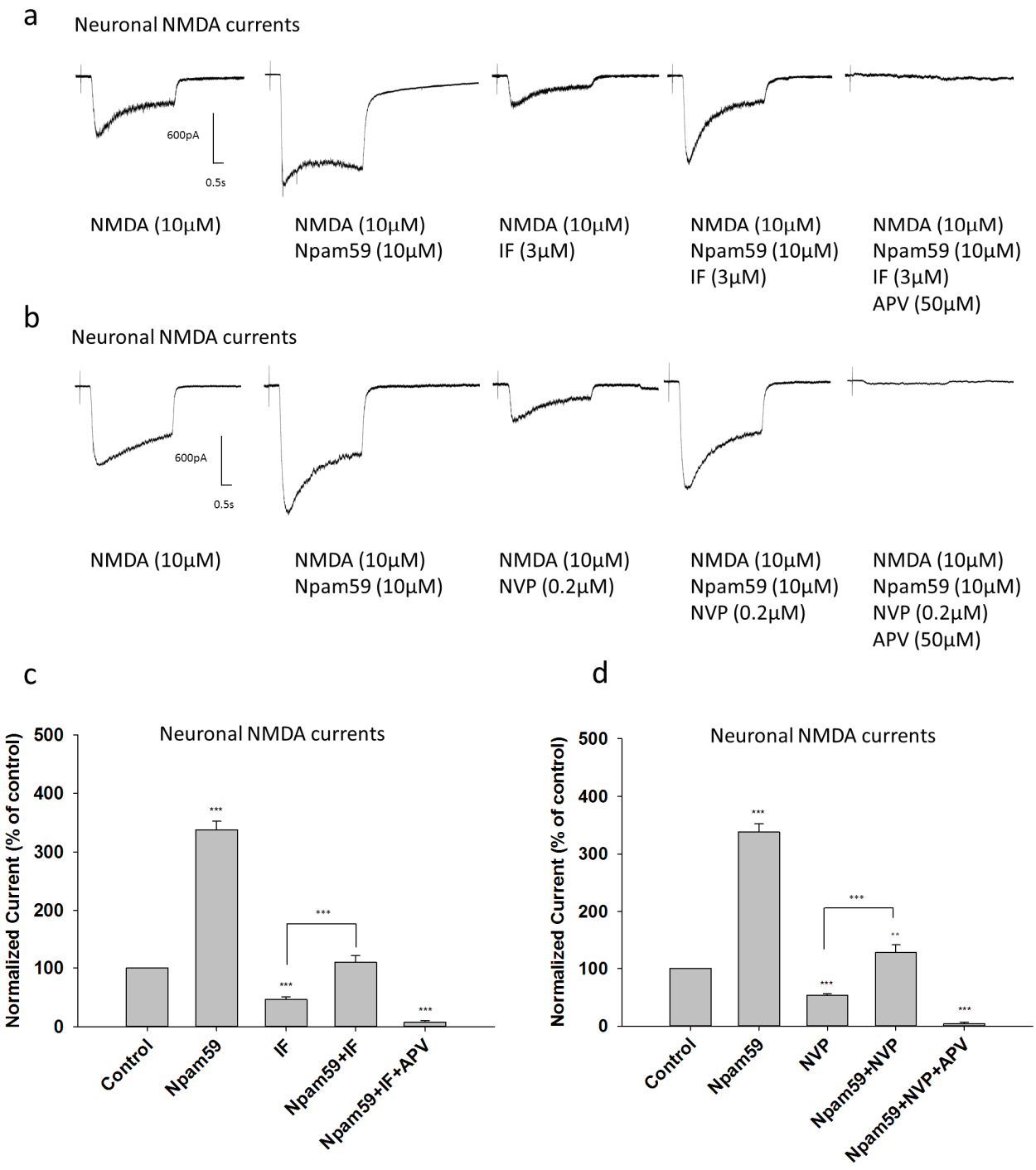

Figure 3.5 Npam59 potentiates pharmacologically isolated GluN1/GluN2A and GluN1/GluN2B currents in hippocampal neurons.

(a) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 potentiated NMDA currents even with application of IF, and that this potentiation was sensitive to blockade by APV. (b) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 potentiated NMDA currents even with application of NVP, and that this potentiation was sensitive to blockade by APV. (c) Quantification of the relative currents of the indicated combination treatments. n=4-14 per group. One-way ANOVA, $F(4,40) = 229.944$, $p<0.001$. (d) Quantification of the relative currents of the indicated combination treatments. n=4-12 per group. One-way ANOVA, $F(4,41) = 138.539$, $p<0.001$.

Data expressed as mean ± SEM. ***$p<0.001$ relative to control.

3.2.3 Npam59 does not potentiate $GABA_A$ or AMPA receptor currents

It is important to evaluate whether Npam59 selectively potentiates only NMDA receptors, or if there are other unexpected off-target effects. Two receptors were chosen for their importance as the primary ionotropic receptors mediating excitatory and inhibitory synaptic transmission: AMPA receptors and $GABA_A$ receptors. AMPA receptors are non-NMDA glutamatergic channels that are the primary excitatory ionotropic receptors responsible for mediating synaptic potentials, and $GABA_A$ ionotropic chloride channels are a key mediator of inhibitory inputs in the central nervous system (CNS). There was no potentiation by Npam59 on GABA-mediated currents ($p=0.976$), and pilot data indicates little, if any effect on AMPA-mediated currents ($p=0.0591$) as determined by two-tailed paired t-test (Figure 3.6).

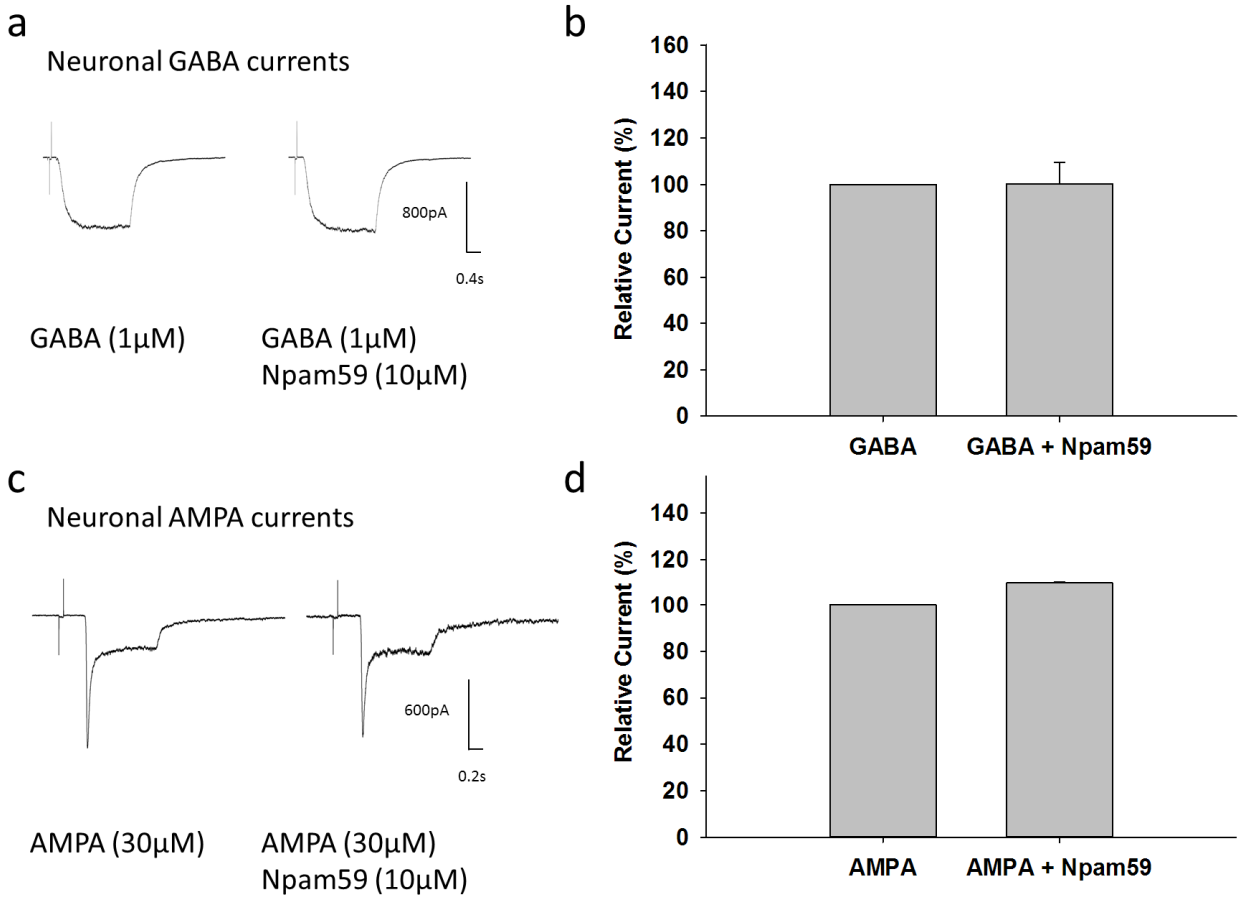

Figure 3.6 Npam59 does not potentiate GABA currents, but may potentiate AMPA currents.

(a) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 did not potentiate $GABA_A$ currents. (b) Quantification of the potentiation by Npam59 (10μM) of $GABA_A$ currents. Relative potentiation is 0.30 ± 9.3%. n=4 neurons. Two-tailed paired t-test p=0.976. (c) Representative traces from whole-cell electrophysiological recordings demonstrating that Npam59 did not potentiate AMPA currents. (d) Quantification of the potentiation by Npam59 (10μM) of AMPA currents. Relative potentiation is 9.87% ± 0.46%. n=2 neurons. Two-tailed paired t-test p=0.0591. Data expressed as mean ± SEM.

Chapter 4: Characterization of effects of Npam59 *in vivo*

4.1 Introduction

In the multi-level characterization of Npam59, we have produced an *in vitro* demonstration of allosteric potentiation of NMDA receptors by Npam59 using whole-cell recordings *in vitro* (see Chapter 3). In order to determine if Npam59 has ability to function as NMDAR potentiator in intact animals *in vivo*, our first goal was to determine if Npam59 could reach the brain following systemic administration. The blood brain barrier (BBB) exists between the capillaries in the brain and the brain itself and acts as a strict, biological filter that regulates the entry of molecules and pathogens into the CSF environment. Unlike other regions in the body, passive diffusion of substances across blood vessels in the BBB is heavily restricted by endothelial cells which lack fenestration and demonstrate limited pinocytotic activity, and are sealed to each other by tight junctions (Oldendorf et al., 1977; Stewart, 2000; Abbott, 2005; Hawkins and Davis, 2005). Any transport of hydrophilic compounds desirable to the brain, such as glucose, amino acids, transferrin, and many other nutrients and hormones is accomplished by active transporter proteins expressed in the endothelial cells (Abbott et al., 2006; Rip et al., 2009). However, small lipophilic compounds can often cross the BBB with little hindrance (Rubin and Staddon, 1999), although possessing too much lipophilicity might trap a drug into the capillary bed without reaching the brain parenchyma (Banks, 2009). As surmounting the BBB has proven to be a major obstacle for many drug development efforts (Neuwelt et al., 2008), the first goal in this chapter was to determine if Npam59 can cross the BBB following a peripheral systemic application at the concentrations required for potentiating the function of NMDA receptors.

Once it could be demonstrated the Npam59 reaches the brain at therapeutically relevant doses, our next goal was to demonstrate that Npam59 could potentiate the function of NMDARs in the brain of intact animals as we observed in the *in vitro* experiments above. As an allosteric modulator, we assumed that Npam59 may not have an overt effect alone under basal, unstimulated conditions. Numerous studies have consistently demonstrated that the d-amphetamine (d-amph) can reliably induce DA release in the nucleus accumbens (NAc) (Kelly et al., 1975; Zetterstrom et al., 1983; Adams et al., 2002; Shoblock et al., 2003) and importantly, that this release is sensitive to modulation by NMDA receptors in the NAc (Bristow et al., 1994; Miller and Abercrombie, 1996; Darracq et al., 2001). We therefore hypothesized that if Npam59 can potentate native NMDA receptors in the brain of intact animals, then we should observe an effect on d-amph-induced DA release in the NAc. Thus, we chose micro-dialysis in the NAc to evaluate the effects of Npam59 on d-amph-induced DA release as an indirect readout of Npam59 allosteric potentiation of NMDARs.

4.2 Results

4.2.1 Npam59 crosses the blood-brain barrier following intraperitoneal and intravenous administration in the rat

Prior to our micro-dialysis studies, we verified that Npam59 could penetrate into the brain. Here, we characterize the bioavailability of Npam59 to the brain and blood by peripherally applying the drug to animals and collecting CSF and serum samples at 0.5h, 1h, 2h, 4h, and 8h (Figure 4.1). Npam59 was detectable by HPLC in blood plasma and cerebrospinal fluid (CSF) at 30 minutes post-i.p. injection, which was the earliest timepoint taken which allows for animals to reach anesthesia and for CSF collection to occur. A 1mg/kg i.p. dose roughly corresponded to a

5.2µM concentration in the CSF at 30 mins post-injection which decayed with a half-life of 1.142h, determined by a nonlinear fit with one phase decay. Intravenous (i.v.) delivery through the tail vein was also tested and was found to be superior, delivering 17.4µM Npam59 at 30 mins to the CSF with a half-life of 2.745 hrs at a dose of 1mg/kg, likely due to bypass of first-pass hepatic metabolism. However, for our purposes, i.p. injections were deemed sufficiently effective, without enough justification for choosing i.v. tail injections or i.v. catheterization surgery given the additional stress on the animals and the significantly increased logistical requirements.

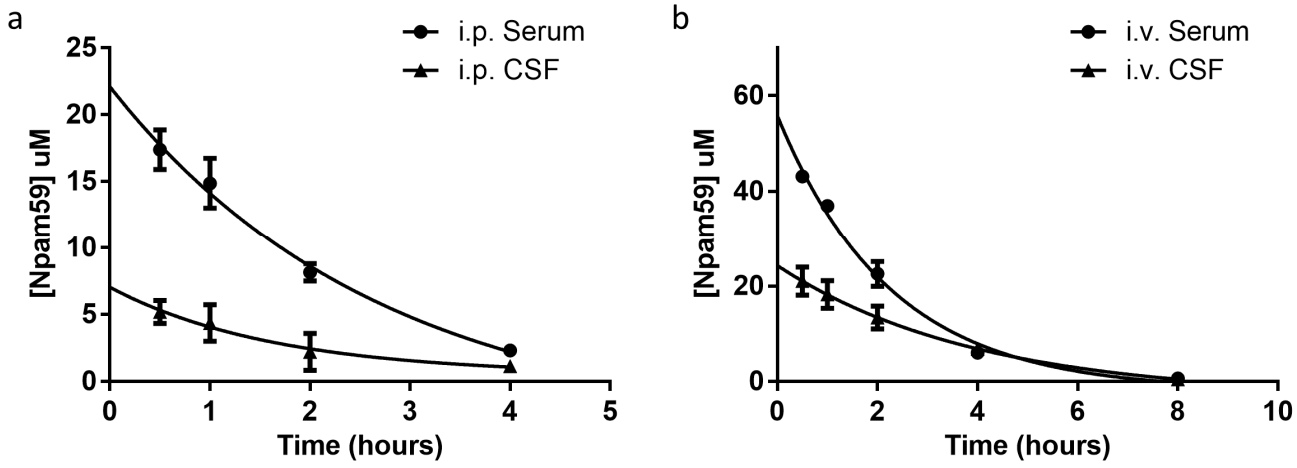

Figure 4.1 CSF and serum concentrations of Npam59 following i.p. and i.v. injection at a dose of 1mg/kg.

Rats were injected with Npam59 either i.p. or i.v. through tail vein, then sacrificed for CSF and blood collection at the indicated time points. (a) Decay of CSF and serum concentrations of Npam59 over time following i.p. injection. A one-phase decay fit found a half-life of 1.863h and 1.142h in Serum and CSF, respectively. n=4. (b) Decay of CSF and serum concentrations of

Npam59 over time following i.v. injection. A one-phase decay fit found a half-life of 1.557h and 2.120h in Serum and CSF, respectively. n=5. Data expressed as mean ± SEM.

We next linearly extrapolated appropriate doses for an i.p. injection from this data. Given that Npam59 reaches saturation near 1μM in neuron electrophysiology and that 1mg/kg injection caused CSF Npam59 concentrations to reach 5.2μM at 30 mins, we determined that an ideal i.p. dose would be 0.25mg/kg, which was estimated to produce a concentration of 5.2μM/4=1.3μM CSF at 30 minutes, allowing for a period of ~0.5-1h post-injection wherein the CSF concentration would remain near 1μM when accounting for the drug's biological half-life. A high dose of 3.0mg/kg was also chosen, which was estimated to deliver Npam59 at a CSF concentration of 5.2μM*3=15.6μM, which is well into the saturation range. This dose was expected to maximize Npam59's duration of action when accounting for its half-life and was also the limit of solubility in the vehicle formulation without using different volumes of injection compared to 0.25mg/kg of Npam59. We also decided to separate the timing of Npam59 injection and d-amphetamine (d-amph) injection by 20 mins to coincide the maximal measured CSF concentration of Npam59 with the delivery of the d-amph.

4.2.2 Npam59 given alone has no effect on dopamine release in the nucleus accumbens

Moving onto micro-dialysis experiments, we first assessed if NPam59 exhibited any effects in the NAc neurochemistry when given alone without d-amph. Animals were implanted with a guide cannula in preparation for micro-dialysis of the NAc. On day of testing, micro-dialysis probes were inserted into the NAc and dialysate samples were collected at 10 minute

intervals for analysis in high performance liquid chromatography. Peaks were measured for DA, DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA). Npam59 was administered via i.p. injection. Consistent with our prediction, as an allosteric NMDAR modulator, Npam59 induced no change in DA level in the NAc at either a low dose (0.25mg/kg) or high dose (3mg/kg) over the course of ~6 hrs (Figure 4.2).

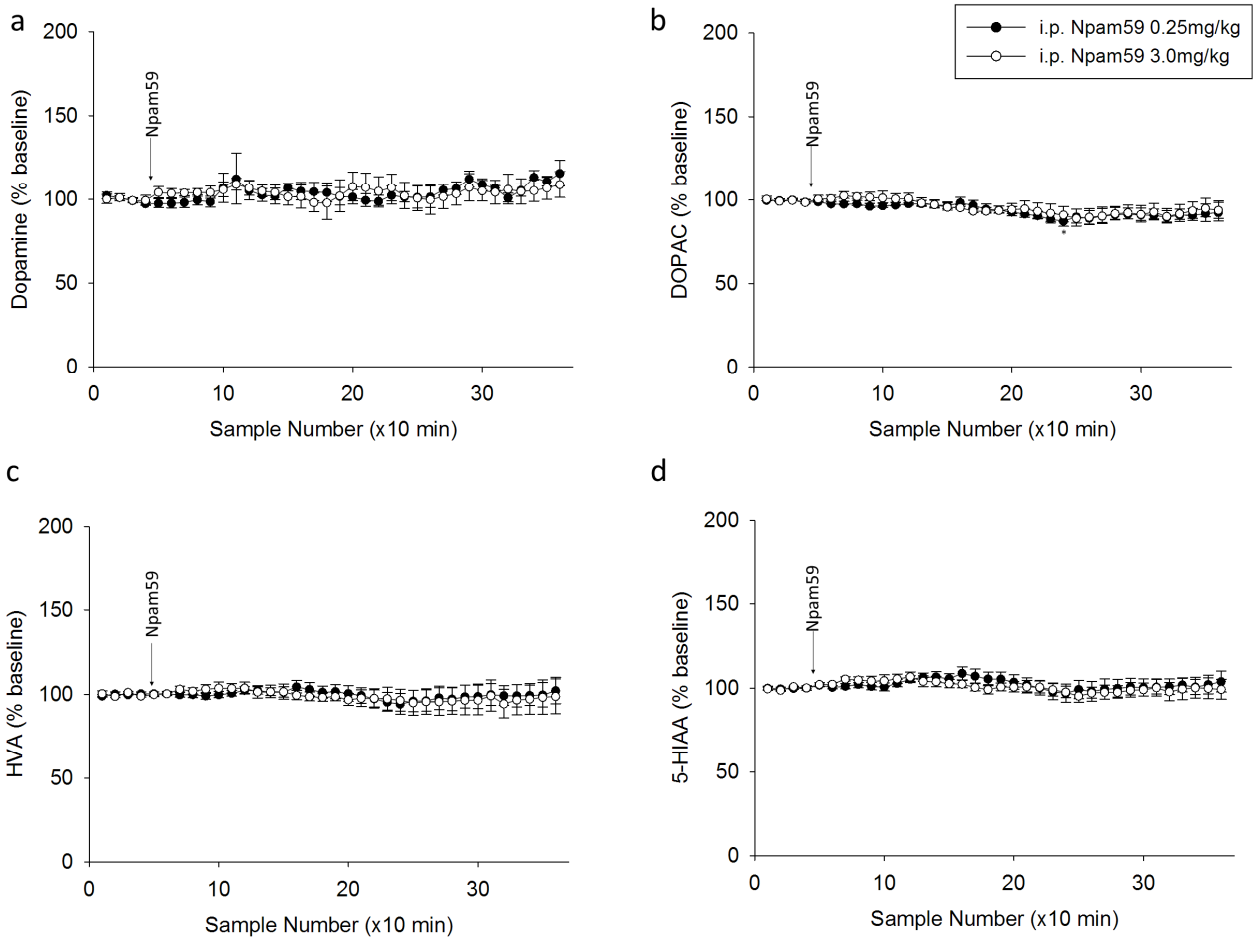

Figure 4.2 Effect of Npam59 on dopamine, DOPAC, HVA, and 5-HIAA release in the NAc.

Animals were injected i.p. with Npam59 at the arrows and at indicated doses. No effect was observed following injection with Npam59 in each of the four readouts. One-way ANOVAs

were performed to determine if there was a difference in response over time. Traces indicate levels of neurotransmitters and metabolites measured from the same dialysate samples. n=5 rats in the 3.0mg/kg treatment group and n=6 rats in the 0.25mg/kg treatment group. (a) Traces for DA levels as a percentage of baseline over time. One-way ANOVA 3mg/kg Npam59, $F(32,164)=0.378$, $p=0.999$. One-way ANOVA 0.25mg/kg NPam59, $F(32,197)=1.139$, $p=0.295$. (b) Traces for DOPAC levels as a percentage of baseline over time. One-way ANOVA 3mg/kg Npam59, $F(32,164)=2.403$, $p<0.001$. Post-hoc analysis did not show any significance in pairwise comparisons between any time points and baseline. One-way ANOVA 0.25mg/kg NPam59, $F(32,197)=2.452$, $p<0.001$. Post hoc analysis shows a significant difference at a single time point with $p=0.015$. *$p<0.05$ compared to baseline. (c) Traces for HVA levels as a percentage of baseline over time. One-way ANOVA 3mg/kg Npam59, $F(32,164)=0.419$, $p=0.997$. One-way ANOVA 0.25mg/kg NPam59, $F(32,197)=0.554$, $p=0.974$. (d) Traces of 5-HIAA over time. One-way ANOVA 3mg/kg Npam59, $F(32,164)=1.274$, $p=0.174$. One-way ANOVA 0.25mg/kg NPam59, $F(32,197)=1.099$, $p=0.342$. Data are presented as means ± SEM.

4.2.3 Npam59 at a lower, but not a higher dose potentiated d-amphetamine-induced release of dopamine in the nucleus accumbens

Next, we used d-amph to artificially increase DA release in the NAc in order to evaluate whether Npam59 can modulate DA release via its effect on NMDA receptors. d-Amph has multiple actions on DA release upon entering the cell following uptake by the dopamine transporter (DAT). Once inside the cell, it reverses DAT (Heal et al., 2013), increases

mobilization of synaptic vesicles from the reserve pool to the readily releasable pool (Covey et al., 2013), and also increases firing of dopaminergic neurons (Underhill et al., 2014). A low dose of 0.25mg/kg amph was chosen in order to elicit a moderate release of DA to optimize sensitivity for modulating effects of Npam59.

We found that Npam59 (0.25mg/kg) significantly potentiated d-amph-induced DA release from an 89% increase to a 165% increase (Figure 4.3a). The potentiation was observed as a large increase in the peak DA release without a subjective change to the timing and shape of the DA release curve over time (Figure 4.2a). Interestingly, when the dose of Npam59 was increased to 3.0mg/kg, no effect on any of the measured variables was observed (Fig 4.3a). A two-way repeated measures ANOVA for time × treatment was conducted on the DA release data, which showed a significant interaction of the two factors ($F(40,335)=2.363$, $p<0.001$). Post-hoc analysis revealed that the timepoints for peak DA release were significantly different between d-amph+vehicle versus combination treatment of Npam59 (0.25mg/kg) and d-amph, but not versus Npam59 (3.0mg/kg).

d-Amph alone also induced a decrease in HVA and DOPAC, and an increase in 5-HIAA over time coinciding with its effects on DA (Figure 4.2b-d). These results were consistent with previous experiments in the lab (not shown) and with results reported elsewhere (Miele et al., 2000). Interestingly, Npam59 at a low dose potentiated d-amph-induced DA release without effects on DOPAC (Two-way repeated measures (RM) ANOVA of time × treatment, $F(40,335)=0.320$, $p=1.000$. No main effect of treatment $F(2,335)=0.0448$, $p=0.956$. Main effect of time $F(20,335)=36.428$, $p<0.001$) or HVA (Two-way RM ANOVA of time × treatment, $F(40,335)=0.429$, $p=0.999$. No main effect of treatment $F(2,335)=0.112$, $p=0.895$. Main effect of time $F(20,335)=5.701$, $p<0.001$). A null effect of Npam59 was also observed for 5HIAA levels

(Two-way RM ANOVA of time × treatment, F(40,335)=1.015, p=0.452. No main effect of treatment F(2,335)=1.173, p=0.341. Main effect of time F(20,335)=5.531, p<0.001)

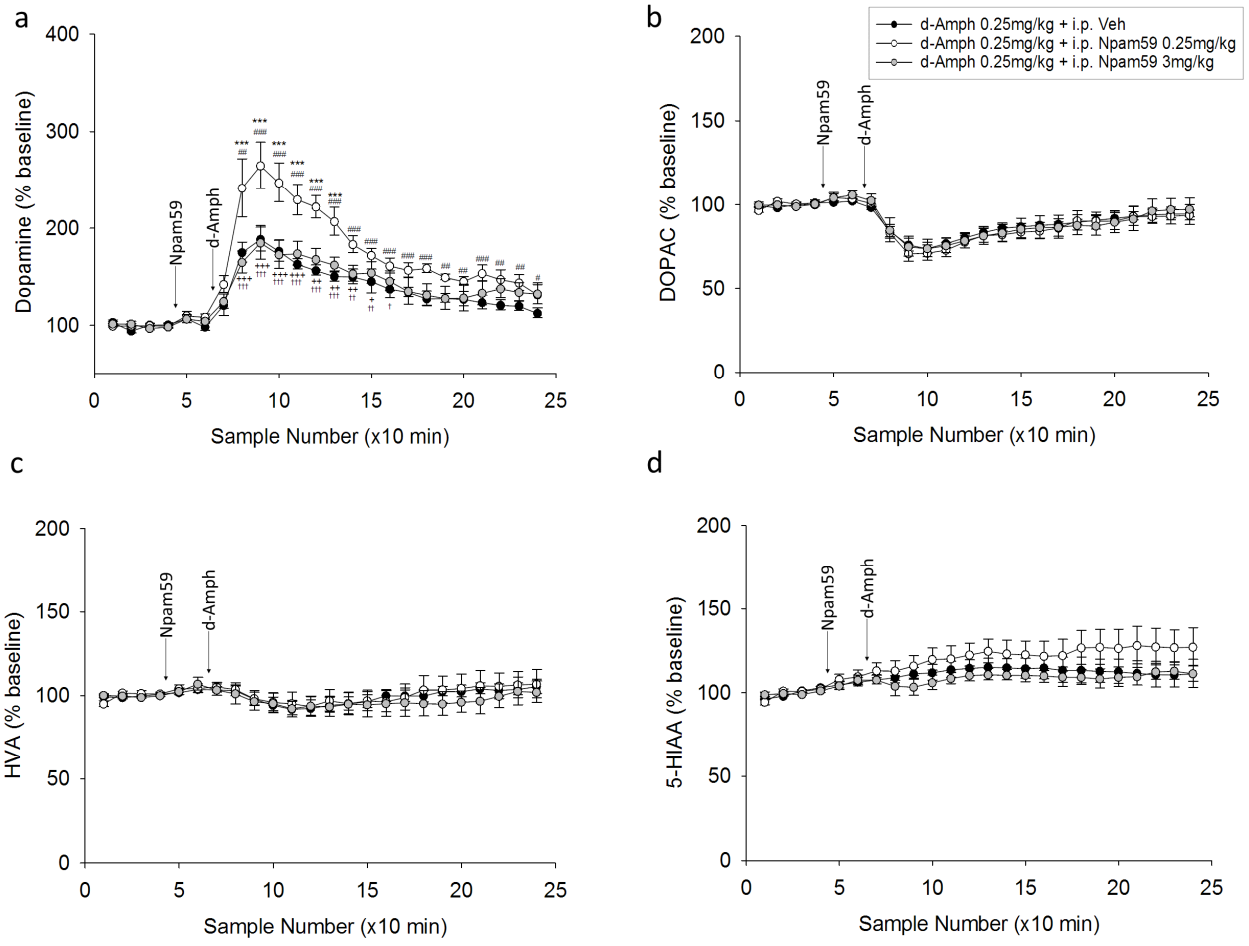

Figure 4.3 Effect of systemic high and low dose Npam59 on d-amph-induced changes in dopamine, DOPAC, HVA, and 5-HIAA levels in the NAc.

Animals were injected i.p. with Npam59 (0.25mg/kg or 3.0mg/kg) or Veh at the first arrows and with d-amph (0.25mg/kg) at the second arrow. Npam59 significantly potentiated d-amph-induced DA release in the NAc, but did not affect DOPAC, HVA, or 5-HIAA. Traces indicate

levels of neurotransmitters and metabolites measured from the same dialysate samples. n=5-6 for each treatment combination. (a) Traces for DA levels as a percentage of baseline over time. Two-way ANOVA, treatment × time, $F(40,335)=0.2.363$, $p<0.001$. Post-hoc reveals significant pairwise comparisons as indicated: Comparisons of treatments vs. d-amph+Veh control within each time bin, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Within treatment differences compared to baseline level in d-amph + 0.25mg/kg Npam59, #$p<0.05$, ##$p<0.01$, ###$p<0.001$. Within treatment differences compared to baseline level in d-amph + 3mg/kg Npam59, † $p<0.05$, † † $p<0.01$, † † †$p<0.001$. Within treatment differences compared to baseline in d-amph+Veh, +$p<0.05$, ++$p<0.01$, +++$p<0.001$. (b) Traces for DOPAC levels as a percentage of baseline over time. Two-way repeated measures (RM) ANOVA of time × treatment, $F(40,335)=0.320$, $p=1.000$. No main effect of treatment $F(2,335)=0.0448$, $p=0.956$. Main effect of time $F(20,335)=36.428$, $p<0.001$. (c) Traces for HVA levels as a percentage of baseline over time. Two-way RM ANOVA of time × treatment, $F(40,335)=0.429$, $p=0.999$. No main effect of treatment $F(2,335)=0.112$, $p=0.895$. Main effect of time $F(20,335)=5.701$, $p<0.001$ (d) Traces for 5-HIAA levels as a percentage of baseline over time. Two-way RM ANOVA of time × treatment, $F(40,335)=1.015$, $p=0.452$. No main effect of treatment $F(2,335)=1.173$, $p=0.341$. Main effect of time $F(20,335)=5.531$, $p<0.001$. All data expressed as mean ± SEM.

The effect of Npam59 on DA efflux evoked by a higher dose of 1.5mg/kg of d-amph was tested to determine the presence and magnitude of potentiation as d-amph dosage is modified. We observed a robust potentiation in the peak DA release from 760% of baseline to 1570% of

baseline (Figure 4.4a). A two-way repeated measures ANOVA was conducted, which showed a significant interaction between time × treatment ($F(20,272)=4.750$, $p<0.001$). Post-hoc analysis revealed that the timepoints for peak DA release were significantly different in animals receiving Npam59 injection. Subjectively, the peak time in DA release for the combination treatment was also sooner. Recapitulating data with 0.25mg/kg of d-amph, there were no significant differences in DOPAC (Two-way RM ANOVA, time × treatment $F(20,272)=0.650$, $p=0.871$. No main effect of treatment $F(1,272)=0.650$, $p=0.871$. Main effect of time $F(20,272)=110.876$, $p<0.001$) HVA (Two-way RM ANOVA, time × treatment $F(20,272)=1.192$, $p=0.263$. No main effect of treatment $F(1,272)=0.0143$, $p=0.907$. Main effect of time $F(20,272)=26.722$, $p<0.001$), or 5-HIAA (Two-way RM ANOVA, time × treatment $F(20,272)=1.213$, $p=0.245$. No main effect of treatment $F(1,272)=1.263$, $p=0.285$. Main effect of time $F(20,272)=37.777$, $p<0.001$) following Npam59 injection in the combination treatment (Figure 4.4b-d).

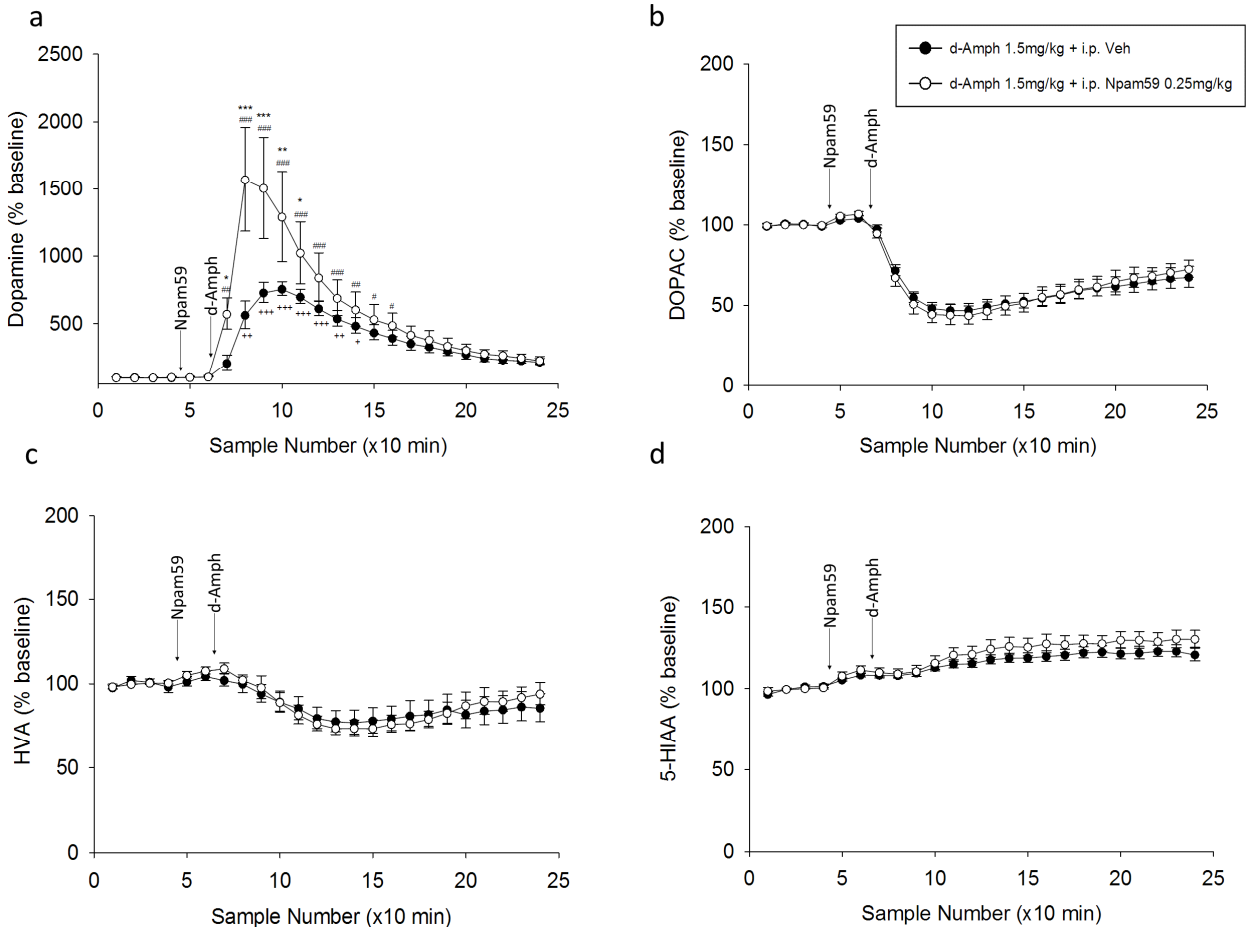

Figure 4.4 Effect of low dose Npam59 on high-dose d-amph-induced changes in dopamine, DOPAC, HVA, and 5-HIAA levels in the NAc.

Animals were injected i.p. with Npam59 (0.25mg/kg) or Veh at the first arrow and with d-amph (1.5mg/kg) at the second arrow. Npam59 significantly potentiated d-amph-induced DA release in the NAc, but did not affect DOPAC, HVA, or 5-HIAA. Traces indicate levels of neurotransmitters and metabolites measured from the same dialysate samples. n=6-7 for each treatment combination. (a) Traces for DA levels as a percentage of baseline over time. Two-way ANOVA, treatment × time, $F(20,272)=4.750$, $p<0.001$. Post-hoc analysis reveals significant pairwise comparisons as indicated: Comparisons of d-amph+Npam59 vs. d-amph+Veh control

within each time bin, *p<0.05, **p<0.01, ***p<0.001. Within treatment differences compared to baseline level in d-amph + Npam59, #p<0.05, ##p<0.01, ###p<0.001. Within treatment differences compared to baseline in d-amph+Veh, +p<0.05, ++p<0.01, +++p<0.001. (b) Traces for DOPAC levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,272)=0.650$, $p=0.871$. No main effect of treatment $F(1,272)=0.650$, $p=0.871$. Main effect of time $F(20,272)=110.876$, $p<0.001$ (c) Traces for HVA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,272)=1.192$, $p=0.263$. No main effect of treatment $F(1,272)=0.0143$, $p=0.907$. Main effect of time $F(20,272)=26.722$, $p<0.001$ (d) Traces for 5-HIAA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,272)=1.213$, $p=0.245$. No main effect of treatment $F(1,272)=1.263$, $p=0.285$. Main effect of time $F(20,272)=37.777$, $p<0.001$. All data expressed as mean ± SEM.

4.2.4 Npam59 reverse dialyzed into the nucleus accumbens potentiated d-amphetamine-induced dopamine release

Given the peripheral route of administration, it was unknown in which brain regions Npam59 might act to potentiate DA release. The presence of dialysis probes in the NAc during neurochemical studies permitted the administration of Npam59 directly into this region of the brain. Therefore, given the presence of NMDA receptors in the NAc as described in the introduction of this chapter, we decided it would be valuable to test if Npam59 would work if applied locally into the NAc by reverse dialysis.

Briefly, Npam59 was dissolved into aCSF dialysis solution at a concentration of 1.25μM, estimated to be equivalent to the peak CSF concentration from an i.p. dose of 0.25mg/kg i.p., based on calculations in Section 4.2.1. Dialysis input solutions were swapped once animals demonstrated a stable baseline in DA, and 20 minutes after the switch, d-amph (0.25mg/kg) was injected i.p. Npam59 was reverse dialyzed over the entire course of this experiment.

Locally applied Npam59 in the NAc potentiated the d-amph-induced release of DA (Figure 4.5a, Two-way RM ANOVA, time × treatment $F(20,293)=2.247$, $p=0.002$), roughly doubling the DA response similar to its effect following i.p. injection, as described in Section 4.2.3. Post-hoc analysis showed that there were differences in the peak DA release, and consistent with all previous observations, there were no significant differences from NPam59 treatment on DOPAC (Two-way RM ANOVA, time × treatment $F(20,293)=1.145$, $p=0.304$. No main effect of treatment $F(1,293)=0.546$, $p=0.474$. Main effect of time $F(20,293)=17.446$, $p<0.001$) HVA (Two-way RM ANOVA, time × treatment $F(20,272)=1.192$, $p=0.263$. No main effect of treatment $F(1,293)=0.701$, $p=0.419$. Main effect of time $F(20,293)=3.660$, $p<0.001$), or 5-HIAA (Two-way RM ANOVA, time × treatment $F(20,272)=1.213$, $p=0.245$. No main effect of treatment $F(1,293)=1.412$, $p=0.256$. Main effect of time $F(20,293)=1.924$, $p<0.012$. Figure 4.5b-d).

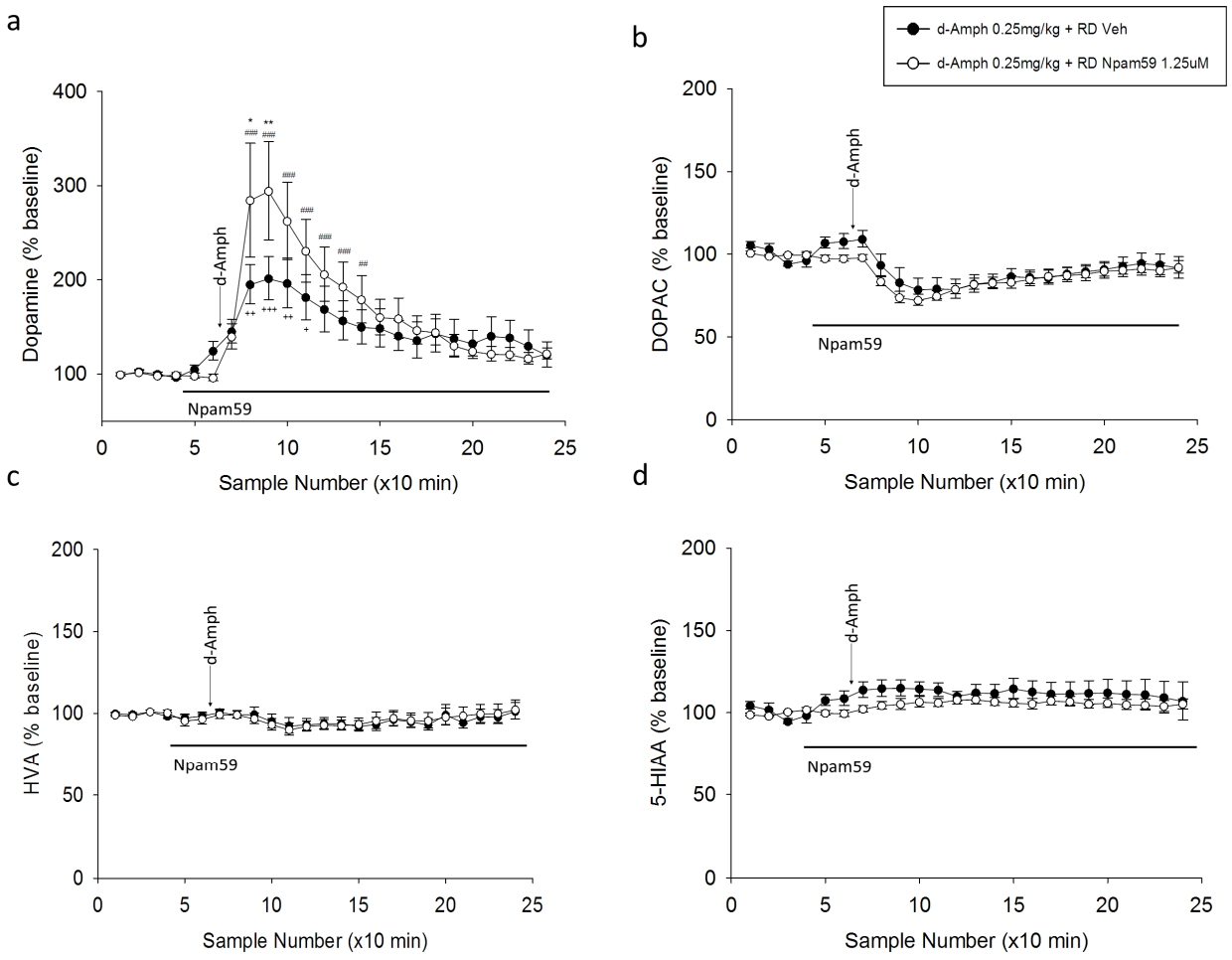

Figure 4.5 Effect of reverse-dialyzed Npam59 (1.25µM) on d-amph-induced changes in dopamine, DOPAC, HVA, and 5-HIAA levels in the NAc.

Animals were reverse-dialyzed with Npam59 (1.25µM) or vehicle for the duration of the black bar and injected with d-amph as indicated by the arrow. Reverse-dialyzed Npam59 significantly potentiated d-amph-induced DA release in the NAc, but did not affect DOPAC, HVA, or 5-HIAA. Traces indicate levels of neurotransmitters and metabolites measured from the same dialysate samples. n=9-11 for each treatment combination. (a) Traces for DA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,293)=2.247$,

p=0.002. Post-hoc analysis reveals significant pairwise comparisons as indicated: Comparisons of d-amph+ RD Npam59 vs. d-amph+RD Veh control within each time bin, *p<0.05, **p<0.01, ***p<0.001. Within treatment differences compared to baseline level in d-amph + RD Npam59, #p<0.05, ##p<0.01, ###p<0.001. Within treatment differences compared to baseline in d-amph+RD Veh, +p<0.05, ++p<0.01, +++p<0.001. (b) Traces for DOPAC levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,293)=1.145$, $p=0.304$. No main effect of treatment $F(1,293)=0.546$, $p=0.474$. Main effect of time $F(20,293)=17.446$, $p<0.001$ (c) Traces for HVA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,272)=1.192$, $p=0.263$. No main effect of treatment $F(1,293)=0.701$, $p=0.419$. Main effect of time $F(20,293)=3.660$, $p<0.001$ (d) Traces for 5-HIAA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,272)=1.213$, $p=0.245$. No main effect of treatment $F(1,293)=1.412$, $p=0.256$. Main effect of time $F(20,293)=1.924$, $p<0.012$. All data expressed as mean ± SEM.

4.2.5 Co-administration of focal MK-801 into the NAc and systemic Npam59 attenuates d-amph-induced dopamine release in the NAc.

To ascertain whether the effect of Npam59 on amph-induced DA release in the NAc is indeed mediated by its allosteric potentiation of NMDARs, in the reciprocal experiment to RD application of Npam59 in the NAc, we applied Npam59 systemically in conjunction with reverse dialysis of MK-801 into the NAc. MK-801 at low doses is thought to exhibit use-dependent blockade of both GluN2A and GluN2B-containing NMDA receptors. Its binding site on the

receptor is only exposed in the open conformation, allowing it to preferentially target receptors receiving the most stimulation (Huettner and Bean, 1988; Rosenmund and Westbrook, 1993). In this way, its function is directly opposed to that of a positive allosteric modulator such as Npam59. Furthermore, reverse dialysis of low-dose MK-801 into the NAc has no effect on DA release in the NAc (Kretschmer, 1999), removing a potential confounding signal which would be present with PCP, APV, or others (Yan et al., 1997; Kretschmer, 1999; Mathe et al., 1999; Hatip-Al-khati et al., 2001), supporting the use of MK-801 as the most preferred NMDA receptor antagonists for use in this experiment.

Results from this experiment were expected to show if any additional brain regions have contributions in potentiating d-amph-induced DA release in the NAc, as well as to demonstrate that the potentiating effects of Npam59 are sensitive to NMDA receptor blockade.

For this next portion of the study, due to resource restraints, we were limited to exclusive usage of a group of animals previously exposed to d-amph from micro-dialysis and a group of animals exposed to the same dose in the behavior experiment in Chapter 5. As a result, our experiment had a two-part design to maximize use of remaining animals. In the first part, we assessed whether MK-801 affected DA or metabolite levels in the NAc when applied by reverse dialysis; in the second, we assessed whether MK-801 could block the potentiating effects of Npam59 on d-amph.

The first experiment showed that MK-801 (1μM) did not affect release of DA before or after d-amph was given in animals with prior history, despite an increase in DA release compared to naïve animals (Figure 4.6a, two-way RM ANOVA, time × treatment $F(20,167)=0.752$, $p=0.765$. No main effect of treatment $F(1,167)=0.152$, $p=0.710$. Main effect of

time $F(20,167)=32.202$, $p<0.001$). Furthermore, MK-801 also had no effect on any of the three metabolites measured (Figure 4.6b-d. DOPAC: Two-way RM ANOVA, time × treatment $F(20,167)=0.627$, $p=0.885$. No main effect of treatment $F(1,167)=0.581$, $p=0.475$. Main effect of time $F(20,167)=30.709$, $p<0.001$. HVA: Two-way RM ANOVA, time × treatment $F(20,167)=0.853$, $p=0.646$. No main effect of treatment $F(1,167)=0.376$, $p=0.562$. Main effect of time $F(20,167)=7.125$, $p<0.001$. 5-HIAA: Two-way RM ANOVA, time × treatment $F(20,167)=0.296$, $p=0.999$. No main effect of treatment $F(1,167)=0.155$, $p=0.707$. No main effect of time $F(20,167)=1.432$, $p=0.120$). A null-effect of MK-801 on the basic phenomenon d-amph-induced DA release allows us to apply it as an NMDA receptor blocker against Npam59 with minimal confound.

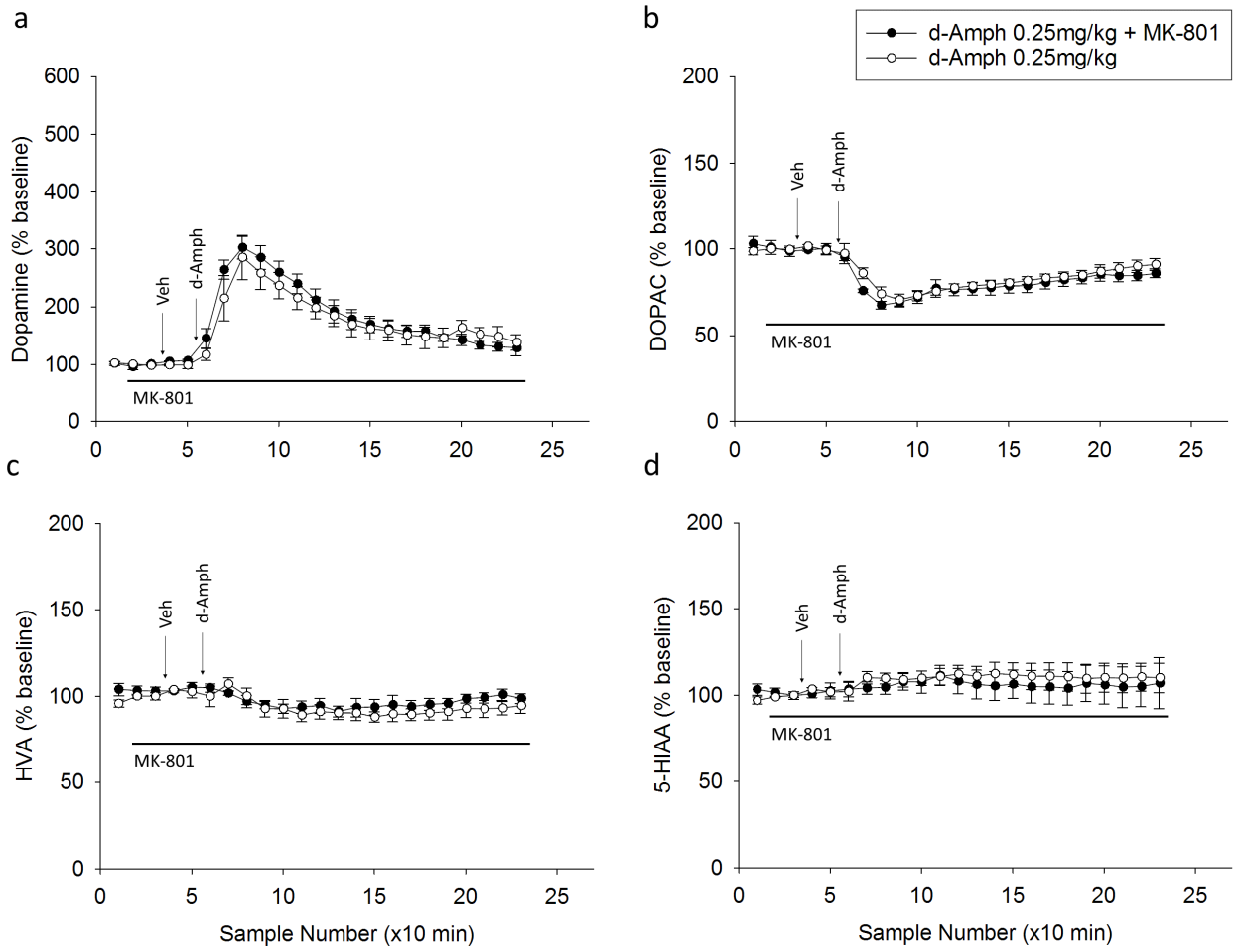

Figure 4.6 Effect of MK-801 on d-amph-induced changes in dopamine, DOPAC, HVA, and 5-HIAA levels in the NAc.

Animals previously exposed to d-amph were reverse-dialyzed with MK-801 (1.0µM) or vehicle (regular aCSF) for the duration of the black bar and injected with Npam59 vehicle formulation i.p. at the first arrow and d-amph (0.25mg/kg) i.p. at the second arrow. MK-801 did not affect levels of DA, DOPAC, HVA, or 5-HIAA in the NAc before or after treatment with d-amph. Traces indicate levels of neurotransmitters and metabolites measured from the same dialysate samples. n=4-5 for each treatment group. (a) Traces for DA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,167)=0.752$, $p=0.765$. No main effect

of treatment F(1,167)=0.152, p=0.710. Main effect of time F(20,167)=32.202, p<0.001. (b) Traces for DOPAC levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment F(20,167)=0.627, p=0.885. No main effect of treatment F(1,167)=0.581, p=0.475. Main effect of time F(20,167)=30.709, p<0.001. (c) Traces for HVA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment F(20,167)=0.853, p=0.646. No main effect of treatment F(1,167)=0.376, p=0.562. Main effect of time F(20,167)=7.125, p<0.001. (d) Traces for 5-HIAA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment F(20,167)=0.296, p=0.999. No main effect of treatment F(1,167)=0.155, p=0.707. No main effect of time F(20,167)=1.432, p=0.120. All data expressed as mean ± SEM.

When Npam59 (0.25mg/kg) was applied to previously-exposed animals with similar history, it significantly potentiated d-amph-induced DA release consistent with our previous findings (Two-way RM ANOVA, time × treatment F(20,125)=2.374, p=0.010). When MK-801 was reverse-dialyzed into the NAc, the potentiation was abolished, demonstrating that Npam59 potentiates DA release in the NAc via NMDA receptors also located in the NAc. (Figure 4.7a). However, while the statistics are in our favor, the experiment requires reproduction with naïve animals and larger sample size for us to make this conclusion with confidence. There were no other effects on DOPAC, HVA, or 5-HIAA by either MK-801, NPam59, or their combination on the changes induced by d-amph (Figure 4.7b-d. DOPAC: Two-way RM ANOVA, time × treatment F(20,125)=0.875, p=0.616. No main effect of treatment F(1,125)=0.724, p=0.484.

Main effect of time $F(20,125)=19.000$, $p<0.001$. HVA: Two-way RM ANOVA, time × treatment $F(20,125)=1.512$, $p=0.131$. No main effect of treatment $F(1,125)=4.105$, $p=0.180$. No main effect of time $F(20,125)=1.652$, $p=0.087$. Two-way RM ANOVA, time × treatment $F(20,125)=1.031$, $p=0.451$. No main effect of treatment $F(1,125)=1.754$, $p=0.316$. No main effect of time $F(20,125)=1.589$, $p=0.105$).

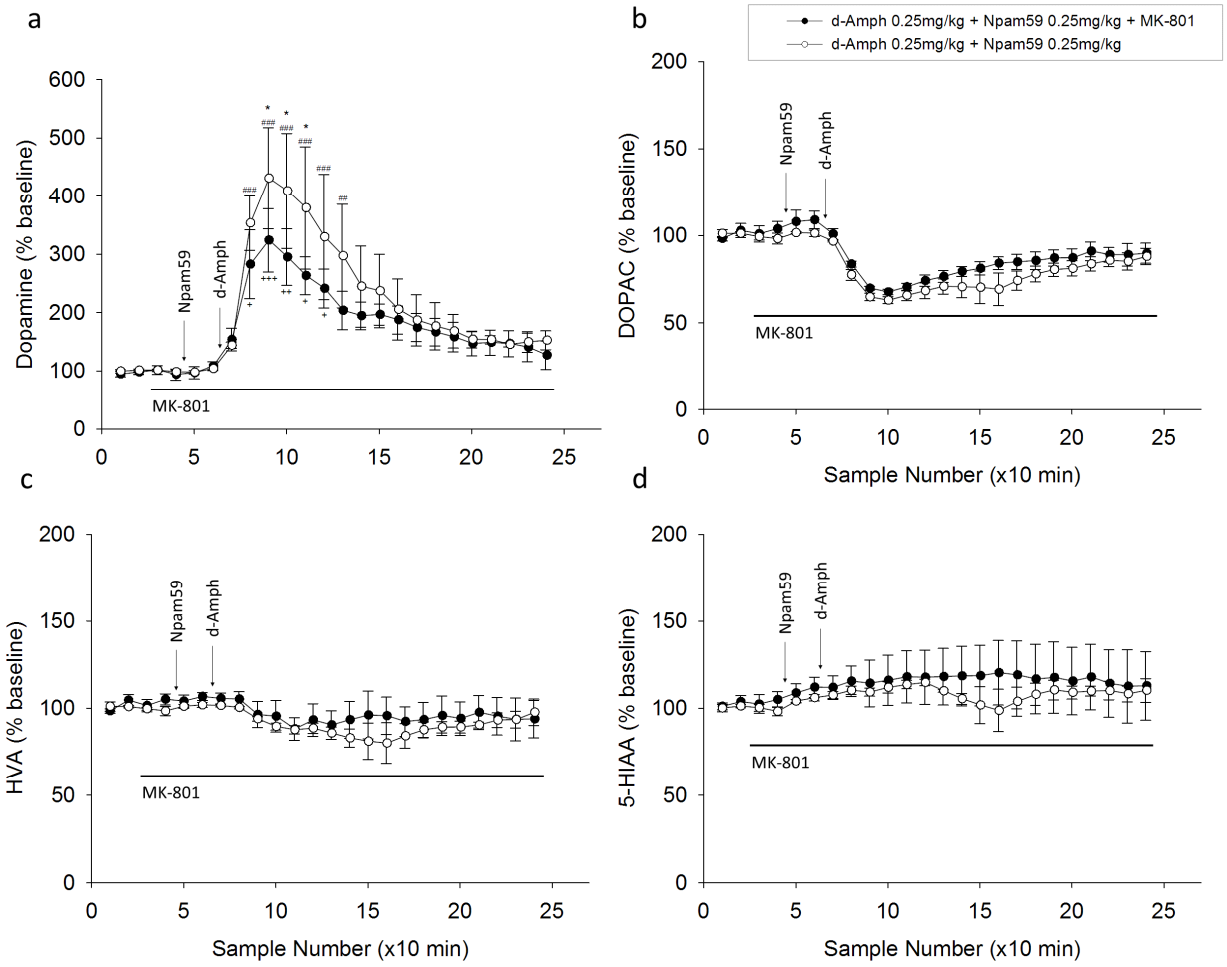

Figure 4.7 Effect of MK-801 on Npam59-potentiated d-amph-induced changes in dopamine, DOPAC, HVA, and 5-HIAA levels in the NAc.

Animals previously exposed to d-amph were reverse-dialyzed with MK-801 (1.0µM) or vehicle (regular aCSF) for the duration of the black bar and injected with Npam59 (0.25mg/kg) or vehicle at the first arrow and d-amph (0.25mg/kg) at the second arrow. MK-801 caused a significant decrease in Npam59-potentiated DA release. Traces indicate levels of neurotransmitters and metabolites measured from the same dialysate samples. 3 animals were measured in a within-subjects design for 6 total observations. n=3 for each treatment group. (a)

Traces for DA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,125)=2.374$, $p=0.010$. Post-hoc analysis reveals significant pairwise comparisons as indicated: Comparisons of d-amph+Npam59 vs. d-amph+Npam59+MK-801 within each time bin, *$p<0.05$. Within treatment differences compared to baseline level in d-amph+Npam59, ##$p<0.01$, ###$p<0.001$. Within treatment differences compared to baseline in d-amph+Npam59+MK-801, +$p<0.05$, ++$p<0.01$, +++$p<0.001$. (b) Traces for DOPAC levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,125)=0.875$, $p=0.616$. No main effect of treatment $F(1,125)=0.724$, $p=0.484$. Main effect of time $F(20,125)=19.000$, $p<0.001$. (c) Traces for HVA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,125)=1.512$, $p=0.131$. No main effect of treatment $F(1,125)=4.105$, $p=0.180$. No main effect of time $F(20,125)=1.652$, $p=0.087$. (d) Traces for 5-HIAA levels as a percentage of baseline over time. Two-way RM ANOVA, time × treatment $F(20,125)=1.031$, $p=0.451$. No main effect of treatment $F(1,125)=1.754$, $p=0.316$. No main effect of time $F(20,125)=1.589$, $p=0.105$. All data expressed as mean ± SEM.

Chapter 5: Npam59 potentiates behaviors under the control of the nucleus accumbens

5.1 Introduction

Our *in vitro* electrophysiological and *in vivo* micro-dialysis data supported our hypothesis that Npam59 can potentiate NMDA receptor function, thereby increasing d-amph induced dopamine release. Seeking further support for functional effects of Npam59 in freely-moving animals, a final experiment examined the effect of Npam59 on the well-characterized induction of exploratory locomotor behavior induced by d-amph. Of the several behaviors mediated by the NAc that are sensitive to d-amph, horizontal locomotion is often employed to understand the functioning of the ventral striatum (Mogenson and Nielsen, 1984; Brundin et al., 1987; Pulvirenti et al., 1989; Layer et al., 1991; Liu et al., 1998).

Various manipulations are understood to cause changes in the horizontal locomotion of an animal. Increasing DA into the NAc through numerous different means, be it by pharmacologically evoked release (Crawley et al., 1985; Koshikawa et al., 1986; Zohar et al., 1989; Kimura et al., 1993; Giros et al., 1996; Mathe et al., 1996; O'Neill and Shaw, 1999) or through direct electrical stimulation of the VTA (Taepavarapruk et al., 2000). One proposed mechanism involves glutamatergic activation of D1-expressing MSNs that project in turn to the substantia nigra to promote locomotor response, while in contrast, glutamatergic activation of D2-expressing MSNs projecting to the pallidum leads to a decrease in locomotion (Millan et al., 1999; David et al., 2004). Since DA increases sensitivity of D1-containing neurons and decreases sensitivity of D2-containing neurons to glutamate, the overall effect is a dual potentiation of

locomotion. Taken together, there is a high likelihood that Npam59 potentiation upon d-amph-induced DA release in the NAc will present as a potentiation of locomotor activity.

There are additional considerations as well for the role of NMDA receptors in the NAc in the modulation of locomotor activation. NMDA receptor antagonists given systemically generally increase locomotion (Adriani et al., 1998; Sukhanov et al., 2004; Rodriguez-Borrero et al., 2006), but NMDA receptor antagonism also blocks locomotor potentiation by amph (Kelley and Throne, 1992; Burns et al., 1994; David et al., 2004). Additionally, NMDA has no effect on locomotor when infused alone into the NAc, but has an inhibitory effect when given together with d-amph (Burns et al., 1994). It is unclear how these conflicting effects may arise or how NMDA potentiation may modify them, but we hope to discover clues from the results which may reconcile these findings.

5.2 Results

5.2.1 Npam59 potentiated locomotor activity by a dose of 3.0mg/kg d-amphetamine

Our general approach for the following experiments was to use the same dosing and treatment schedules as those in Chapter 4 for the micro-dialysis studies. As such, we continued administering two injections separated by 20 mins to deliver Npam59 with d-amph. The dose of Npam59 was kept at 0.25mg/kg in combination with separate doses of d-amph (0.25, 0.75, 1.5, or 3.0 mg/kg). Animals were habituated in the arenas for 30 mins on each of two days prior to testing to minimize stress effects and exploratory behavior from the novelty of the arenas. On test day, rats were injected with Npam59 and d-amph and placed into the boxes for 2h, and locomotor activity was video tracked and measured by computer software.

As anticipated, an initial locomotor increase from exploratory behavior observed in all groups, including controls, confounded the first 15 minutes of data. Therefore, data from the first 15 mins of each locomotor session were omitted from the data analyses. Locomotor distance traveled was summed across the entire trial and analyzed with a one-way ANOVA. There was a main effect of treatment ($F(9,94)=49.499$, $p<0.001$) on total distance traveled. Pairwise comparisons in post-hoc analysis revealed that doses 0.75mg/kg of d-amph and higher produced more locomotion than saline vehicle, with degree of significance as indicated (Figure 5.1). Npam59 given alone had no effect relative vehicle ($p=0.901$). No inverted-U response was observed that may indicate hyperstimulation into a state of stereotypy. However, there was a significant potentiating effect of Npam59 on 3mg/kg of d-amph, with a relative increase of 30% more distance traveled ($p<0.001$), but there was no observed potentiation by Npam59 at any other dose of d-amph.

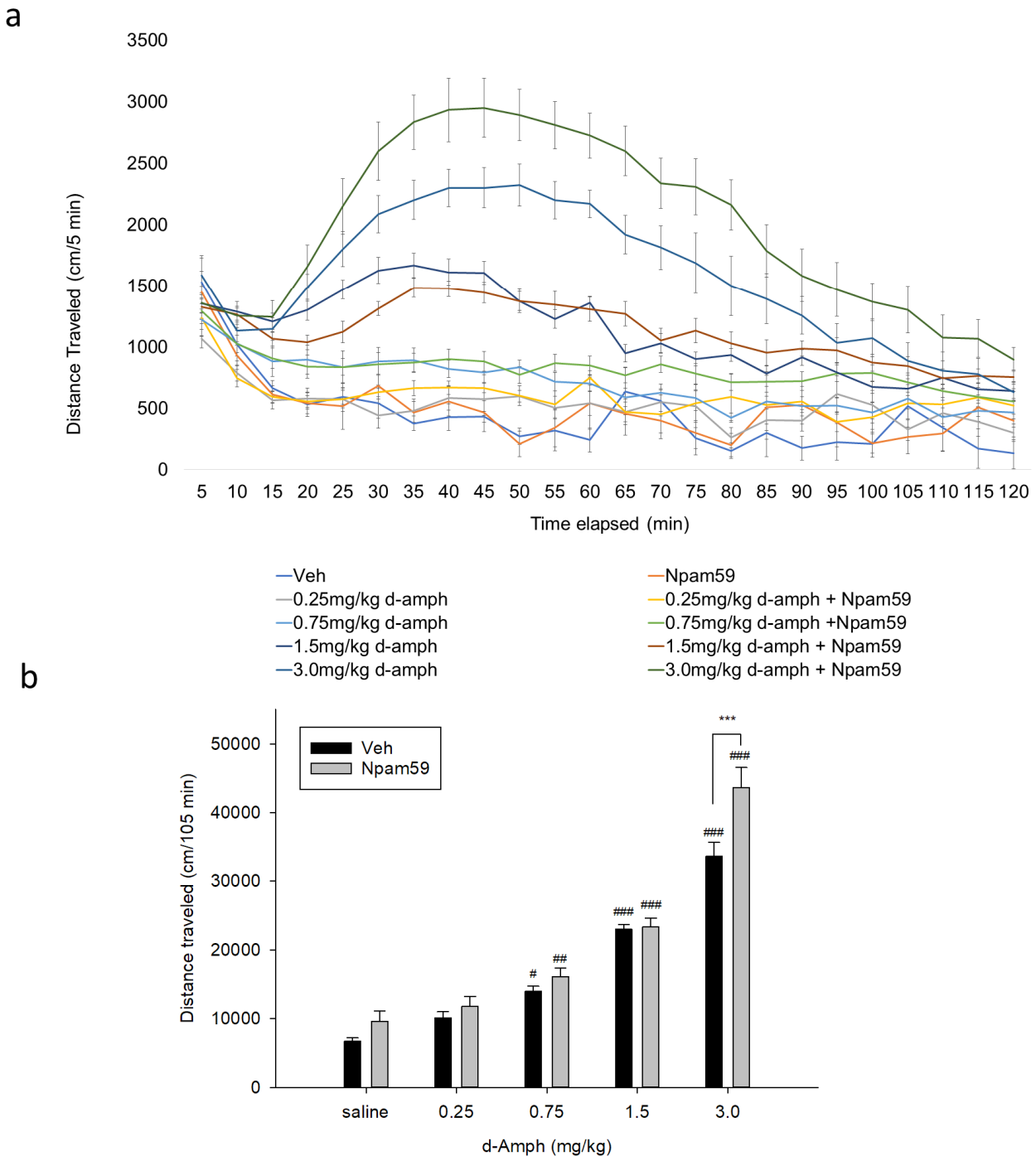

Figure 5.1 Effect of Npam59 on d-amph-induced hyperlocomotion.

Rats were injected i.p. with Npam59 (0.25mg/kg) or Veh in combination with the indicated doses of d-amph. Npam59 potentiated locomotion in conjunction with a 3mg/kg dose of d-amph. n = 6-18 rats per group (a) Locomotor counts over time presented as locomotor counts in 5 min bins. (b) Locomotor scores were summed over 15-120min. One-way ANOVA $F(9,94)=49.499$, $p<0.001$. Post-hoc analysis revealed significant effects of d-amph at and above 0.75mg/kg. #$p<0.001$. #$p<0.05$, ##$p<0.01$, ###$p<0.01$ in comparison to the vehicle + saline treatment. ***$p<0.001$ comparing with or without Npam59 within the same dose of d-amph. Data presented as mean ± SEM.

Chapter 6: Discussion

6.1 Overview

The goal of this project was to identify and characterize a new NMDA allosteric modulator that has selectivity at both GluN2A- and GluN2B-containg NMDA receptors. These receptors are involved in myriad facets of normal brain function, and their dysfunction has been linked to an increasing list of neurological disorders such as ADHD (Findling et al., 2007; Biederman et al., 2017), schizophrenia (Schwartz et al., 2012), and perhaps even Alzheimer's disease (Ulas and Cotman, 1997; Newcomer et al., 2000; Huang et al., 2012). Previously, efforts have been made towards increasing glycine co-agonism on the NMDA receptor, either through GLYT-1 inhibitors or NMDA glycine site agonists, as a strategy for upregulating NMDA receptor function. However, as already discussed, there are issues with specificity, bioavailability, and safety that create significant handicaps for these approaches. By developing a selective allosteric modulator compound, we can avoid these issues and target NMDA receptor hypofunction in a more direct manner.

The importance of designing an Npam capable of modulating both GluN2A and GluN2B subunits was twofold. First, because these receptors mediate opposing functions on neuronal plasticity, interventions that cause too great a change in their ratio of relative signaling might have unintended consequences that might limit the development of these Npams as therapeutics. For example, chronic potentiation of GluN2A without GluN2B may lead to inappropriate LTP that might present as inappropriate perseveration of behaviors. Conversely, chronic potentiation of GluN2B without GluN2A may cause synaptic depression or even increase the rate of age-related neuronal degeneration. Second, from the current literature related to NMDA receptor

hypofunction, it is unclear as to which subtypes, if any, are predominantly responsible for pathological decreases in NMDA receptor signaling. Coupled with new arguments suggesting that triheteromeric GluN1/GluN2A/GluN2B receptors might constitute a significant portion of all NMDA receptors in vivo (Rauner and Kohr, 2011; Tovar et al., 2013; Cheriyan et al., 2016; Lu et al., 2017), it seems prudent to develop an allosteric compound that is agnostic to the importance of any one specific diheteromeric NMDA receptor. On a more pragmatic note, GluN2A-selective Npams have been developed and their functional properties have been characterized recently as part of a broader project on Npams (Axerio-Cilies, 2016; Hackos et al., 2016). However, there has been little development of other types of Npams that may be more specific for GluN2A/GluN2B and GluN2B subunits. Developing a dual potentiator would not only be useful in its own right, but it may also serve as a transitional entity that may lead to the development of a GluN2B-specific allosteric modulator, which would also be first of its kind. In summary, for practical and theoretical reasons, there are numerous justifications for the development of an Npam for GluN2A- and GluN2B-containing receptors.

The novel research presented in this thesis describes the discovery and characterization of such a compound, with the demonstration of efficacy at the cellular, the neurochemical, and behavioral levels of brain function. As such, we now have an important pharmacological tool with which to understand better the roles of NMDA receptors in the control of synaptic plasticity and its functional consequences in health and disease.

6.1.1 Npam59 as a selective dual modulator of NMDA receptor currents

The data gathered in our electrophysiological experiments demonstrate that Npam59 can potentiate both GluN2A- and GluN2B-containing receptors, fulfilling the crucial first aim of the

thesis. Npam59 potentiated NMDA currents with an EC50 in the low micromolar to high nanomolar range and was capable of potentiating currents by up to 300% in neuron culture. Interestingly, the maximal potentiation on GluN1/GluN2A receptors expressed in HEK293 cells was dramatically higher than that of GluN1/GluN2B receptors also in HEK293 cells. A possible explanation might be differences in the expression efficiencies of the different plasmids, or simply different potency of Npam59 on the different NMDA receptor subtypes. However, when recapitulating the data in neuron culture, the difference in potentiation by subtype was dramatically smaller, and in fact almost identical when comparing effects on isolated GluN2A- and GluN2B-specific currents, suggesting that the discrepancy may be due to the artificial nature of the HEK293 cell system.

The specificity of NPam59 was briefly tested by attempting to potentiate $GABA_A$ and AMPA currents. Interestingly, there was no effect on $GABA_A$ or AMPA receptors by Npam59, whereas Npam43 did have a mild potentiating effect on $GABA_A$ receptors (Axerio-Cilies, 2016). These data provide indications that simple modifications upon the functional groups of Npam compounds may allow greater selectivity against off-target effects with further potential to increase the specificity of these compounds. Of course, as small molecules inevitably have unintended off-target effects, further characterizations would need to be done to confirm that there are no non-specific effects outside the tight pharmacological isolation used in electrophysiological recording, and to apply further optimizations as needed to our Npams.

6.1.2 Npam59 as a potentiator of d-amphetamine-induced dopamine release in the nucleus accumbens

Consistent with our electrophysiological findings showing that Npam59 allosterically modulated, but did not directly activate, NMDA receptors *in vitro,* NPam59 did not have an

effect on DA, DOPAC, HVA, or HIAA levels in the NAc. When d-amph was injected alone, a robust, dose-dependent increase in DA was observed, with corresponding decreases to DOPAC and HVA and a slight increase in 5-HIAA. When Npam59 was applied in conjunction with d-amph, we saw a dramatic potentiation, generally doubling the peak DA concentration. Interestingly, this potentiation occurred in the absence of changes to DOPAC and HVA, which may provide clues as to the modality by which the DA efflux is enhanced. Production of DOPAC from DA depends on monoamine oxidase (MAO) in the cytosol (Okada et al., 2011), which is sensitive to the rate of reuptake of D into the cytosol by DAT. When d-amph is given, DAT is reversed and reuptake cannot occur, which decreases DOPAC, and eventually HVA levels. Potentiated DA efflux by Npam59 may have occurred through a mechanism that does not expose DA to the cytosol. Accordingly, enhanced release was likely via increased exocytosis of synaptic vesicles. In future studies, this could be verified by using calcium-free aCSF to block vesicular release in the NAc. Other micro-dialysis data confirmed that 0.25mg/kg Npam59 potentiated d-amph-induced DA release without affecting DOPAC levels, despite increasing the dose of the d-amph or applying Npam59 through RD instead of i.p.

The experiment wherein MK-801 was reverse dialyzed into the NAC while d-amph and Npam59 were delivered i.p. were conducted near the end of this portion of the study and used animals with prior d-amph exposure that may have induced sensitization (Paulson and Robinson, 1995; Fiorino and Phillips, 1999). This modified the baseline response of DA to d-amph, shifting the peak height from an expected 200% for 0.25mg/kg d-amph to 300%. While these animals cannot be compared to the naïve ones in the preceding experiments, inferences could still be made by internal comparisons. MK-801 did not potentiate or otherwise affect DA release in the NAc when given alone. While previous literature has described that MK-801 may potentiate the

effects of d-amph-induced DA release (Miller and Abercrombie, 1996), another study has shown that the dose of MK-801 is of critical importance, with a lower dose having no effect on DA release in conjunction with d-amph (Wolf et al., 1994). It is possible that in our hands, applied in reverse dialysis, we were at a dose below which causes changes to DA levels, but sufficient to block effects of Npam59.

When Npam59 was tested in combination with d-amph + RD MK-801 or vehicle, we first observed a significant potentiation in DA release up to a peak of ~420% from ~320%, and this potentiation was abolished by application of MK-801 in RD. Although the statistical analyses confirmed a significant inhibitory effect of MK-801, the variance is high due to small sample sizes and the data are confounded by the use of non-naïve animals. Therefore, these results require replication with a greater number (8-10) of naïve subjects per group before we can make any confident conclusions. Taking the result at face value for now, we can make a powerful insight into the mechanism of Npam59. In concert with the previous finding that Npam59 potentiates DA release when applied locally in the NAc, this experiment implicated NMDA receptors in the NAc, to the exclusion of NMDA receptors elsewhere, as the principle mediator of the potentiation of d-amph-induced DA release in the NAc. Clearly this experiment should be replicated in the near future to verify that the potentiation of DA release by Npam59 is indeed through the action of NMDA receptors and that this potentiation depends on activation of NMDA receptors exclusively in the NAc.

There are two other interesting general observations that arise from the micro-dialysis data. First, it was demonstrated that Npam59 applied systemically could cause potentiation of d-amph-induced DA release. However, according to the glutamate hypofunction hypothesis of schizophrenia, it is hypofunction of NMDA receptors on cortical GABAergic interneurons that

causes disinhibition of pyramidal neurons, leading to excessive glutamatergic stimulation to the VTA and resulting in hyperdopaminergia in the ventral striatum (Schwartz et al., 2012). Even in a wild-type animal, potentiation of the NMDA receptors on cortical interneurons would be expected to increase inhibition on cortical functioning and counteract the potentiation in dopaminergic efflux. This possibility may still be valid if we consider that a high dose of 3.0mg/kg Npam59 may exactly neutralize its own local potentiation in the NAc by potentiating inhibition in the cortex. Of course, an exact neutralization at one of two doses we tested is highly speculative and it is difficult to imagine that negating a doubling of DA efflux from 200% to 300% baseline would not otherwise have overt observable effects in the behavior of animals. This remains an open question that requires further investigation. Despite these important caveats, it is appropriate to note that our studies of the allosteric modulator Npam59 are the first in its class to demonstrate an observable neurochemical readout in an intact, awake animal.

6.1.3 Npam59 as a potentiator of nucleus accumbens-mediated behavior

Npam59 dramatically potentiated the locomotor response in animals treated with 3.0mg/kg of d-amph, thereby providing the first evidence that allosteric potentiation of NMDA receptors can result in behavioral consequences. In keeping with its role as an allosteric potentiator, it also had no effect on locomotion in the absence of d-amph.

Surprisingly, the drug had no effect on locomotion induced by lower doses of d-amph. This finding is puzzling if one assumes a direct relationship between the magnitude of DA efflux in the NAc induced by d-amph and the corresponding increase in locomotor behavior. It has long been understood that dopamine increases in the NAc correlate to locomotor response in a dose dependent manner (Creese and Iversen, 1973; Kelly et al., 1975; Davis et al., 1991), but DA

always works through modulating the post-synaptic neuron's sensitivity to glutamate. Our context is different from the classical AIL experiments because glutamatergic modulation is occurring at the same time. Indeed, it has been shown that locomotor output is modulated in unexpected ways by NMDA receptor antagonism. Moderate dose MK-801 given alone potentiates locomotion even if DA is depleted by reserpine (Carlsson and Carlsson, 1989). APV, which normally potentiates locomotion when given alone, inhibits amph-induced locomotion (Burns et al., 1994). Clearly, there are unknown mechanisms responsible for these seemingly contradictory findings. As Npam59 is an allosteric modulator that is first-of-its-kind, it is uniquely poised as a powerful tool in the study of mechanisms that may explain these data.

6.1.4 Implications of current findings for future directions involving Npam59

One guiding consideration throughout this thesis has been the necessity to verify with clear evidence that Npam59 has an effect in the intact brain. As this is a new compound belonging to a family of molecules with little *in vivo* data beyond neuroprotection in stroke (Axerio-Cilies, 2016), our primary objective was to maximize the ability to observe a clear readout, which led us to choose the robust neuropharmacological phenomenon caused by d-amph and observed in the NAc. In one way, this strategy succeeded by producing convincing results that Npam59 has effects at the cellular, *in vivo*, and behavioral levels, but we have been left with relatively few indicators for the clinical usefulness of Npam59. Future experiments should examine its effects on PFC dopamine levels as a correlate for schizophrenia hypofrontality. In short, we have provided the foundation for future research into the applications of Npam59, but a great amount of subsequent research will be required to further characterize its development as a research tool and therapeutic strategy.

6.2 Potential applications of Npam59

6.2.1 Npam59 as a therapeutic prototype

In our work, d-amph was used primarily for its ability to inducing a state of increased synaptic activity rather than as a drug we sought to improve. However, our data shows that combination therapy with d-amph and Npam59 may be useful in clinical situations in which amphetamines are used routinely. Psychostimulant drugs including amphetamines are used principally in the management of ADHD (Heal et al., 2013) and have been shown to have disease-modifying effects in brain development based on fMRI studies (Frodl and Skokauskas, 2012; Hart et al., 2013; Spencer et al., 2013). While it is unknown how treatment with d-amph can decrease abnormalities in focal brain structures, it is well understood that short-term therapeutic effects stem from increasing DA and norepinephrine signaling (Bidwell et al., 2011). Given that dopaminergic signaling can modify the expression of LTP and LTD (Sheynikhovich et al., 2013), we might expect that the disease-modifying effects of d-amph occur through normalizing plasticity in affected brain regions.

Importantly, Npam59 might be expected to accelerate these changes when given as an adjunct, or simply by potentiating DA release in the brain. Additionally, there are hints that NMDA receptor hypofunction may be involved in ADHD (Lehohla et al., 2004; Chang et al., 2014), for which Npam59 would in theory serve as an ideally poised adjunct therapy. However, expectations for how effective Npam59 might be must be tempered given our behavioral results which show a potential for unanticipated effects on a well-understood drug-induced phenomenon.

Schizophrenia is another obvious target for Npam59, given the strength of evidence supporting the NMDA receptor hypofunction hypothesis of the disorder's pathogenesis (Stahl, 2007; Moghaddam and Javitt, 2012; Poels et al., 2014). While in our data Npam59 had not demonstrated any effect when given alone, please note that we did not assess its effects in any animal models with predictive validity for schizophrenia. The possibility that Npam59 may act as an antipsychotic still exists, but with a caveat. We have demonstrated that Npam59 potentiates d-amph, which can cause a drug-induced psychosis very similar to schizophrenic psychosis (Jones et al., 2011). Furthermore, repeated administration of d-amph is often used to produce animals that model positive symptoms of schizophrenia (Featherstone et al., 2007). Extrapolating, we might erroneously expect that Npam59 would exacerbate the positive symptoms in a schizophrenic patient. However, one key difference between the d-amph-based models of schizophrenia and true schizophrenia is the mechanism behind increased DA to the striatum, which is responsible for the positive symptoms. d-Amph simply reverses DA reuptake, evoking widespread DA release (Eshleman et al., 1994) without necessarily affecting NMDA receptor function, while in schizophrenia, DA release is secondary to loss of inhibition of cortical neurons, which subsequently drives both DA increase in the striatum and DA decrease in the cortex (Stahl, 2007). Due to these differences, Npam59 might be expected to have therapeutic, and not exacerbating effects when applied to schizophrenia or a model more closely modeling its pathophysiology. Of course, further experiments must be conducted to verify this hypothesis.

Lastly, there are considerations to be had for when NMDA receptor potentiation may be detrimental to health. As discussed in Chapter 1, there are diseases with contributions of excitotoxicity mediated by the GluN2B receptor such as stroke, Huntington's disease, Parkinson's disease, and Alzheimer's disease. It would be expected that potentiation of these

receptors during excessive glutamate spillover would exacerbate cell death. Application of a specific GluN2A potentiator such as Npam43 would likely be a superior choice in these cases.

6.2.2 Npam59 as a research tool

Npams can be applied in mechanistic studies in ways that NMDA receptor antagonists and agonists cannot. NMDA receptor agonists activate all receptors they diffuse to in the brain, and do not give information about the endogenous signaling state of an NMDA-containing glutamatergic synapse system, while antagonists can only infer the normal function of receptors by blockade. Npams might give new insights into the role and function of NMDA receptors by potentiating their natural functions and studying the consequences.

Prior to the characterization of Npam59, there were no known compounds capable of allosterically potentiating both GluN2A- and GluN2B-containg receptors simultaneously. Furthermore, development of a GluN2B-specific allosteric potentiator may be possible, and new efforts in our lab are already underway with compounds showing strong potentiation of NR2B-containing receptors and moderate potentiation of NR2A-containing receptors. Together with NR2A-specific potentiators such as Npam02 and Npam43, we are close to producing a full toolkit of Npams that may be powerful tools in determining not only the functional roles of NMDA receptors in the brain, but also the important roles played by subtypes of these receptors.

6.3 Insights into dopamine-glutamate signaling mechanisms in the nucleus accumbens

While the primary goal of this thesis was the characterization and proof of effect of an Npam specific for GluN2A- and GluN2B-containing receptors, we have also obtained data describing an interaction between NMDA receptors and dopamine release in the NAc, providing an example of how an Npam might be applied for mechanistic studies. Examination of the

mechanism(s) by which Npam59 could potentiate d-amph-induced DA release and hyperlocomotion may provide new insights into the regulation of NAc DA release by glutamate. Here, we propose a model that may harmonize our findings with the existing literature. One caveat about the following discussion is that our work did not include a screen of possible effects Npam59 may have by nonspecific binding to any number of the involved proteins. However, we do have some preliminary data showing some specificity at AMPA and $GABA_A$ receptors and a finding suggestive that NMDA receptor blockade in the NAc by MK-801 can prevent Npam59 from potentiating dopamine release. For the sake of discussion, we will assume that the effects seen in our *in vivo* and behavior data are due to NMDA receptor allosteric potentiation.

6.3.1 Physiological dopamine release

Before discussing the role of Npam59 further, we will first review mechanisms and pathways by which DA signaling is regulated. In the present experiments, we studied the mesolimbic pathway, in which VTA projections into the NAc are the key dopaminergic component. These projections release DA onto primarily GABAergic medium spiny neurons (MSNs) in the NAc, which comprise over 90% of the region's neuron population, with the remaining fraction composed of various subtypes of inhibitory GABAergic interneurons (Russo and Nestler, 2013). Additionally, there is evidence that VTA projections release both DA and glutamate into the NAc (Hnasko et al., 2010; Stuber et al., 2010; Hnasko et al., 2012), although there still appears to be segregation of dopaminergic and glutamatergic varicosities in these neurons (Zhang et al., 2015).

It is important to note that DA signaling is entirely metabotropic, and therefore has no effect on the post-synaptic neuron's firing when acting alone. Rather, DA works in combination

with glutamate to regulate synaptic activity (Seamans and Yang, 2004). Post-synaptic DA receptors are categorized into either D1-like receptors, which include D1 and D5 receptors, or D2-like receptors, which include D2, D3, and D4 receptors. D1-like receptor activation by DA causes increased adenylyl cyclase activity, a buildup of cAMP, and a variety of signaling sequelae that result in an increased sensitivity to excitatory stimuli. Conversely, D2-like receptors inhibit adenylyl cyclase upon activation and additionally activate phospholipase C, reducing cAMP while increasing cytosolic calcium and producing the second messengers diacyl glycerol and inositol triphosphate. Through activation of one type of receptor or the other, DA can indirectly increase or decrease the firing of downstream neurons.

D2 auto receptors also exist as an alternative "short" isoform (D2s), which differ from the "long" isoform of post-synaptic D2 receptors (D2L) (De Mei et al., 2009). These presynaptic D2s receptors enable negative feedback at the level of the presynaptic terminal by three mechanisms. First, activation of the D2s increases activation of Kv1.2 channels and blocks activation of voltage-gated calcium channels, hyperpolarizing the membrane potential and reducing intracellular calcium. The net effect of these changes hinders vesicular release of DA by restricting calcium to the calcium-dependent synaptotagmins (Sudhof, 2012). Second, D2s drive events that result in the phosphorylation and inactivation of tyrosine hydroxylase (TH), reducing the replenishment synthesis of DA from its precursors (Lindgren et al., 2003). Third, D2S increases membrane insertion of the DA transporter (DAT), increasing cellular reuptake following efflux (Chen et al., 2013). The combined actions of these pathways exert a tonic suppression on DA release in vivo, and blockade of the D2s is known to cause dramatic increases in DA efflux from the VTA, which is demonstrated by increased DA efflux following

application of haloperidol (Pehek, 1999) or raclopride (Wiker et al., 2005), among other D2 antagonists.

6.3.2 Dopamine release in the NAc following application of d-amphetamine

Modulation of DA release from the VTA can occur at the presynaptic terminal or by inputs into the VTA and d-Amph is capable of increasing DA release by influencing both modalities. Once administered, amphetamine enters presynaptic terminals of dopaminergic neurons via transport across the dopamine transporter (DAT) (Heal et al., 2013). Within the cell, it acts upon the DAT to reverse its function (Eshleman et al., 1994; Heal et al., 2013), causing it to efflux rather than re-uptake DA to the synaptic space. Additionally, d-amph causes the liberation of synaptic vesicles from the reserve pool to the readily releasable pool (Covey et al., 2013), while simultaneously inhibiting the proton transporter on these vesicles (Covey et al., 2013). The loss of the proton gradient reverses function of vesicular monoamine transporter (VMAT), driving the cotransporter to pump dopamine into, rather than away from, the cytosol. The net effect is an increase of non-activity dependent DA efflux that is not sensitive to blockade by application of the voltage-gated sodium channel blocker tetrodotoxin (TTX) (Benwell et al., 1993). The massive amount of DA efflux triggers strong negative feedback through activation of the D2s presynaptic auto-receptors (Pehek, 1999), which likely include receptors located outside the synapse (Sesack et al., 1994) following DA spillover.

d-Amph also increases DA release by increasing the firing rate of VTA neurons (Xue et al., 1996). One mechanism involves the d-amph-induced endocytosis of post-synaptic EAAT3 on VTA neurons, which normally have a role in glutamate transport out of the synapse (Underhill et al., 2014). Accordingly, endocytosis of EAAT3 through a RhoA-dependent mechanism increases

the duration and intensity of glutamatergic stimulation on VTA neurons (Underhill et al., 2014), increasing synaptic release of DA from their axons. Another mechanism may involve increased synaptic output through activation of α1 adrenergic receptors indirectly by d-amph (Shi et al., 2000; Shi et al., 2007), which would similarly increase release of DA from the VTA.

6.3.3 Glutamate release in the NAc following application of d-amphetamine

It has been demonstrated that d-amph can also increase the extracellular levels of glutamate in the NAc as measured by micro-dialysis (Reid et al., 1997; Dalia et al., 1998) through a D1 and D2 receptor-mediated mechanism. d-Amph-induced glutamate release could be attenuated by D1 or D2 antagonists, or induced by a concurrent application of quinpirole, a D2 agonist, and SKF 38393, a D1 agonist (Dalia et al., 1998). Cocaine, a DAT blocker, also increases release of glutamate in the NAC (Reid et al., 1997; Dalia et al., 1998). While it remains unclear how D1 and D2 receptor activation in the NAc leads to increased glutamate release, it is possible that the mechanism is in-part mediated through a polysynaptic loop increasing glutamatergic inputs from elsewhere in the brain, such as the frontal cortex (Dalia et al., 1998).

A sub-population of dopaminergic neurons in the VTA also exhibit co-release of glutamate (Hnasko et al., 2010; Stuber et al., 2010; Tecuapetla et al., 2010), with distinct micro-domains for DA and glutamate synapses within the same axon (Zhang et al., 2015). Given that d-amph increases firing of VTA neurons in part through induced endocytosis of EAAT3 (Underhill et al., 2014) and through activation of α1 adrenergic receptors (Shi et al., 2000; Shi et al., 2007) as described above, we would predict increased glutamate co-release alongside increased DA release in the NAc.

6.3.4 Proposal of a presynaptic mechanism explaining the ability of Npam59 to potentiate dopamine release in the nucleus accumbens

Given that Npam59 administered only into the NAc is sufficient for potentiating d-amph-induced DA release, the mechanism must be at least in part mediated by local effects in this brain region. One possible mechanism is illustrated in Figure 6.1. A presynaptic varicosity of a VTA neuron co-releasing DA and glutamate synapses upon neurons in the NAc. This varicosity is also under presynaptic control by NMDA receptors of currently unknown subtype (Cheramy et al., 1986; Krebs et al., 1991) and AMPA receptors (not shown) that receive inputs from the cortex (Cheramy et al., 1998). Under normal conditions, DA and glutamate cycle in and out of the synaptic clefts, and presynaptic NMDA receptors may be activated by cortical inputs as indicated by the solid arrows. Since NMDA receptors are under tonic Mg^{2+} blockade, glutamatergic regulation may be limited by the requirement of concurrent depolarizing events at normal conditions.

When d-amph is given, increased firing and neurotransmitter release from the VTA neuron coupled with loss of reuptake create a situation where there is spillover of both DA and glutamate, as indicated by the dotted arrows. Concurrently, Mg^{2+} is repelled more frequently, increasing the population of activatable NMDA receptors. These receptors are activated by spill-over and cortical glutamate, contributing to local depolarization and calcium influx, further potentiating fusion of synaptic vesicles to the synaptic membrane in a positive feedback loop. Application of Npam59 greatly potentiates the currents passing through the presynaptic NMDA receptors and results in an even greater release of DA and glutamate, which may explain the potentiation we have observed. While it has been suggested that only a small fraction of VTA neurons corelease DA and glutamate (Yamaguchi et al., 2007), Stuber, et al. have demonstrated

significantly increased glutamate efflux following exclusive optogenetic stimulation of neurons expressing dopaminergic and glutamatergic markers, additionally commenting that current detection limits may be too low to accurately quantify the proportion of co-releasing VTA neurons (Stuber et al., 2010). Alternatively, multi-synaptic pathways activated by DA can increase NAc glutamate input from other brain regions (Reid et al., 1997; Dalia et al., 1998), which may provide the glutamate input necessary for Npam59 to potentiate DA release.

Another possible mechanism cannot be ruled out whereby potentiation of post-synaptic NMDA receptors expressed on MSNs increases their synaptic output and initiates either LTP or LTD. Some of these MSNs reciprocally innervate the VTA to inhibit dopaminergic output, while others activate VTA neurons through disinhibition (Russo and Nestler, 2013). However, it is unclear what the net sum of these effects would be following treatment with d-amph and Npam59, making the effects on and by these MSNs difficult to predict.

6.4 Concluding thoughts

NMDA receptor allosteric potentiators represent novel and powerful tools in the infancy of their development. Characterizing work is of critical importance as we move forward with increasingly specific and effective compounds capable of potentiating receptors with subtype specificity. Npam59 is a dual potentiator of GluN2A- and GluN2B-containing receptors demonstrated here to have substantial effects at the cellular, neurochemical, and behavioral level, and despite being a first-generation prototype, already shows potential as a research tool for mechanistic studies and may be valuable as a therapeutic agent. Unique opportunities are present with application of Npam59 in conjunction with optogenetic techniques to resolve the reliance on d-amph to trigger the allosteric potentiation.

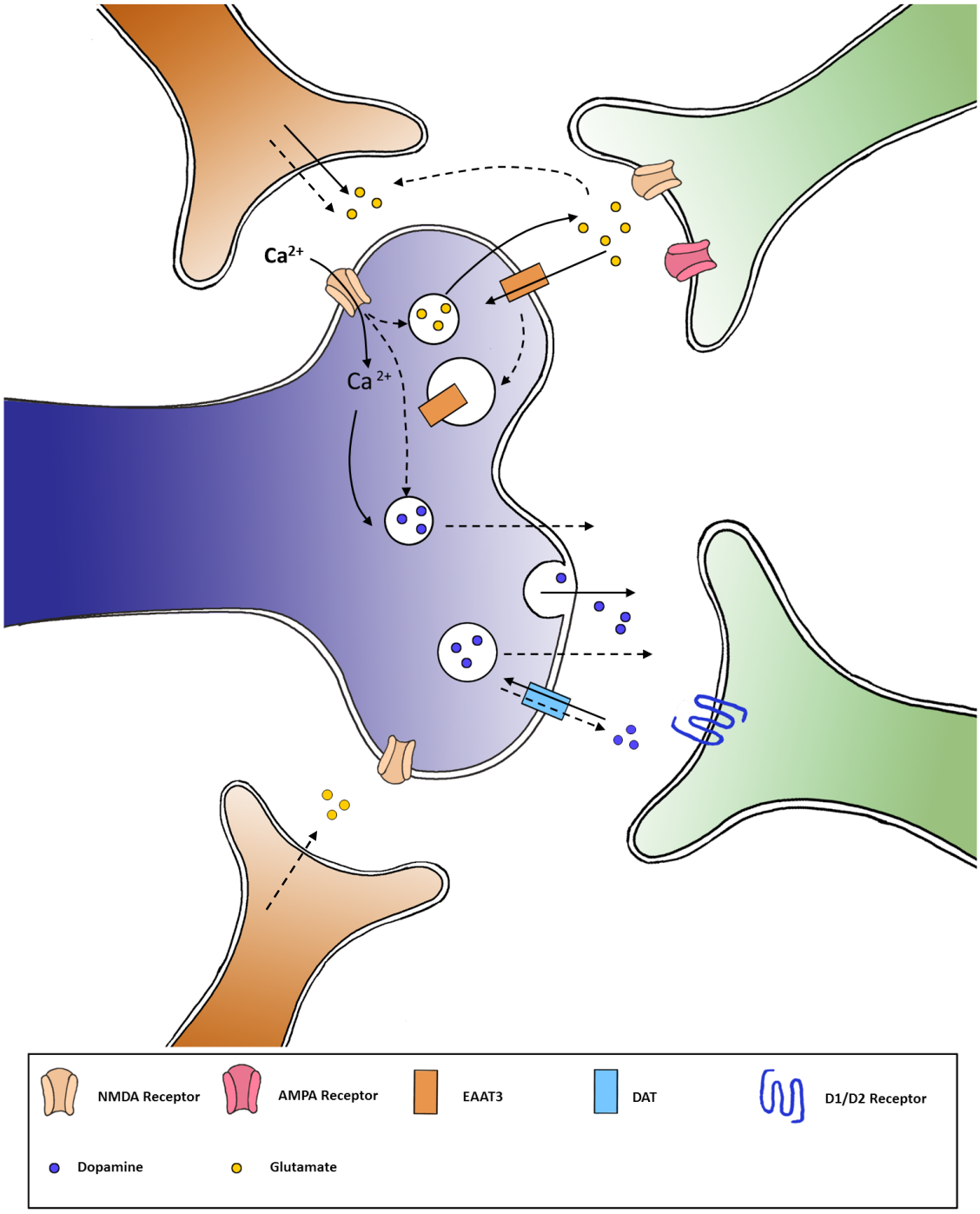

Figure 6.1 Proposal of a presynaptic mechanism for Npam59 potentiation of d-amph-induced DA release.

A synaptic varicosity from a VTA dopaminergic neuron that co-releases glutamate is shown in purple. Dendritic spines of MSNs are shown in green. Glutamatergic inputs which do not originate from the VTA, such as cortical axons, are shown in orange, which exert presynaptic influence. Solid arrows indicate physiological processes. Dotted arrows indicate changes which occur following treatment with d-amph, which set up the system for potentiated DA release by Npam59.

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Appendix

Appendix A

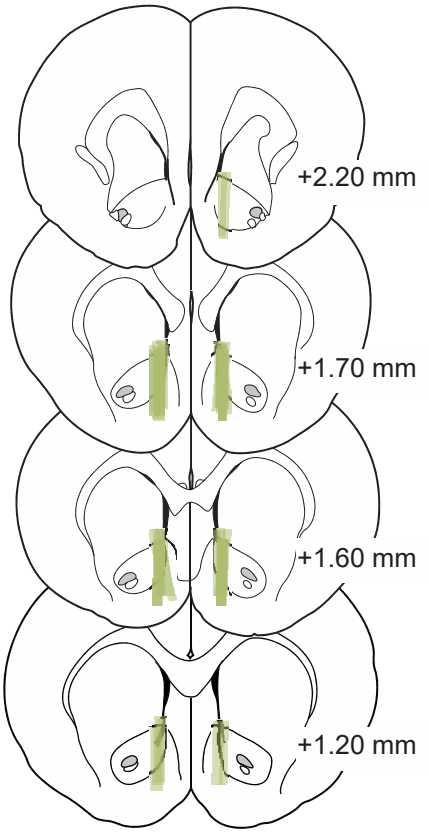

Figure A.1 Verification of correct micro-dialysis probe placement in the brain.

At the end of each micro-dialysis experiment, animals were sacrificed and brains were sectioned to verify the correct placement of dialysis probes within the NAc. Green rectangles represent the position of a probe, sampled randomly from all experimental cohorts.