DISCOVERY OF A SELECTIVE GLUN2A-CONTAINING N-METHYL-D-ASPARTATE GLUTAMATE RECEPTOR POSITIVE MODULATOR AS A NOVEL POST-STROKE NEUROPROTECTIVE THERAPY

by

Peter Axerio-Cilies

B.S., The University of British Columbia, 2006

M.S., The University of London, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2016

© Peter Axerio-Cilies, 2016

Abstract

N-methyl-d-aspartate glutamate receptors (NMDARs) are fundamental to many normal brain functions such as cognition and memory; however, NMDAR over-activation can cause neuronal death as a result of excitotoxicity. Although the mechanisms underlying these paradoxical roles of NMDARs remain unclear, accumulating evidence from both in-vitro and invivo studies suggested that GluN1/GluN2B-NMDAR subtypes mediated signaling may contribute cell death, while GluN1/GluN2A-NMDARs signaling promote pro-survival outcomes. Employing an extensive drug discovery pipeline process, we identified and characterized a class of novel small molecules that specifically potentiate GluN1/GluN2A NMDARs in an allosteric manner. This new class of molecules is referred to as NMDAR positive allosteric modulators (Npams) with the Npam43 being the lead compound. Mutational analysis demonstrates that Npam43 binds to a novel binding site on the N-terminal domain (NTD) at the interface between the GluN1 and GluN2A subunits. Functional characterizations in *in-vitro* show that Npam43 activates cell survival signaling, increasing phosphorylated CREB levels, and thereby protects neurons against NMDAR-mediated excitotoxicity and NMDA-independent H₂O₂ oxidative stress. Moreover, Npam43 potentiates GluN1/GluN2A-mediated synaptic currents, and facilitates the induction of long-term potentiation (LTP) in hippocampal slices acutely prepared from mouse brain. Using a rat focal ischemia model of stroke in-vivo, we show that systemic administration of Npam43 not only modulates GluN1/GluN2A containing NMDARs but also substantially reduces neuronal damage and improves behavioral outcomes. Together, our study not only develops a novel class of Npams for GluN1/GluN2A NMDARs, but also demonstrates their therapeutic potential as novel neuroprotectants for stroke. In addition, the

present work also provides strong evidence supporting a critical role of GluN1/GluN2A subtype of NMDARs in promoting cell survival.

Preface

Chapter 1: All figures in Chapter 1 were re-printed with permission. Please refer to legend for details.

Chapter 2: Materials and Methods

Chapter 3-9: A version of this material will be assembled into a manuscript for submission. Peter Axerio-Cilies preformed and analyzed all the data presented from Chapter 3-8. Chapter 9 was performed in collaboration with Dr. Woei-Cherng Shyu at the Translational Medicine Research Center and Centers for Neuropsychiatry, China Medical University Hospital in Taiwan. More specially, Peter Axerio-Cilies initiated the study, screened compounds, electrophysiological recordings, chemical synthesis, discovery and development of drugs, preformed biochemical experiments, designed the study with regards to the drug discovery process, pharmacological *in-vivo* profile of drugs, Dr. Steven Conner preformed the slice recordings, and Dr. Woei-Cherng Shyu group in Taiwan performed the stroke model *in-vivo*. Dr. Yu Tian Wang designed the study, supervised the overall project and provided thoughtful advice for the biological design of the study.

Chapter 10: Discussion of the overall thesis.

Experiments were performed in compliance with regulations at the University of British Columbia. Animal experiments were performed in compliance with institutional requirements at the University of British Columbia and in accordance with guidelines set forth by the Canadian Council on Animal Care. Animals were obtained and handled according to University of British Columbia ethics protocols A13-0139.

Table of contents

Abstract	ii
Preface	iv
Table of c	ontentsv
List of tab	les xiii
List of figu	ıres xiv
List of abb	previations xviii
Acknowle	lgements xxi
Dedication	ı xxiii
Chapter 1	: Introduction1
1.1 0	Overview of the N-methyl-d-aspartate glutamate receptors (NMDARs) 1
1.1.1	NMDARs as calcium (Ca ²⁺) channels 1
1.1.2	NMDAR channel properties
1.1.3	NMDAR composition and structure
1.1.4	NMDAR distribution
1.2 N	MDAR pharmacology7
1.2.1	Agonists of NMDARs7
1.2.2	GluN2 subunit selectivity of glutamate binding site ligands
1.2.3	Glycine GluN1 subunit agonists for NMDARs 12
1.2.4	Allosteric modulatory sites for NMDARs 12
1.2.5	Zinc (Zn ²⁺) as a NMDAR modulator
1.2.6	Final remarks for the pharmacology of NMDARs 16
	V

1.3	NMDAR and its relationship with excitotoxicity	18
1.4	Important signaling pathways for NMDARs	20
1.4	.1 Pro-death signaling pathways downstream of NMDARs	20
1.4	.2 Pro-survival signaling pathways downstream of NMDARs	23
1.5	The failure of NMDAR antagonists in clinical trials	27
1.6	Opposing roles of GluN2A and GluN2B-containing NMDARs	27
1.7	Crystal structure of GluN1/GluN2B NMDARs	34
1.8	Stroke	37
1.9	NMDAR-mediated excitotoxicity and its relationship with an ischemic stroke even	it. 38
1.10	The evolution of an ischemic infract damaged area	40
1.11	Current treatments for ischemic stroke	49
1.12	Computer-aided drug discovery (CADD)	54
1.13	Rationales, hypotheses and specific aims	55
Chapte	r 2: Methods and methodology	59
2.1	Homology model	59
2.2	Chemical library	59
2.3	Virtual Screening (docking) for selective modulators for GluN1/GluN2A-containing	ng
NMD	OARs	61
2.4	Analog search	61
2.5	Chemicals	62
2.6	Chemical synthesis	63
2.7	Determination of compound purity	64
		vi

2.8	Buffers and media	64
2.9	Plasmids	65
2.10	HEK293 cell culture and plasmid transfection	65
2.11	Electrophysiology	66
2.12	Slice recordings	67
2.13	Primary culture of cortical neurons	68
2.14	Data analysis	69
2.15	Site-directed mutagenesis	70
2.16	Neuronal culture	70
2.17	Measurement of Ca^{2+} in rat cortical cultures using a Ca^{2+} -sensitive dye	71
2.18	Slice preparation for probing pCREB	72
2.19	NMDA-induced excitotoxicity and H ₂ O ₂ -induced cytotoxicity	72
2.20	Lactate dehydrogenase (LDH) assay	73
2.21	Immunoblotting	74
2.22	Formulation	75
2.23	Reagents	75
2.24	Instrumentation and chromatographic conditions	75
2.25	Sample preparation	76
2.26	Cerebrospinal fluid (CSF), serum extraction and HPLC-ECD analysis	76
2.27	Cerebral ischemia	77
2.28	Magnetic resonance image	78
2.29	Neurological behavioral tests	78
		vii

Chapter 3: Structure-based modeling, target site identification and drug screening on

GluN1	/GluN2A NMDARs79
3.1	Introduction79
3.2	Identification of a druggable interface between the GluN1 and GluN2A subunits in the
N-tei	minal domain (NTD)
3.3	Initial drug screening using GluN1/GluN2A or GluN1/GluN2B cDNA transiently
trans	fected in human embryonic kidney (HEK293) cells and tested by whole-cell patch clamp
recor	rdings
Chapte	er 4: Hit identification and characterization of Npam02 as a selective GluN1/GluN2A
positiv	e allosteric modulator (PAM) that targets the N-terminal domain (NTD)93
4.1	Introduction
4.2	Evaluation of the potentiation effect of Npam02 in mature cortical wild-type and
GluN	V2B-lacking neurons using whole-cell voltage clamp recordings
4.3	Npam02 had no visible modulation effects on AMPAR and GABAR-mediated currents
in ma	ature cortical neurons
4.4	Npam02 binding position in the interface site of the GluN1/GluN2A 103
4.5	Site-directed mutagenesis of the predicted binding site in the N-terminal domain
(NTI	D)105
4.6	Structure-activity relationships (SARs) of Npam02 and its closely related analogs to
ident	ify chemical features that are responsible for selectivity

Chapte	r 5: Hit optimization of Npam02 using a rational drug design approach and	
develop	oment of SARs to select a potent positive allosteric GluN2A-type modulator112	2
5.1	Introduction112	2
5.2	Rational drug design to discover selective positive allosteric modulators of GluN2A-	
conta	ining NMDARs	4
5.3	Chemical synthesis of Npam compounds 110	6
5.4	Does the ortho-substituent on ring R1 (positions 21 in Figure 4.14) elicit selectivity	
towa	rds GluN1/GluN2A containing NMDARs110	5
5.5	Structure-activity relationships (SAR) for the lead family of compounds 119	9
5.6	Selection of a lead compound	7
Chapte	r 6: Identification and characterization of Npam43 as a selective and potent PAM	
for the	GluN2A-containing NMDARs13	0
6.1	Introduction130)
6.2	Npam43 selectively potentiates GluN1/GluN2A-containing NMDAR-mediated	
curre	nts in transiently transfected <i>HEK293</i> cells	1
6.3	Identification of critical amino acid residues at the GluN1/GluN2A interface in the N-	
termi	nal domain (NTD) required for formation of the Npam43 binding pocket	6
6.4	Modulation of Npam43 on GluN1/GluN2A-containing NMDARs potentiates	
NME	OAR-mediated currents in cultured rat hippocampal neurons	3
6.5	The GluN2A component of synaptic transmission is enhanced by Npam43	1

6.6	The GluN2A component of LTP is enhanced by Npam43 153
6.7	Npam43 specificity for the GluN1/GluN2A may come from subtle differences in the
relativ	ve positions of amino acid residues from the interface
6.8	Npam43 increases intracellular Ca ²⁺ via GluN1/GluN2A-containing NMDARs 158
Chapte	r 7: Npam43 activates GluN2A-NMDAR mediated cell survival signaling, and
thereby	protects cultured neurons <i>in-vitro</i> against neuronal death161
7.1	Introduction161
7.2	Npam43 increased CREB phosphorylation that was prevented through GluN2A
antag	onism in cortical neurons 164
7.3	Npam43 increased CREB phosphorylation that was prevented through GluN2A
antag	onism in hippocampal slices
7.4	Npam43 is neuroprotective against NMDA-mediated excitotoxicity in primary neuron
cultur	es
7.5	Npam43 protects neurons against oxidative stress in primary neuron cultures 170
Chapte	r 8: Pharmacology- pharmacokinetics of Npam43 <i>in-vivo</i>
8.1	Introduction174
8.2	Characterization of the soluble state of Npam43 in water 176
8.3	Formulation of Npam43177
8.4	Toxicity of vehicle
8.5	Detection of Npam43 using HPLC-ECD

8.	6	Npam43 crosses the BBB and has a ~3 hours half-life via an IV-injection in mature	
ra	.ts		2
Cha	pter	9: Npam43 protects neurons against ischemic injuries in a mouse model of focal	
stro	ke <i>in</i>	- <i>vivo</i> 18	7
9.	1	Introduction	7
9.	2	Systemic application of Npam43 reduces neuronal damage in a MCAo model of focal	
ise	chem	ia in rat	9
9.	3	Npam43 reduces infarct volume post-ictus as evaluated by 7 day magnetic nuclear	
re	sona	nce (MRI) imaging <i>in-vivo</i>	2
9.	4	Post-stroke treatment with Npam43 improves motor behavior performance after focal	
ise	chem	ic brain insult <i>in-vivo</i>	4
Cha	pter	10: Discussion19	8
10).1	Background 19	8
10).2	Targeting GluN2A-containing NMDARs	2
10).3	Novel modulation binding site on the GluN1/GluN2A NMDARs	0
10).4	Drug development of Npam43	4
10).5	The efficacy <i>in-vivo</i> for Npam43	5
10).6	Clinical relevance	8
Bibl	iogra	aphy22	6
Арр	endi	ces28	3
A	ppen	dix A	3
A	ppen	dix B	6
		2	ĸi

B.1	Potentiation and inhibition effects of the "hit" compounds of initial screen validat	ted
in <i>HER</i>	K293 cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs via whole-cel	11
voltage	e clamp recordings	286
B.2	Positive modulation effects (potentiation) of different chemically synthesized	
analog	s for Npam02 on NMDAR-mediated currents in cortical neurons and their SARs	
analysi	is based on closely related analogs	290

List of tables

Table 4.1 Hit compounds identified in the first screen that showed a positive modulation of the
GluN1/GluN2A NMDARs or/and inhibition effect of GluN1/GluN2B NMDARs 110
Table 6.1 Npam43-induced potentiation of the GluN1 or GluN2A mutants

List of figures

Figure 1.1 Unique subpopulations of the NMDAR mediate neuronal death and survival
Figure 3.1 In-silico pipeline developed to identify potential GluN2A-containing NMDARs
binders from the pre-filtered ZINC database
Figure 3.2 Targeting the dimer interface of the homomeric GluN1/GluN2A NMDARs in the N-
terminal domain (NTD)
Figure 3.3 Potentiation and inhibition effects of the "hit" compounds of initial screen validated in
HEK293 cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs via whole-cell voltage
clamp recordings
Figure 3.4 Npam02 potentiated GluN1/GluN2A-mediated NMDAR currents via direct binding in
HEK293 cells transfected with GluN1/GluN2A subunits and did not change GluN1/GluN2B-
mediated NMDAR currents
Figure 3.5 Structural differences between Npam04 and Npam02

Figure 4.1 Npam02 enhanced neuronal NMDAR function in cultured hippocampal neurons and
the potentiation effect was blocked by GluN2A antagonist
Figure 4.2 Npam02 application in genetic deletion of GluN2B subunit cortical culture neurons
potentiates GluN2A NMDARs mediated currents 100
Figure 4.3 Npam02 does not affect current responses induced by AMPA and GABA in neuronal
cultures
Figure 4.4 2D chemical structure of Npam02 105
Figure 4.5 GluN2A F_{177} and Q_{111} form the Npam02 binding pocket between the GluN1 and
GluN2A interface of NMDAR receptors in the NTD 107
Figure 5.1 Modulation effects of different analogs for Npam02 in HEK293 cells expressing
GluN1/GluN2A and Glu1/GluN2B NMDARs
Figure 5.2 Potentiation effects of different chemically synthesized R1 and R2 ring modifications
based on the structure of Npam02 in cortical neurons
Figure 5.3 Chemically synthesized analogs of Npam43 enhanced neuronal NMDAR function in
cultured hippocampal neurons
Figure 6.1 Npam43 potentiated GluN1/GluN2A-mediated NMDAR currents via direct binding in
HEK293 cells transiently transfected with GluN1/GluN2A subunits
Figure 6.2 Npam43 dose response curve and glutamate dose response curve in the presence of
Npam43135
Figure 6.3 Npam43's chemical structure and its binding to the NMDAR
Figure 6.4 GluN1 L ₁₃₅ or GluN2A F ₁₇₇ , P ₇₈ , F ₁₁₄ , Q ₁₁₁ , and P ₁₇₈ form the Npam43 binding pocket
between the GluN1 and GluN2A interface of NMDAR receptors in the NTD

Figure 6.5 Dose-response of glutamate in <i>HEK293</i> cells expressing wild-type GluN1/GluN2A,
mutant GluN1 (L ₁₃₅ Q) and mutant GluN1 (L ₁₃₅ Q)/GluN2A (F ₁₁₄ S)
Figure 6.6 Npam43 dose-dependently enhanced neuronal NMDAR function in cultured
hippocampal neurons
Figure 6.7 Npam43 targets specifically GluN2A-containing NMDARs in cultured hippocampal
neurons
Figure 6.8 Npam43 does not affect current responses induced by AMPA but modestly enhanced
current responses induced by GABA at higher concentrations in neuronal cultures 149
Figure 6.9 NMDA dose-dependent curve in the presence and absence of Npam43 150
Figure 6.10 The GluN2A component of synaptic transmission is enhanced by Npam43 152
Figure 6.11 Npam43 facilitates the induction of LTP
Figure 6.12 Npam43 docks into the NTD of GluN1/GluN2B; the analogous binding site of
GluN1/GluN2A
Figure 6.13 Npam43 increases Ca ²⁺ influx in primary culture neurons in a dose-dependent
manner
Figure 7.1 Npam43 enhancement of CREB phosphorylation in cortical neurons
Figure 7.2. Npam43 increased pCREB levels in hippocampal slices
Figure 7.3 Npam43 protects against NMDA excitotoxicity and H ₂ O ₂ cytotoxicity 171
Figure 8.1 Turbidity assay of Npam43 in water 176
Figure 8.2 The vehicle used to dissolve Npam43 for <i>in-vivo</i> testing did not exhibit toxicity in
primary neurons
Figure 8.3 Npam43 was detectable using an HPLC-ECD and the response was concentration-
dependent
xvi

Figure 8.4 Npam43 detection in rat CSF (rCSF) using HPLC-ECD. Dose responsive effect of
Npam43 post-1 hour extraction of rCSF 184
Figure 8.5 Npam43 effectively crosses the BBB triggering the pro-survival pathway in-vivo 185
Figure 8.6 Npam43 passes the BBB and exhibits a ~3 hours half-life
Figure 9.1 Npam43 reduces the infract volume of ischemic brain <i>in-vivo</i>
Figure 9.2 Post-stoke treatment of Npam43 promotes a long-lasting reduction of neuronal
damage and improves behavioral performances after a focal ischemic brain insult 193
Figure 9.3 Post-stoke treatment of Npam43 improves behavioral performances after a focal
ischemic brain insult
Figure 10.1 Schematic representation of the drug discovery pipeline used for the screening
process, hit selection, and compound validation <i>in-vitro</i> and <i>in-vivo</i>
Figure 10.2 Lead optimization from a high μM hit compound (Npam02) to a high nanomolar
lead compounds
Figure 10.3 Structural architecture of the predicted GluN1/GluN2A NMDA receptor complex in
the presence and absence of Npam43 at the NTD 213

List of abbreviations

AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPAR	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartic acid receptor
GABA	γ-Aminobutyric acid
GABA _A R	A type γ-Aminobutyric acid receptor
CNS	Central nerves system
APV	(2R)-amino-5-phosphonovaleric acid
TTX	Tetrodotoxin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
ECS	Extracellular solution
Glu	Glutamate
Amox	Amoxicillin
PNG	Penicillin-G
BBB	Blood-brain-barrier
LGIC	Ligand-gated ion channel
PS	Pregnenolone sulphate
mGluRs	Metabolic glutamate receptors
LTP	Long-term potentiation
OGD	Oxygen and glucose deprivation
NTD	N- terminal domain
LBD	Ligand-binding domain

ТМ	Transmembrane	
MCAo	Middle cerebral arterial occlusion	
TTC	2,3,5-Triphenyltetrazolium chloride	
Npam	NMDA positive allosteric modulator	
PAM	Positive allosteric modulator	
NAM	Negative allosteric modulator	
HEK293	Human embryonic kidney 293 cells	
DMSO	Dimethyl sulphoxide	
NMR	Nuclear magnetic resonance	
CREB	cAMP response element binding protein	
pCREB	Phosphorylated cAMP response element binding protein	
IF	Ifenprodril	
NVP or NVP-AAM077 PEAQX tetrasodium hydrate		
GluN2A	Glutamate receptor ionotropic, N-methyl-D-aspartic acid receptor 2A	
GluN2B	Glutamate receptor ionotropic, N-methyl-D-aspartic acid receptor 2B	
ESI	Electrospray ionization mass spectrometry	
GluN1	Glutamate receptor ionotropic, N-methyl-D-aspartic acid receptor 1	
HPLC	High-performance-liquid-chromatography	
ECD	Electrochemical detection	
CSF	Cerebrospinal fluid	
MCA	Middle cerebral artery	
LC	Liquid chromatography	
IS	Internal standard	

TLC	Thin-layer chromatography	
TCN201	3-Chloro-4-fluoro-N-[4-[[2-	
(phenylcarbonyl)hydrazino]carbonyl]benzyl]benzenesulfonamide		
hERG	ether-a-go-go-related gene (hERG) potassium channels	
LDH	Lactate dehydrogenase	
¹ H-NMR	Proton nuclear magnetic resonance	
EC ₅₀	Concentration that gives half-maximal response; Effective concentration	
H_2O_2	Hydrogen peroxide	
H-bond	Hydrogen bonding	
rCSF	Rat cerebrospinal fluid	
i.v.	Intravenous	
MRI	Magnetic resonance imaging	
LTD	Long-term depression	
HT	Hemorrhagic transformation	
DWI	Diffusion weighted imaging	
CSF	Cerebrospinal fluid	
μΜ	micromolar	
nM	nanomolar	

Acknowledgements

It is a genuine pleasure to express my deep sense of thanks and gratitude to my mentor, Dr. Yu Tian Wang. His dedication and keen interest and his overwhelming positive attitude to help his students was an integral part in the successful accomplishment for completing my work. With his patience, passion, knowledge and scientific insight, he supported me throughout my PhD study and helped me overcome many of the challenges faced when working in science. One particular attribute about him that has helped me to succeed in this project was that Dr. Yu Tian Wang trusted and believed in me right from the beginning and this allowed me to use my previously acquired knowledge and skills and integrate them to this PhD study. I will be internally grateful to have him as a role model for my scientific career. I would also like to acknowledge Dr. Anthony Phillips and Dr. Terry Snutch, for their continuous support and helpful suggestions during my project. They have also given me scientific guidance but also emotional support which was important ingredients in succeeding in a multi-year scientific project.

I would like to extend my appreciation to all of the members in the Wang Lab because over so many years we can consider ourselves one big family. I would like to give a special appreciation to Dr. Steven Conner and Dr. Allen Chan who has collaborated with me on this project but also encouraged me. They are two amazing scientists! I would like to extend a special deep sense of gratitude to Dr. Jie Lu, who has been supporting me throughout the project. Not only, has he helped me scientifically but also given me motivation and emotional support during the years. The emotional support he has given me was critical for succeeding in this project especially during the dark days and when I first started. He pushed me through it and if was not for him, xxi

this project would not have progressed so far. I would like to thank them for providing valuable discussion and advice for my work. I would like to thank Ms. Yuping Li, Dr. Soyon Ahn, Mrs. Giada Vacca, Ms. Maya Nesbit, Mr. Yang Ge, and Mrs. Ya Wen for the support throughout the years for the continuous technical assistance, moral support and scientific discussions. I would like to give a special thanks to UBC and Heart and Stroke Foundation of BC/Yukon and the Prostate Cancer Canada for their sustained financial supports for my PhD study and research. I would also extend my deep gratitude to Mr. Rodney Baker who has provided emotional support right from the beginning and who has kindly proofread this tome. I would like to thank him for his moral support and especially for his encouragement. His 'no problem' attitude helped me to face any challenges without fear. I additionally would like to thank my 'second' family (Mr. David and Mrs. Joanne North) who have stood by me in the bad and good times right from the beginning when I was only 19.5 inches tall. They were integral part of my success over the years and especially during my difficult years shortly after 2002. Personally, I'd like to thank my brother, Dr. John (Berdeax) Axerio-Cilies, and my mother, Anna (Ceppi) Axerio-Cilies for their unconditional love and support and the only reason why I did the PhD in the first place. This work could never be done without their great moral and financial support. Their patience and love have given me the motivation and strength to do something no one would have ever imagined and almost literally impossible. Finally, I would like to thank my father, Peter Axerio-Cilies, who inspired me to be the best who I can be. Unfortunately, he was not able to see it happen but he was in my heart and my fuel. I can only hope that he would have been proud of me in completing the PhD study. I am sure he would have enjoyed seeing me reach this endpoint. I hope I fulfilled a promise.

Dedication

To my beloved family and to my father.

Chapter 1: Introduction

1.1 Overview of the N-methyl-d-aspartate glutamate receptors (NMDARs)

1.1.1 NMDARs as calcium channels

N-methyl-D-aspartate receptors (NMDARs) are sub-family of ionotropic glutamate receptors in the brain that have critical roles in mediating brain functions such as learning and memory (Tang et al., 1999), and also in the pathogenesis of disorders including acute brain insults such as stroke (Y. Liu et al., 2007), brain trauma (Shohami & Biegon, 2014), chronic brain degenerative diseases such as Alzheimer's disease (Paoletti, Bellone, & Zhou, 2013), Huntington's disease (M. M. Fan & Raymond, 2007), and Parkinson's disease (Schmidt, Hochman, & MacLean, 1998). The NMDARs are described as tertrameric transmembrane channels composed of combinations of the obligatory GluN1 (previously also known as NR1) subunit with GluN2 (A-D; previously also known as NR2 A-D) and/or GluN3 (A & B) subunits (Collingridge, Olsen, Peters, & Spedding, 2009).

The principal excitatory NMDAR in the central nervous system is composed of two obligatory GluN1 subunits and two GluN2 subunits (A-D), which could be di-heteromeric (2 parts GluN1 & 2 parts GluN2A) or tri-heteromeric (ie. 2 parts GluN1 & 1 part GluN2A & 1 part GluN2B) (Hansen, Ogden, Yuan, & Traynelis, 2014). Activation of NMDARs requires the two co-agonists; L-glutamate and D-serine or glycine to bind to the different subunits of the receptor; two of each are thought to be necessary for maximum activation of the receptor, where each of the two GluN1 subunits are occupied by one molecule of D-serine or glycine, and each of the two GluN2 subunits associate with one molecule of L-glutamate (Chang & Kuo, 2008; Papouin

et al., 2012). Different GluN2 subunits (GluN2A – D) confer distinct electrophysiological and pharmacological properties on the receptor complexes and couple them with different signaling machineries (T. Bliss & Schoepfer, 2004; Seeburg, 1993a, 1993b).

NMDARs are ionotropic receptors which allow Na⁺ and Ca²⁺ ions to influx and let K⁺ ions to efflux (MacDermott, Mayer, Westbrook, Smith, & Barker, 1986; Voglis & Tavernarakis, 2006). Ca²⁺ influx through NMDARs is thought to be essential in synaptic plasticity, a cellular mechanism for learning and memory (MacDermott et al., 1986; Voglis & Tavernarakis, 2006). Under basal conditions, their high capacity for Ca²⁺ permeability (Jahr & Stevens, 1993) contributes strongly to the triggering of Ca²⁺-dependent intracellular signaling pathways downstream that ultimately control different forms of synaptic plasticity (Luscher & Malenka, 2012) (L. Liu et al., 2004) such as long-term potentiation (LTP) and long-term depression (LTD) (Shipton & Paulsen, 2014). Conversely, under certain pathological conditions, the overactivation of NMDARs causes an excessive influx of Ca²⁺ triggering apoptotic death signaling pathways which ultimately contributes to neuronal damages during acute brain insults such as ischemic stroke (Lai, Zhang, & Wang, 2014) and chronic neurodegenerative conditions such as Huntington's disease (L. Li et al., 2003).

1.1.2 NMDAR channel properties

Unarguably one of the most important physical properties of NMDARs, namely the one that confers its role upon learning and memory (Tang et al., 1999), is the tonic blockade of NMDARs in the channel pore by Mg^{2+} ions in a voltage-dependent manner (Blanke & VanDongen, 2009; Mayer, Westbrook, & Guthrie, 1984; L. Nowak, Bregestovski, Ascher,

Herbet, & Prochiantz, 1984). At resting potential, NMDARs are blocked by Mg^{2+} but during excitatory synaptic input depolarization of the neuron ultimately releases the Mg^{2+} blockade (Mayer et al., 1984; L. Nowak et al., 1984; Voglis & Tavernarakis, 2006). Accordingly, those NMDARs which have L-glutamate and glycine or D-serine bound in the appropriate binding sites, will open and allow Ca^{2+} to pass through the channel (Blanke & VanDongen, 2009; Papouin et al., 2012).

Importantly, NMDARs can act as coincidence detectors of both pre- and post-synaptic excitation (Luscher & Malenka, 2012). The GluN2 subunits are responsible for controlling the biophysical properties and characteristics of NMDA receptors, including their responsiveness to Mg²⁺ cations, channel conductance, decay time or deactivation kinetics, developmental expression levels, and subcellular distribution in neurons for instance extrasynaptic or synaptic regions (Evans et al., 2012). When the two co-agonists (D-serine/glycine and glutamate) are bind to their binding sites on NMDARs the receptor exhibits a relatively high single channel conductance approximately (~50pS) when compared to other glutamate receptor subtypes like α amino-3-hydroxy-5-methyl-4-isoazole-proprionic acid (AMPAR) (~2-20pS) but show slow kinetics with a rise times of (5-15ms) versus (0.2-0.8ms) for AMPAR (Cheffings & Colquhoun, 2000; Gebhardt & Cull-Candy, 2006; Hestrin, Sah, & Nicoll, 1990; Howe, Cull-Candy, & Colquhoun, 1991; Kleppe & Robinson, 1999; Papouin et al., 2012; Wyllie, Livesey, & Hardingham, 2013). NMDARs also deactivate with a much slower time course when compared to AMPARs (Pilati et al., 2016; Vance, Simorowski, Traynelis, & Furukawa, 2011; Wyllie et al., 2013). It has been suggested that the quick decay time attributed to GluN2A-containing NMDARs, make them superior as coincidence detectors of pre- and post- synaptic excitation,

whereas GluN2B-containing NMDARs have a more gradual decay time and therefore are more important for excitatory postsynaptic potential (EPSP) summation, which permits neurons to reach the firing ceiling more quickly (R. Li, Huang, Abbas, & Wigstrom, 2007; Tong & MacDermott, 2014).

1.1.3 NMDAR composition and structure

The GluN1 subunit gene expresses eight functional splice variants, while separate genes produce four types of GluN2 (A-D) and two types of GluN3 (A & B) (Wyllie et al., 2013). It is known that the type of splice variant of GluN1 controls the type of pharmacological properties of NMDARs, such as the inhibitory effects from proton (H^+) binding to the receptor (Masuko et al., 1999). The structural architecture of NMDARs can be characterized as having an extracellular N-terminal domain (NTD), three transmembrane domains (M1, M3, M4) with a pore channelforming "re-entrant" loop, a bi-lobed ligand-binding domain (LTD) formed by a distal fragment after the NTD (named the S1 domain) and a large extracellular loop linking M3 and M4 (named the S2 domain) plus the intracellular C-terminal domain (CTD) (Paoletti et al., 2013; Traynelis et al., 2010). The pair of S1 and S2 regions together are characterized as D-serine or glycine binding sites on GluN1 and the S1 and S2 regions of GluN2, constituting of the L-glutamate binding sites (Blanke & VanDongen, 2009; Papouin et al., 2012). The M2/p-loop is defined as the pore-facing region that contains an extremely important asparagine residue that endows NMDARs with the properties of Mg^{2+} blockade and Ca^{2+} flux (Blanke & VanDongen, 2009; Mayer et al., 1984; L. Nowak et al., 1984). The C-terminus of NMDARs contains areas that are recognized by a plethora of proteins that in turn act as channel modulators, enhancers and scaffolding proteins that stabilize the receptor to the synaptic and extrasynaptic sites for instance

(Ryan, Emes, Grant, & Komiyama, 2008). It has been shown that the CTD truncation in NMDARs did not change the subunit rise time but increased the decay time of glutamateactivated currents and stipulated that the CTD of GluN2 has a modulating role in NMDAR gating (Punnakkal, Jendritza, & Kohr, 2012). The similarity between GluN2 (A versus B) is quite high of 70% due to highly similar secondary structure but most of the differences come from the CTD (X. M. Zhang & Luo, 2013) and may suggest that the CTD-mediated intercellular signaling endows the differences seen for GluN2A versus GluN2B in synaptic plasticity (Massey et al., 2004).

1.1.4 NMDAR distribution

Unlike GluN1 mRNA, which is expressed in the majority of central neurons, the GluN2 subunits display distinct patterns of expression in the developing and adult brain (T. Bliss & Schoepfer, 2004), (Seeburg, 1993a), (Seeburg, 1993b). In the early postnatal and immature brain, the GluN2B subunit is the predominant subtype with the highest expression levels but as the brain matures GluN2B decreases and becomes largely confined to the forebrain (Ewald & Cline, 2009; Lohmann & Kessels, 2014). In contrast, in early development the expression of GluN2A is low and gradually increases as the brain matures at post-natal day 15 and expression levels GluN2A subunits overtakes GluN2B subunits in the adult brain (mainly forebrain; called the 'developmental switch') (Ewald & Cline, 2009; K. Williams, Russell, Shen, & Molinoff, 1993). This transformation has been explained by three proposed paradigms: 1) dramatic surge in synaptic GluN2A in conjunction with a decrease of GluN2B subunits (X. B. Liu, Murray, & Jones, 2004) (K. Williams et al., 1993); 2) extrasynaptic shift of GluN2B away from synapses with an augmentation in GluN2A levels (Dupuis et al., 2014); 3) increase in protein population

of GluN2A subunits overwhelming the effect GluN2B NMDARs without decreasing the absolute number of GluN2B receptors (X. B. Liu et al., 2004). As in the case with GluN2A, in early development the GluN2C subunits are extremely sparse, but as the brain reaches adulthood GluN2C subunit expression is augmented in the cerebellum (Wenzel, Fritschy, Mohler, & Benke, 1997). Similar to GluN2B, the GluN2D subunit is more prominent in the immature brain than the adult brain, and these receptors are mostly confined to the diencephalon and midbrain (Wenzel, Villa, Mohler, & Benke, 1996). As for the GluN3 class subunits of NMDARs, they have distinct properties that set them apart from their GluN2 counterparts (Low & Wee, 2010). The GluN3-containing NMDARs show several differences in receptor properties compared with the conventional GluN2 counterparts (Henson, Roberts, Perez-Otano, & Philpot, 2010).

In contrast, recombinant receptors composed of GluN1 and GluN3A or GluN3B are glycine sensitive channels, but lack glutamate binding sites, and thus can be activated by glycine alone (Henson et al., 2010). They are classified as glycine-gated cation-permeable ion channels but are insensitive and thus impermeable to Ca²⁺, insensitive to Mg²⁺ blockade and do not respond to traditional NMDAR antagonists such as ifenprodril; the GluN2B selective antagonist (Henson et al., 2010). Regions with the highest levels of GluN3A are spinal cord, thalamus, hypothalamus, brainstem, CA1, amygdala, and certain parts of the cortical area (Henson et al., 2010). On the other hand, GluN3B expression is confined to a few regions such as the spinal cord, brain stem, cerebellum, and hippocampus (Henson et al., 2010). NMDARs were considered to be expressed exclusively in neurons but more recent findings have demonstrated functional NMDARs in brain microglia, astrocytes, and in oligodendrocytes (Dzamba et al., 2015; Henson et al., 2010; Jimenez-Blasco, Santofimia-Castano, Gonzalez, Almeida, & Bolanos, 2015; Kaindl

et al., 2012). In neurons, the NMDARs are found at both pre- and post-synaptic sites and in the post-synaptic membrane of excitatory neurons (Luscher & Malenka, 2012) and in this study we will mainly focus on studying NMDARs in hippocampal and cortical neurons.

1.2 NMDAR pharmacology

1.2.1 Agonists of NMDARs

Early structure-activity relationship (SAR) studies established that L-glutamate is very efficient and effective for activating NMDARs demonstrated by its low EC₅₀ (0.4-1.8 μ M). (Cserep et al., 2012; Monaghan & Jane, 2009). It also was observed that excitatory activity needs one positive charge coming from the amino group (NH₃⁺) from L-glutamate and two negative charges coming from the two end termini carboxylic acid groups (COO⁻) (Monaghan & Jane, 2009; Traynelis et al., 2010). Furthermore, the positive charge amino group (NH₃⁺) should be attached α to the carboxyl group and the two negative charged terminal ends (COO⁻) need to be separated by four aliphatic carbon-carbon bond lengths for proper and efficient binding as longer or shorter carbon chains lose considerable affinity (Monaghan & Jane, 2009; Traynelis et al., 2010). Moreover, the α carbon to the carbonyl group should have an L or S (stereochemistry) geometry (the most predominant in the body) as its counterpart (D or R: stereochemistry) lost activity from EC₅₀ of 3.30 to 249 μ M for the GluN2A NMDAR which was similar to the GluN2B NMDAR complex (2.86 to 156 μ M) (Erreger et al., 2007; Monaghan & Jane, 2009).

These findings were later confirmed by co-crystallized GluN1/GluN2A structure with the two co-agonists L-glutamate and glycine in their respective binding sites in the ligand-binding domain (LBD) in between the S1 & S2 domains, with glycine binds to GluN1 and L-glutamate

interacts with GluN2 subunit (Storey, Opitz-Araya, & Barria, 2011; Traynelis et al., 2010). The discovery of NMDAR arise from the synthesis and study of a molecule called NMDA and different closely related antagonists such as D-AP5 (a phosphate derivative of NMDA) (Watkins, 1981). NMDA is a small molecule that mimics L-glutamate and is also known to activate the NMDAR selectively but with lower affinity than glutamate (30-100 µM vs 2.86-3.30 µM) (Erreger et al., 2007; Monaghan & Jane, 2009). The difference between glutamate and NMDA is that it is one carbon shorter and has a methylated amino group (-NH-CH₃) making it selective for NMDAR. This particular molecule is commonly used to study NMDAR pharmacology as it selectively activates NMDAR in the µM range whereas glutamate is known to be unspecific as it activates other glutamate receptors such as AMPA/kianate receptors (Clements, Feltz, Sahara, & Westbrook, 1998; Monaghan & Jane, 2009). However, the potency of NMDA is significantly weaker than L-glutamate and the selectivity to NMDARs seems to be attributed to an aspartate residue of GluN2 subunit which is able to engage with glutamate molecule within the binding site through H-bonding interactions (P. E. Chen & Wyllie, 2006; Monaghan & Jane, 2009). However, in AMPAR the corresponding residue is a glutamate and therefore unable to engage with NMDA due to the charge repulsion from two negative charges coming from NMDA and glutamate therefore prevents it from binding (Monaghan & Jane, 2009). This difference is enough to allow NMDA, which is one methylene group shorter than glutamate, to fit easily into the GluN2 subunit while also providing sufficient area to accommodate the -NH-CH₃ from NMDA (Monaghan, Irvine, Costa, Fang, & Jane, 2012; Monaghan & Jane, 2009; Traynelis et al., 2010).

1.2.2 GluN2 subunit selectivity of glutamate binding site ligands

The only structural feature that separates the NMDAR subtypes comes from the GluN2 subunit; as the GluN1 subunit is characterized as obligatory among all NMDARs (Wyllie et al., 2013). Therefore, subtype selective pharmacological targeting, would to a large degree come from the GluN2 subunit rather than from the GluN1 subunit (Ogden & Traynelis, 2011). Furthermore, a pharmacological intervention can also be aimed at interfaces that make up the GluN1/GluN2A complex versus GluN1/GluN2B combination that could be structurally diverse, or alternatively the area in between the interface of the subunits which are spatially different (Traynelis et al., 2010; Wyllie et al., 2013). This spatial difference may arise due to the differences on how the two subunits (GluN1 and GluN2A compared to GluN1 and GluN2B) would assemble together (Traynelis et al., 2010; Wyllie et al., 2013). This group of subunits are all formed by four unique genes, each coding for slight differences in their glutamate binding sites and in the N-terminal domain (NTD) regulatory sites (Marchand et al., 2012; Monaghan & Jane, 2009; Wyllie et al., 2013). As, GluN2 subunits also confer distinct physiological and biochemical properties to NMDARs, the selective blockade of differing GluN2 subunit types should yield compounds with distinct therapeutic and pharmacological profiles (Paoletti et al., 2013; Wyllie et al., 2013). In this subunit, glutamate binds in between the S1 and S2 domains and physiological studies have demonstrated that both co-agonists glutamate and D-serine or glycine-binding sites must be occupied at the same time to allow the receptor to be completely activated (Monaghan & Jane, 2009; Papouin et al., 2012; Wyllie et al., 2013).

Agents that target the NTD would regulate activity via domain-domain interactions, and therefore their actions may be dependent on the specific complex or alternatively, the subunit in the heteromeric assembly may alter the pharmacological specificities of the nearby subunits (Krieger, Bahar, & Greger, 2015; Traynelis et al., 2010). For example, the glycine-site antagonist CGP 61594 has a ~10-times higher efficacy in a receptor containing GluN2B subunits than those containing GluN2A, which advocates that the adjacent GluN2 subunit may alter the pharmacological specificity of the GluN1 subunit (Honer et al., 1998; Monaghan & Jane, 2009; Tu & Kuo, 2015). Since glutamate binding sites in the GluN2 subunit have a highly conserved area of residues among all the subtypes, there are currently no glutamate-binding site antagonists that display a significant degree of GluN2-subunit selectivity (Monaghan & Jane, 2009; Wyllie et al., 2013). D-APV (a L-glutamate mimic composed of a phosphate terminal group) would be one of these antagonists that targets the glutamate binding site but is completely unselective towards any particular NMDAR subunit but it is used regularly as a tool to study NMDAR pharmacology *in-vitro* (Strong, Jing, Prosser, Traynelis, & Liotta, 2014) and will be used in this study.

On the other hand, antagonists that incorporated several ring topologies have shown varied selectivity patterns confirmed by recombinant receptors (Monaghan & Jane, 2009). For instance, LY233536, a bicyclic decahydroisoquinline, showed a preferential selectivity for GluN2B over GluN2A containing NMDAR (Buller & Monaghan, 1997; Monaghan & Jane, 2009; Morley et al., 2005). However, this selectivity was too low and consequently limited their pharmacological use to achieve inhibition for a particular subunit composition as this 10-times selectivity would be hard to control towards GluN2B NMDARs (Monaghan & Jane, 2009) (Wyllie et al., 2013)(Monaghan & Jane, 2009; Morley et al., 2013)(Monaghan & Jane, 2009; Morley et al., 2005). The multitude of chemical alterations of the ring backbone and continued SAR studies have led to the identification of an

inhibitor that showed 100-times selectivity for the GluN2A-containing NMDARs over GluN2Bcontaining NMDARs (Irvine et al., 2012; Monaghan & Jane, 2009). A quinoxaline 2-3 dione based antagonist (NVP-AAM007), which has been used extensively to study the pharmacological intervention of blocking GluN2A-containing NMDARs (de Marchena et al., 2008; Wu, Xu, Ren, Cao, & Zhuo, 2007). NVP-AAM077 also has high efficacy for GluN2C subunits and lower affinity for GluN2D-containing receptors and thus is modestly selective for GluN2A and GluN2C subunits (He, Shao, Wang, & Bausch, 2013; Monaghan et al., 2012; Monaghan & Jane, 2009). Overall, the major challenge in developing agents to distinguish between GluN2 subunits is attributed to the highly conserved regions among GluN2 subunits, especially if the area of interest is the glutamate binding site core (a highly conserved area among all subtypes). As a result of their careful analysis described above, many groups have started to postulate that selective targeting for a particular subunit composition will most likely come from distal regions of the glutamate binding site core (Monaghan et al., 2012; S. Zhu & Paoletti, 2015). This was deducted due to the fact that the glutamate and D-serine or glycine binding site residues are highly conserved among all NMDARs and thus difficult to target a particular subtype and therefore has forced the pharmacologist to target other distal regions from the core LBD. Therefore distal sites away from these regions were explored as interesting targets for modulation for NMDARs and will be explored in this study.

1.2.3 Glycine GluN1 subunit agonists for NMDARs

Similar to glutamate binding to the S1 & S2 site on the GluN2 subunit; D-serine or glycine binds to the S1 & S2 site on the GluN1 subunit and is an obligatory co-agonist for activation of NMDARs (Furukawa & Gouaux, 2003; Papouin et al., 2012; Wyllie et al., 2013). Initially, it was postulated that endogenous levels of extracellular glycine is enough to saturate the D-serine/glycine binding site on NMDARs; however, later studies report that extracellular glycine levels are not adequate to saturate the D-serine/glycine binding sites (Collingridge et al., 2013; Papouin et al., 2012). This stimulated interest in the development of positive modulators of NMDAR function via an interaction of the D-serine/glycine binding sites (Papouin et al., 2012; Strong et al., 2014). Amino acids such as D-alanine or D-serine display high affinities for the glycine site and behave as full agonists and recently Papouin et al. have showed that the more potent D-serine versus glycine might be the major agonist for synaptic NMDARs whereas the glycine agonist is the major species for extrasynaptic sites (Blanke & VanDongen, 2009; Papouin et al., 2012). It was also found that compounds that were geometrically constrained cyclic mimics of the glycine molecule such as ACPC, a cyclopropyl analogue, and ACBC, a cyclobutane mimic, were partial agonists with different ranges of potency (Erreger et al., 2007; G. Nowak, Li, & Paul, 2000).

1.2.4 Allosteric modulatory sites for NMDARs

A surprising number of pharmacological agents bind and inhibit NMDAR activity specifically at GluN2B-containing receptors but they mostly fall within the same subclass (Karakas, Simorowski, & Furukawa, 2011). This family of inhibitors have a characteristic phenylethanolamine backbone which are known to bind at sites distinct from the glutamate- and D-serine/glycine-binding sites (Amico-Ruvio, Paganelli, Myers, & Popescu, 2012). One of these prototypes, ifenprodil (IF) which is comprised of this phenylethanolamine pharamcophore exhibits greater selectivity for GluN2B over GluN2A containing receptors and very low affinity at GluN2C and GluN2D-containing receptors (Monaghan et al., 2012; Paoletti et al., 2013; K. Williams, 2009). The ifenprodil binding site is located on the NTD region and involves amino acid residues distinct from (to some extent overlapping) residues that contribute to polyamine binding (Mony, Kew, Gunthorpe, & Paoletti, 2009; Perin-Dureau, Rachline, Neyton, & Paoletti, 2002). The GluN1 insert (exon 5), which alters polyamine modulation of NMDARs has no effect on ifenprodil inhibition of NMDAR activity (Williams 2009)(Wyllie et al., 2013)(Kasawar & Farooqui, 2010; Mony et al., 2009). This advocates that the glycine-independent polyamine binding sites on NMDARs are located in a separate area when compared to the ifenprodil binding site (Perin-Dureau, Rachline et al. 2002). A variety of other compounds show GluN2B selectivity, including haloperidol, CP-101,606, and Ro 25-6981 which are all ifenprodril mimics (Karakas et al., 2011; Mutel et al., 1998). These compounds display the highest degree of subtype selectivity among the different classes of NMDAR antagonists (Mutel, Buchy et. al 1998). SAR analysis of ifenprodil-like compounds has been explored extensively and multiple series of compounds have been optimized for selective high affinity binding (Mony, Triballeau, Paoletti, Acher, & Bertrand, 2010). A lot of work has already gone into modifying these if enprodril-like drugs structurally to avoid any interactions with the α -1 adrenergic receptor and/or human ether-a-go-go (hERG) K⁺ channel as blocking these receptors unintentionally can lead to serious side effects including cardiac arrhythmias (Monaghan & Jane, 2009; Traynelis et al., 2010). Several lead compounds provided new preclinical insights regarding the role of GluN2B subunits in neuropathic pain and excitotoxicity (Qu et al., 2009); (Y. Liu et al., 2007).
The pharmacophore structure of the ifenprodil can be described by two aromatic rings separated by a linker with a basic nitrogen in the middle. Commonly, each ifenprodil-like compound has a 4-benzyl-piperidine group that is connected to one aromatic ring and the basic nitrogen atom (Borza & Domany, 2006). This moiety is then linked to a second aromatic ring system that optimally has a hydrogen bond donor such as an hydroxyl group (-OH)(Borza and Dormany 2006). Thus, the potency of ifenprodil is reduced by removal of its phenol hydroxy group (Borza & Domany, 2006; Masuko et al., 1999). This general structure is similar to those of the well-characterized GluN2B antagonists, Ro-25,6981 (Fischer et al., 1997) and CP-101,606 (Layton, Kelly, & Rodzinak, 2006). Medicinal chemistry efforts of the initial pharmacophore structure showed that the removal of an aromatic ring or nitrogen is tolerable (Monaghan & Jane, 2009)(Ma, Yeo, Farooqui, & Ong, 2011). The phenol ring was able to be translated to heterocyclics such as a benzimidazole (McCauley et al., 2004), benzimidazolone (Wright et al., 2000), benzoxazole-2(3H)-one (Wright et al., 2000), indole-2-carboxamides (Borza et al., 2003), and aminotriazole (Gregory et al., 2000), especially if they contain an H-bond donor substituent (Gurusamy et al., 2011). Of particular note, the nitrogen within the linker was not deemed to be critical towards the activity for GluN2B NMDARs (Gurusamy et al., 2011; Tamiz et al., 1999). It was found that the incorporation of an acidic nitrogen helped to reduce hERG and α -1 NE activity (Tamiz et al., 1999). Taken together all these modifications on the pharmacophore structure has helped to understand the structure-activity relationships (SARs) and has also helped to define the structural determinants that are responsible to NMDAR binding.

1.2.5 Zinc (Zn²⁺) as a NMDAR modulator

Zinc (Zn^{2+}) ion displays subunit-specific actions at recombinant NMDARs (Paoletti, Ascher, & Neyton, 1997). It displays a voltage-dependent inhibition of NMDAR responses in heteromeric GluN1/GluN2A and GluN1/GluN2B receptors (Paoletti et al., 1997). At lower concentrations (IC₅₀ in the nM range), it shows a voltage-independent inhibition of GluN1/GluN2A receptors compared to IC₅₀ in the µM range for GluN1/GluN2B (Amico-Ruvio, Murthy, Smith, & Popescu, 2011); (Christine & Choi, 1990; Paoletti et al., 1997). The GluN2A selectivity accounts for observations that the addition of heavy metal chelators significantly potentiates GluN1/GluN2A but not GluN1/GluN2B receptor responses (Paoletti et al., 1997). This result may be due to chelation of contaminant traces of heavy metals in solutions that tonically block GluN1/GluN2A NMDAR responses (Paoletti, Ascher et al. 2011). Two effects were observed for Zn^{2+} in cultured cortical neurons (Christine & Choi, 1990) where it induced a voltage-independent reduction in channel open probability at lower concentrations (Brouns et al., 2010; Ong et al., 2010) and conversely in the presence of Zn^{2+} at a high amounts (10 to 100 μ M) caused a voltage-dependent reduction in single channel amplitude which accompanied an increase in channel noise and showing a relatively quick channel block (Paoletti, Ascher et al. 2011). Since Zn^{2+} is co-released with glutamate from pre-synaptic terminals, Zn^{2+} modulation of NMDARs may be physiologically relevant (Aniksztejn, Charton, & Ben-Ari, 1987) (Assaf & Chung, 1984).

A combination of *in-silico* molecular modeling and experimental site-directed mutagenesis indicated that the NTD region forms a bi-lobed structure with a binding cavity in the center, which is geometrically similar to the D-serine/glycine or glutamate binding S1 & S2

domains (Paoletti et al., 2000). In GluN2A, specific histidine residues are located in this region and are the main residues engaged with Zn^{2+} blockage (Paoletti, Ascher et. al. 2011). Interestingly, these sites contour both sides of the binding pocket in the NTD structure, suggesting that Zn^{2+} binding may cause the domain to turn into its closed conformation geometry and this rearrangement can be transmitted downward towards the S1 and S2 domains in the LTD as a signal that would inhibit the receptor (Paoletti, Ascher et. al. 2011).

1.2.6 Final remarks for the pharmacology of NMDARs

Selective NMDAR antagonists for a particular subtype have been used as tools over the years to study the receptor-related physiological and pathological processes (Neyton & Paoletti, 2006; Paoletti & Neyton, 2007). The traditional and most used inhibitors for NMDARs would be MK801 and D-APV (Ogden & Traynelis, 2011). MK801 blocks NMDARs by blocking the channel pore in an activity-dependent manner (Sobolevsky & Yelshansky, 2000), whereas D-APV is a glutamate mimic and a competitive inhibitor for NMDA molecule at the L-glutamate binding site of the GluN2 subunit (M. Benveniste & Mayer, 1991). For GluN2A subunit selectivity, NVP-AAM007 can be used (Shipton & Paulsen, 2014), which also binds to the glutamate binding site on GluN2 (Auberson et al., 2002; Feng et al., 2004; B. D. Lee et al., 2005). TCN201 has been recently discovered to be a GluN2A selective antagonist that employs its effect via a novel modulation site located near glutamate or D-serine/glycine binding sites of the ligand-binding domain (LBD) (Hansen, Ogden, & Traynelis, 2012). Moreover, ifenprodril and its derivatives selectively target GluN1/GluN2B by binding to near the polyamine site located in the N-terminal domain (NTD) in a non-competitive manner and these are used extensively for selective inhibition GluN2B NMDARs (Shipton & Paulsen, 2014).

However, to date, there has been very little advancement in pharmacological enhancement of the function of NMDARs using a strategy of positive modulation of the receptor channel to study its effects on downstream signaling pathways. The pharmacology of NMDARs can be highly diverse mainly due to the involvedness of the different types of subunit composition of NMDARs (Paoletti et al., 2013). Despite many years of discovery efforts in the development of drugs that interact selectively with NMDAR complexes, only memantine, a low molecular weight and low affinity channel blocker, has successfully made it through clinical trials and onto the market due its fast kinetic binding which helps to reduce adverse effects by inhibiting NMDARs (Perng et al., 2006; Rainer et al., 2011; Rammes, Danysz, & Parsons, 2008). Nevertheless, recent advances in solving the X-ray crystal structures of drug binding towards GluN1 and GluN2 subunits will greatly assist in the development of selective agonists, antagonists, negative and positive modulators for NMDARs (Hackos & Hanson, 2016; Volgraf et al., 2016; S. Zhu & Paoletti, 2015). These discoveries of subunit-selective pharmacological modulators for NMDARs will facilitate the understanding of NMDAR function and their subunit selective roles they mediate in the CNS such as LTP and LTD in learning and memory paradigms or their roles in Alzheimer's diseases (Hackos & Hanson, 2016; Regan, Romero-Hernandez, & Furukawa, 2015; S. Zhu & Paoletti, 2015). Therefore, these developments are likely to generate new prospects for treating a variety of CNS pathologies in which NMDARs might play important roles (Hackos & Hanson, 2016, Regan, Romero-Hernandez, & Furukawa, Zhu & Paoletti, 2015).

1.3 NMDAR and its relationship with excitotoxicity

Excitotoxicity is defined as the collateral damage caused by a pathological overactivation and an over-accumulation of intercellular calcium (Ca^{2+}) ions in neurons due to abnormal release of excitatory neurotransmitters such as glutamate (Kristian & Siesjo, 1998; Lo, Dalkara, & Moskowitz, 2003). Excitotoxicity neurotransmitters like glutamate accumulate in the extracellular space (Dong, Wang, & Qin, 2009; Mark et al., 2001). The build-up of glutamate results in an over-activation of AMPAR and NMDAR-type glutamate receptors on other neurons, which will cause an influx of Na⁺, Cl⁻ and Ca²⁺ ions through the channels gated by these receptors (Dong et al., 2009; Kristian & Siesjo, 1998; Lo et al., 2003; Mark et al., 2001). Consequently, these neurons will become depolarized, which will cause a further increase of intracellular Ca²⁺ and more glutamate release triggers the initial step of a local ischemic event (Dong et al., 2009; Kristian & Siesjo, 1998; Lo et al., 2003). A consequence of this accumulation of intracellular Ca²⁺ ions would be that water passively follows the ion influx, resulting in cvtotoxic swelling and the increased Ca^{2+} levels initiates a series of cytoplasmic and nuclear pathological actions, among which generation of free radicals and activation of Ca²⁺-dependent enzymes are common events intracellularly (Dong et al., 2009; Kristian & Siesjo, 1998; Lo et al., 2003).

This change in intracellular Ca²⁺ is the primary causal factor for the activation of a plethora of destructive enzymes such as proteases, lipases and endonucleases that release inflammatory cytokines and other mediators, causing a significant loss of cellular integrity (Jaffer, Morris, Stewart, & Labhasetwar, 2011). These include proteolytic enzymes that degrade the integrity of cytoskeletal proteins and extracellular matrix proteins, as well as phospholipase

A2, calpains, endonucleases, adenosine triphosphatase, cyclooxygenase, and nitric oxide synthase type 1 resulting in a detrimental effect to the integrity of the cell and consequently releasing free radicals formed by the mitochondria (Brouns & De Deyn, 2009; Kalogeris, Baines, Krenz, & Korthuis, 2012). The formation and the release of free radicals/nitrogen-containing species and activation of degradative enzymes leads to acute cell death through necrosis but excitotoxic mechanisms can also initiate molecular paths that lead to apoptosis (Dong et al., 2009; Elmore, 2007; Festjens, Vanden Berghe, & Vandenabeele, 2006; Kalogeris et al., 2012). Finally, the intracellular signaling pathways activated during the excitotoxic event will initiate gene expression that can trigger inflammation, which is another mechanism that contributes to ischemic injury (Brouns & De Deyn, 2009; Lakhan, Kirchgessner, & Hofer, 2009; Lo et al., 2003).

The NMDAR is fundamental to many normal brain functions such as cognition and memory (T. V. Bliss & Collingridge, 1993; Collingridge, Isaac, & Wang, 2004). However, as already noted, its overactivation can cause neuronal death as a result of excitotoxicity (Collins, Dobkin, & Choi, 1989; S. A. Lipton & Nicotera, 1998). Although NMDAR-mediated excitotoxicity is thought to be a common pathological event leading to neuronal injury in many neurological disorders (Choi, 1988; S. A. Lipton & Nicotera, 1998), the evidence for their destructive roles in ischemic neuronal damage has been particularly strong (M. Chen et al., 2008; Dong et al., 2009; Lai et al., 2014; Sattler & Tymianski, 2000). Prolonged NMDA stimulation results in both necrotic and apoptotic neuronal death and NMDAR blockade protects neurons from ischemic neuronal injuries *in-vitro* (M. Aarts et al., 2002; Strominger, Slamovits, Herskovitz, & Lipton, 1994) (Rothman, 1983) (Rothman & Olney, 1995) (Y. Wang et al., 2004) (Y. T. Wang & Linden, 2000) and *in-vivo* (M. Aarts et al., 2002; Hewitt & Corbett, 1992; S. A.

Lipton & Rosenberg, 1994; Palmer, Miller, Cregan, Gendron, & Peeling, 1997; Simon, Swan, Griffiths, & Meldrum, 1984; Sorkin & Waters, 1993). Interestingly, NMDARs have also been demonstrated to have strong actions in promoting cell survival (Hardingham, Fukunaga, & Bading, 2002; Yano, Tokumitsu, & Soderling, 1998). Although the mechanisms underlying these paradoxical roles of NMDARs remain unclear, a recent study reports that subcellular localization of the receptors (synaptic vs. extrasynaptic) may be a determining factor; activation of synaptic NMDARs promotes neuronal survival whereas stimulating extrasynaptic NMDARs leads to neuronal death (Dick & Bading, 2010; Hardingham & Bading, 2010; Hardingham et al., 2002). However, as detailed below, using a combination of pharmacological and molecular biological techniques, it was demonstrated that the subunit composition of the NMDARs may also play a critical role of NMDARs in promoting neuronal survival and death.

1.4 Important signaling pathways for NMDARs

1.4.1 Pro-death signaling pathways downstream of NMDARs

It is well-established that elevated and prolonged levels of glutamate kill neurons (P. Lipton, 1999; Mattson, 2008). This process is initially governed by the over-activation of NMDARs but important questions remain including why is it so damaging and which downstream signaling pathways are involved? Neuronal cultures can be used to study how neurons respond to high activity levels of NMDARs, following their stimulation by application of amino acid L-glutamate or small molecule NMDA. These neurons experience delayed Ca²⁺ deregulation that precedes and causes necrotic cell death (Nicholls, 2009; Ward, Kushnareva, Greenwood, & Connolly, 2005; Ward, Rego, Frenguelli, & Nicholls, 2000). Several mechanisms are implicated in cell death triggered by Ca²⁺ influx through the NMDAR (Papadia &

20

Hardingham, 2007; Shu, Pei, & Lu, 2014). Firstly, mitochondrial dysfunction occurs in response to excessive Ca^{2+} uptake by the mitochondria and this causes the membrane to be depolarized, which in turn inhibits ATP production and diminishes cytosolic ATP, due to reversal of the mitochondrial ATPase (G. Cheng, Kong, Zhang, & Zhang, 2012; Nicholls, 2009; Ward et al., 2000). The loss of ATP has detrimental effects to the neuron's ability to regulate intracellular Ca^{2+} levels (Papadia & Hardingham, 2007; Uttara, Singh, Zamboni, & Mahajan, 2009). Most notably, the Ca^{2+} uptake in the mitochondrial can stimulate reactive oxygen species (ROS) formation and consequently discharge cytochrome c, necessary for the creation of the apoptosome and triggering the activation of caspases and consequently producing apoptosis; a form of cell death (Circu & Aw, 2010; Tait & Green, 2013; C. Wang & Youle, 2009).

Furthermore, toxic levels of NMDAR activation impairs Ca^{2+} efflux (G. J. Wang, Jackson, & Thayer, 2003). In neurons, Ca^{2+} efflux is attained through the plasma membrane Ca^{2+} ATPase pump and Na⁺/Ca²⁺ exchangers (NCXs) (Nikoletopoulou & Tavernarakis, 2012; Papadia & Hardingham, 2007). Furthermore, the rise of Ca^{2+} in the cell leads to activation of calpains which consequently cleave a major isoform of the plasma membrane Na⁺/Ca²⁺ exchanger (NCX3) thereby affecting the proper function of cerebellar granule neurons (Araujo et al., 2007; Bano et al., 2005; Brustovetsky, Bolshakov, & Brustovetsky, 2010; Papadia & Hardingham, 2007; Parnis et al., 2013). Undoubtedly, an event that is more damaging, the plasma membrane, Ca^{2+} ATPase, which depends on the energy from ATP hydrolysis are effected and compromised by excitotoxic insults via mechanisms that are associated by caspases and calpains (Brini & Carafoli, 2011; Brustovetsky et al., 2010; Zaidi, 2010). Another causal effect from the large escalation of Ca^{2+} is the over-activation of the Ca^{2+} -dependent nNOS (neuronal nitric oxide

synthase) which also affects cytotoxic downstream signaling including mitochondrial dysfunction, p38 mitogen-activated protein kinase signaling and consequently the activation TRPM (transient receptor potential melastatin) channel (Hardingham, 2009; Lai et al., 2014; Papadia & Hardingham, 2007; Soriano & Hardingham, 2007). nNOS activation is closely dependent to the nitric oxide (NO) formation, which under high levels can be toxic both by itself and when it is combined with other ROS such as superoxides to generate ONOO⁻ (peroxynitrite) species (Forstermann & Sessa, 2012; Lai et al., 2014; Pacher, Beckman, & Liaudet, 2007; Uttara et al., 2009).

Both NO and ONOO⁻ species are extremely toxic species and detrimental to cellular machineries, by inhibition of mitochondrial chain enzymes and triggering mitochondrial depolarization (Gao, Laude, & Cai, 2008; Uttara et al., 2009). Another pathway that is affected during the over-activation of NMDARs is NO release and its effect on TRPM7 cation channels (M. M. Aarts & Tymianski, 2005; Bae & Sun, 2013; Papadia & Hardingham, 2007). During excitotoxic conditions Ca^{2+} influx triggers both NO discharge via nNOS activation and superoxide formation via an uptake of Ca^{2+} , which combines with ONOO⁻ a species capable of activating the cation channel TRPM7 which also allows for detrimental Ca^{2+} influx and thus has been deemed an interesting new target to block for reducing cell death in cerebral ischemia (M. M. Aarts & Tymianski, 2005; Bae & Sun, 2011; Hardingham, 2009; Lai et al., 2014; Papadia & Hardingham, 2007; Sun et al., 2009).

Another kinase that is involved due to the detrimental effects of Ca²⁺ in the cell and mediate cell death, is stress-activated protein kinases (SAPKs)(Woodgett, Avruch, & Kyriakis,

1996; Zanke et al., 1996). In cerebellar granule neurons, NMDAR-dependent cell death depends on p38 activation, progressing to caspase-independent cell death (Cao et al., 2004; Hardingham & Bading, 2010; Papadia & Hardingham, 2007). This class of SAPK located in cortical neurons are the c-Jun N-terminal kinases (JNKs) which have been known to cause death *in-vitro* and *invivo* (Irving & Bamford, 2002; Mehan, Meena, Sharma, & Sankhla, 2011; R. S. Morrison et al., 2002; Papadia & Hardingham, 2007). Other new pathways are implicated during excitotoxic conditions which include Rho, a member of the Rho-family of GTPases, which were discovered to afflict to p38α mitogen-activated protein kinase-dependent excitotoxic neuronal death as well (Porras et al., 2004; Semenova et al., 2007).

1.4.2 Pro-survival signaling pathways downstream of NMDARs

Ca²⁺ influx through the NMDAR subtype of ionotropic glutamate receptors plays a paradoxical role in the CNS (Carvajal, Mattison, & Cerpa, 2016; Traynelis et al., 2010). On the one hand it mediates excitotoxic death triggered by stroke and other acute brain traumas, but there is also growing evidence that physiological levels of NMDAR activity promote cell survival (Hardingham, 2006; Hardingham & Bading, 2010; Papadia et al., 2008). In comparison to the destructive effects of over-activation of NMDARs, physiological stimulation of synaptic NMDAR activity promotes neuronal survival via the activation of cAMP response elementbinding (CREB) and its co-activator CREB-binding protein (CBP) that triggers gene transcription of many CREB-responsive genes (Hardingham, 2009; Hardingham & Bading, 2002; Papadia et al., 2008). CREB is activated by phosphorylation at the serine-133 (Ser133) position which can occur through a plethora of other mechanisms but also through the activation of NMDARs (Hardingham & Bading, 2002; Lai et al., 2014; B. Lee, Butcher, Hoyt, Impey, & Obrietan, 2005). This event involves the brief transient calmodulin kinase IV (CaMKIV) and the longer-lasting initiation of downstream targets of pERK, mitogen and stress-activated kinase 1/2 (MSK1/2) and the p90 ribosomal S6 kinase 2 (RSK2), which will translocate in the nucleus after phosphorylation by pERK1/2 in the cytoplasm or can be activated by ERK1/2 in the nucleus (Bengtson & Bading, 2012; Hardingham & Bading, 2002; Hardingham et al., 2002; Wayman, Lee, Tokumitsu, Silva, & Soderling, 2008). The ERK1/2-facilitated CREB phosphorylation (pCREB) event potentially are implicated to endow a critical role in extending the phosphorylation event of CREB at Ser133 after the brief transient phosphorylation by CaMKIV (Hardingham, Arnold, & Bading, 2001; Impey, Obrietan, et al., 1998; Impey, Smith, et al., 1998; Lai et al., 2014).

Overall, the activity-dependent CREB activation through NMDARs depends on the CaMKIV pathway within a 1 hour timeframe and the ERK-mediated pathway prolongs the CREB activation even longer at greater than 1 hour (Hagenston & Bading, 2011; Hardingham & Bading, 2002; Hardingham et al., 2002). It has been demonstrated that NMDAR-mediated CREB signaling depends on the ERK pathway and that the NMDAR-mediated ERK survival is highly dependent on CREB (Hardingham & Bading, 2010). Moreover, the protective outcomes seen after a brief stimulation of synaptic NMDAR activity is long-lived which suggests that neuroprotection is dependent on CREB signaling (Dick & Bading, 2010; Hardingham, 2009). Co-activator CREB binding protein (CPB) is also susceptible to a persistent increase in nuclear Ca^{2+} levels, which transactivates CPB by phosphorylation at serine-301 by a CaMKIV-dependent pathway and consequently triggers CPB/CREB-mediated transcription (S. C. Hu, Chrivia, & Ghosh, 1999; Lonze & Ginty, 2002; West, Griffith, & Greenberg, 2002). The

activation of CREB-mediated transcription is a necessary event for NMDAR-mediated neuronal survival and other targets such as the neurotrophin brain-derived neurotrophic factor (Bdnf)-dependent cell survival, which can make neurons more invulnerable to excitotoxic events and other types of negative stimuli which can cause cell death mechanisms (Hardingham & Bading, 2010; Lai et al., 2014; D. Lau, Bengtson, Buchthal, & Bading, 2015).

Among a plethora of nuclear Ca²⁺-regulated genes, a set of genes known as activity controlled inhibitors of death (AID) genes, have been shown to provide neurons with a sort of "safeguard" effect both *in vitro* and *in vivo* (Hardingham & Bading, 2010; S. J. Zhang et al., 2009). More specifically, CREB can not only control its own set of genes, but can also control expression of these AID genes like encoding activating transcription factor 3 (Atf3), B-cell translocation gene 2 (*Btg2*), B-cell lymphoma 6 (*Bcl6*), growth arrest and DNA-damage-inducible 45 beta (*Gadd45b*), *Gadd45g*, inhibin beta A (*Inhba*), interferon activated gene 202B (*Ifi202B*), neuronal PAS domain protein 4 (*Npas4*), nerve growth factor-induced gene B (*Nr4a1*; also known as nuclear receptor subfamily 4, group A, member 1) and serine protease inhibitor B2 (*Serpinb2*) (Hardingham & Bading, 2010; S. J. Zhang et al., 2009).

These were likely to be deemed the CREB target genes capable of promoting protective effects through a process that make the mitochondria less susceptible to stressful and cell-deathcausing stimuli (Hardingham, 2009; Hardingham & Bading, 2010; Lonze & Ginty, 2002). Another piece of evidence that CREB is a key player in the pro-survival signaling is the observation that under stressful environments such as oxidative stress and hypoxia, CREB becomes phosphorylated, which strongly suggests that it could be a defense mechanism of the cell against an event that is detrimental or unpleasant (Fulda, Gorman, Hori, & Samali, 2010; Lonze & Ginty, 2002). Moreover, a similar event was observed in the hippocampus, when a temporary ischemia initiated CREB phosphorylation (pCREB) that is transient in CA1 neurons but is sustained in dentate gyrus (DG) neurons (Bender, Lauterborn, Gall, Cariaga, & Baram, 2001; Lonze & Ginty, 2002). These two particular temporal changes in CREB phosphorylation between the hippocampus and the DG pushed the idea that the neuroprotective outcome in the DG was arbitrated through a CREB-dependent pathway (Bender, Lauterborn et al. 2001, Lonze and Ginty 2002).

This protective outcome appears to be dependent on CREB-dependent gene expression because administration of CRE decoy oligos, worsened the apoptotic event following the excitotoxic stimulation (Kitagawa, 2007; Lonze & Ginty, 2002). Similarly, in PC12 cells, under oxygen deprived conditions prompted the expression of the CREB-dependent pro-survival gene bcl-2, which is known to help make cells more impervious to the outside stimuli (Beitner-Johnson, Rust, Hsieh, & Millhorn, 2000; Lonze & Ginty, 2002; Maroto & Perez-Polo, 1997). All of these observations strongly suggest that insults are capable of triggering two main signals, one that stimulates the cell death pathway and the other activates CREB-directed pro-survival mechanism (Hardingham & Bading, 2002; Lonze & Ginty, 2002). It is conceivable to deduce that the destiny and the health of the cell depend strongly on which pathway is more dominant at a given time point (Lonze & Ginty, 2002; Soriano et al., 2006).

1.5 The failure of NMDAR antagonists in clinical trials

Although molecular and experimental animal studies have consistently demonstrated that over-activation of NMDARs is the primary step leading to neuronal injury following insults of stroke and brain trauma, (Arundine & Tymianski, 2004; J. M. Lee, Zipfel, & Choi, 1999; S. A. Lipton & Rosenberg, 1994; Mattson, 1997), several large-scale clinical trials have completely failed to get the necessary efficacy of NMDAR antagonists in reducing brain injuries (Gladstone, Black, Hakim, Heart, & Stroke Foundation of Ontario Centre of Excellence in Stroke, 2002; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002; J. M. Lee et al., 1999). Plausible explanations for this apparent contradiction between basic research results and clinical trials have been proposed. These include, but are not limited to, the inability to use the NMDAR antagonists at doses necessary for neuroprotection due to side effects, an inability to administer these drugs within their neuroprotective windows, inability to use the antagonists at protective doses due to potential blocking of normal brain function and neuronal survival, poor experimental designs, and also heterogeneity in the patient population (Albensi, Igoechi, Janigro, & Ilkanich, 2004; Corbett & Nurse, 1998; Gladstone et al., 2002; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002). As mentioned above, it is important to consider the opposing roles of NMDAR subtypes in promoting cell survival and cell death. Accordingly, we hypothesize that inhibition of NMDAR-mediated cell survival actions by some of the NMDAR blockers used in these clinical trials may also be an important contributing factor.

1.6 Opposing roles of GluN2A and GluN2B-containing NMDARs

Different GluN2 subunits (GluN2A – D) confer distinct electrophysiological and pharmacological properties on the NMDARs, therefore the incorporation of specific GluN2

subunits may be responsible for differential functions of NMDARs (T. Bliss & Schoepfer, 2004; Seeburg, 1993b; Seeburg et al., 1995). The subunit composition of the NMDAR confers distinct channel kinetics, agonist affinity, and sensitivity to inhibitors (C. G. Lau & Zukin, 2007; Paoletti et al., 2013). Moreover, NMDARs are expressed in different brain regions depending on their subunit composition (Bar-Shira, Maor, & Chechik, 2015). Previous reports, demonstrate that GluN2A- and GluN2B-containing NMDAR subtypes have opposing roles in dictating the direction of synaptic plasticity where GluN2A-containing NMDARs mediate LTP and GluN2Bcontaining NMDARs are mediating LTD (Kaufman et al., 2012; L. Liu et al., 2004). Genetic deletion of the GluN1 in mice completely abolishes NMDAR activity, and the animals do not survive (Myers, Dingledine, & Borges, 1999). In the case of GluN2A knockout mice, these animals survive but show compromised synaptic plasticity with regards to long-term potentiation (LTP) of synaptic strength along with an abnormal spatial memory formation (Brigman et al., 2008).

GluN2B knockout mice are not viable but experiments can be done when the knockout is specific to the pyramidal neurons of the cortex and the CA1 hippocampus, in which case they show a compromised long-term depression (LTD) and abnormal neuronal development (Brigman et al., 2010; C. Chen & Tonegawa, 1997; Lai et al., 2014). In addition, many of the physiological roles of the NMDAR subunits are related to unique regions of the cytoplasmic tails, and genetic deletion of these C-terminal domains causes many of the cellular features observed when the whole receptor is knockout (X. Fan, Jin, & Wang, 2014; Lai et al., 2014; Ryan et al., 2008; Wyllie et al., 2013). The protein machinery required for synaptic plasticity, including proteins required for LTP and LTD, have been demonstrated to interact directly with

28

the C-terminal domains of the GluN2A and GluN2B subunits of the NMDAR (Collingridge, Peineau, Howland, & Wang, 2010; X. Fan, Jin, & Wang, 2014; Lai et al., 2014; Shipton & Paulsen, 2014). The subunit composition of NMDARs is specific not only to different regions of the brain but also to different subcellular positions (extracellular versus synaptic compartments) and changes dynamically during the development of the brain and also during responses to different stimuli (Lai et al., 2014; Paoletti et al., 2013; Shipton & Paulsen, 2014).

For instance, the hippocampal and cortical neurons in the neonatal forebrain express GluN2B-containing NMDARs in synapses and extra-synaptic compartments, whereas the adult brain cortical neurons display an increased amount of synaptic GluN2A-containing NMDARs (Paoletti et al., 2013; Papouin & Oliet, 2014; Petralia, 2012). It was observed that when the rat brain reaches adulthood between P39–P92, the hippocampus shows a significant decrease in the amount of functional GluN2B-containing NMDARs which was proved by the application of GluN2B antagonists to block these receptors (Lai et al., 2014; Magnusson, 2012; Paoletti et al., 2013; Shipton & Paulsen, 2014). Moreover, the particular localization of NMDAR subunit types (GluN2A versus GluN2B) can be a major contributing factor of the distinct mechanistic effects from synaptic or extrasynaptic receptors (Hardingham & Bading, 2010; Wyllie et al., 2013). A strong example for this observation is that synaptic and extrasynaptic NMDARs have been shown to govern different directions of synaptic plasticity where synaptic NMDARs have shown to mediate the LTP and extrasynaptic NMDARs mediate the opposing LTD (L. Liu et al., 2004). One possible explanation for this observation could be attributed to the different NMDAR subtypes that are present in these compartments, with the GluN2A NMDAR-subtype mediating the LTP and the GluN2B NMDAR-subtype mediating the LTD (Lai et al., 2014; L. Liu et al.,

29

2004; Luscher & Malenka, 2012; Papouin & Oliet, 2014; Shipton & Paulsen, 2014)(Figure 1.1). Along the lines with this concept, the differential roles of synaptic and extrasynaptic NMDARs in neuronal death and survival can also be described by the existence of distinct NMDAR subunits in these sub-cellular locations (Hardingham & Bading, 2002; Hardingham et al., 2002; Y. Liu et al., 2007; Luo, Wu, & Chen, 2011). More specifically, GluN2B-containing NMDAR specific antagonists like Ro 25-6981 or ifenprodril are highly neuroprotective in models of cerebral ischemia particularly pertaining to an excitotoxic event (Y. Liu et al., 2007). They also have shown protective effects toward other neurodegenerative diseases (ie. Huntington's disease, Alzheimer's disease)(N. W. Hu, Klyubin, Anwyl, & Rowan, 2009; Verhagen Metman et al., 2002), whereas selective antagonism toward GluN2A NMDARs are awful and not effective neuroprotective agents and in some experimental studies they have actually exacerbated neuronal death suggesting blocking the function for these receptors do not block cell death and may have implications to cell survival by interfering with the cells' ability to signal the pro-survival pathway (Hardingham & Bading, 2010; Hardingham et al., 2002; L. Liu et al., 2004)(Figure 1.1).

All things considered the finding that GluN2A and GluN2B are the predominant GluN2 subunits in the adult forebrain, where stroke most frequently occurs, we hypothesize that GluN2A- and GluN2B-containing NMDARs may have differential roles in supporting neuronal survival and mediating neuronal death (L. Liu et al., 2004; Y. Liu et al., 2007), and hence have opposing consequences on excitotoxic brain damage following acute brain insults such as stroke and brain trauma, irrespective of the subcellular location of them (Y. Liu et al., 2007). Synaptic GluN2B-containing NMDARs appear to be sufficient for the induction of neuronal death, whereas synaptic GluN2A-containing NMDARs protects neurons against excitotoxic neuronal

death mediated by synaptic GluN2B-containing NMDARs (Hardingham & Bading, 2002, 2010; Hardingham et al., 2002; Hetman & Kharebava, 2006; Lai et al., 2014; Y. Liu et al., 2007; Zhou & Sheng, 2013)(Figure 1.1). This is also evident by the fact that NMDARs employ their subtypespecific actions via their unique C-terminus tails, swapping the C-terminal of GluN2B with the GluN2A C-terminal stopped greatly GluN2B NMDAR-mediated excitotoxic events both shown *in-vitro* and *in-vivo* (Martel et al., 2012; Sprengel et al., 1998). On the other hand, exchanging the C-terminus of GluN2A with the GluN2B C-terminus transformed the pro-survival GluN2Acontaining NMDAR into a receptor that now instilled an excitotoxic mechanism (Martel et al., 2012).

Overall, these observations suggest that since expression levels of GluN2A-containing NMDARs in the adult brain neurons are substantially increased and consequently play an important role in synaptic activity-dependent survival signaling, whereas GluN2B-containing NMDAR in extrasynaptic compartments play an important role in excitotoxicity cell death not only for under stroke conditions but also for other diseases associated with excitotoxic events such as Huntington's disease or traumatic brain injuries (TBI) (Hardingham & Bading, 2002; Hardingham et al., 2002; Lai et al., 2014; Wyllie et al., 2013). There are numerous studies have found that, in mature cortical cultures, activation of either synaptic or extrasynaptic GluN2B-containing NMDARs results in excitotoxicity, increasing neuronal apoptosis (Hardingham & Bading, 2010; Hardingham et al., 2002; Lai et al., 2014; Papadia et al., 2008; Zhou & Sheng, 2013). In contrast, activation of either synaptic or extrasynaptic GluN2A-containing NMDARs promotes neuronal survival and exerts a neuroprotective action against both NMDAR- and non-NMDAR-mediated neuronal damage (Hardingham & Bading, 2010; Hardingham et al., 2002; Y.

Liu et al., 2007; Papadia et al., 2008; Zhou & Sheng, 2013). These results strongly suggest that the two receptor subpopulations have opposing roles in promoting cell survival (GluN2A activation) and cell death (GluN2B activation) (Y. Liu et al., 2007). However, the neuroprotective effect of a GluN2B blockade has a relatively narrow window of efficacy as GluN2B antagonism and not suitable in a clinical setting (Y. Liu et al., 2007). replacing the Cterminus of GluN2A with the GluN2B C-terminus made the pro-survival GluN2A-containing NMDAR into a receptor that now mediates an excitotoxic mechanisms (Carvajal et al., 2016; Davalos, Shuaib, & Wahlgren, 2000). Glutamate is rapidly taken up by the cells and normal levels can be re-established in as little as 30 min following stroke insult (H. Benveniste, Drejer, Schousboe, & Diemer, 1984). At this time point, GluN2B is no longer to be activated and hence blockade of the receptor would not be expected to remain useful and completely ineffective to block cell death (X. Fan, Jin, & Wang, 2014; Y. Liu et al., 2007). In comparison, selective activation of GluN2A-containing receptors would initiate cell survival promoting signals, protecting neurons against ischemic damage irrespective of the time of activation in relation to the stroke event, and may therefore be anticipated to have a much wider therapeutic window and therefore more clinically relevant at time points greater than 3 hours (X. Fan, Jin, & Wang, 2014; Y. Liu et al., 2007).





Figure 1.1 Unique subpopulations of the NMDAR mediate neuronal death and survival

(a) During basal conditions, synaptic activity sustains neuronal survival via activation of the synaptic NMDARs which are mainly GluN2A-containing NMDARs. This pro-survival effect is dependent on the Ca^{2+} influx through these receptors. (b) Shortly after stroke or under excitotoxic conditions, a massive release of glutamate (glutamate cascade) into the synapses and extrasynaptic compartments which causes activation of both subpopulations of NMDARs at both synaptic and extrasynaptic sites (Hardingham & Bading, 2002, 2010). This spillover causes neuronal death via activation of extrasynaptic GluN2B-containing NMDARs (Hardingham & Bading, 2002, 2010; Hardingham et al., 2002; Lai et al., 2014).

1.7 Crystal structure of GluN1/GluN2B NMDARs

In 2005, Gouaux et al. crystallized the S1 & S2 domains of the GluN1 and GluN2A subunits in the ligand-binding domain (LBD), clearly demonstrating the L-glutamate and D-serine/glycine binding sites (Furukawa, Singh, Mancusso, & Gouaux, 2005; Papouin et al., 2012;

Shleper, Kartvelishvily, & Wolosker, 2005). More recently, this domain was used to cocrystallize a selective antagonist of GluN2A-containing NMDARs (TCN201), which showed an ability to bind a modulatory interface in between the S1 & S2 domains of the GluN1 and GluN2A subunits (Volgraf et al., 2016). At present there is no known crystal structure for the Nterminal and C-terminus domains for the GluN2A-containing NMDARs. On the other hand, the GluN1/GluN2B combination complex initially was crystallized in 2009, where the N-terminal domain was resolved in the presence of the GluN2B antagonist ifenprodril (Karakas, Simorowski, & Furukawa, 2009). In 2014, the X-ray crystal structure of the Xenopus laevis GluN1/GluN2B NMDAR was obtained with the allosteric inhibitor, Ro25-6981, partial agonists and the ion channel blocker, MK801 (C. H. Lee et al., 2014). This structure exposed that allosteric antagonist-bound GluN1/GluN2B structure has an overall 2-fold symmetry with a layered dimer-of-dimers arrangement of subunits (C. H. Lee et al., 2014). They showed that the interlinked connections and linkages of the R2 lobes of the NTDs to the LBDs allows for possible molecular paths for transmission of an allosteric signal to the D-serine/glycine and glutamate-binding LBD layer (C. H. Lee et al., 2014). This could suggest that NTD could be a suitable target that could endow structural rearrangements capable to cause movements that are transmitted to the LTD and potentially facilitate glutamate or D-serine/glycine binding and thus induce an allosteric signal (C. H. Lee et al., 2014).

It has been demonstrated that even though closure of the LBD 'clamshells' S1/S2 domains attributed to partial agonists has been observed, the ion channel gate is in a closed-blocked state, which gave the initial interpretations into the structural makeup for allosteric inactivation of a NMDAR complex (C. H. Lee et al., 2014). All the crystallized work completed

to this point was focused on crystallizing the NMDAR in the presence of a negative allosteric modulator that causes the conformational changes which leads to a closure of the LBD clamshell-like structure, consequently causing the receptor to be in its closed/blocked state (C. H. Lee et al., 2014). However, the crystal structure of NMDAR in the absence of a negative allosteric modulator and co-crystallized with L-glutamate and glycine in their respective binding sites, remained unknown and was expected to have different configurations when characterized receptor in its active form (C. H. Lee et al., 2014; Traynelis et al., 2010). Very recently, Zhu et. al. crystallized such a structure, revealing for the first time the active conformation of the activated GluN1/GluN2B structure complex with no inhibitor (S. Zhu et al., 2016). They reported the conformational changes in the domains associated to the activation of mammalian GluN1/GluN2B NMDARs (C. H. Lee et al., 2014; S. Zhu & Paoletti, 2015; S. Zhu et al., 2016).

Specifically, the activation mechanism requires opening of the bi-lobed architecture of the GluN2B NTD and re-positioning of the heterodimeric organization in the GluN1/GluN2B NTD (S. Zhu et al., 2016). They also showed that these changes lead to rotated GluN1/GluN2B heterodimeric pairs in both the NTD and LBD causing dilation of the gating ring (Zhu, Stein et al. 2016). Importantly, this was the first study to show the complexity of the dynamics of these receptors with respect to the different activation paradigms or arrangements controlled by the conformational changed states that occur when L-glutamate and D-serine/glycine bind to the receptor and particularly how the movements of the NTD can transmit down to the LBD and potentially cause allosteric effects (S. Zhu & Paoletti, 2015; S. Zhu et al., 2016).

1.8 Stroke

Stroke is the fifth and third leading cause of death for Americans and Canadians respectively and the leading cause of disability and cognitive impairment (Ovbiagele et al., 2013)(Public Health Agency of Canada.(2011) Tracking Heart Disease and Stroke in Canada – Stroke Highlights 2011. http://www.phac-aspc.gc.ca/cd-mc/cvd-mcv/sh-fs-2011/index-eng.php). However, the risk of having a stroke is heavily reliant on race, ethnicity and age (Ovbiagele et al., 2013; Trimble & Morgenstern, 2008). Approximately 130,000 people die each year from stroke which translates 1 out of every 20 deaths (2015; Go et al., 2013; Mozaffarian et al., 2015) and approximately annually, 800,000 people in the United States have been diagnosed with a stroke episode (Go et al., 2014; Go et al., 2013; Mozaffarian et al., 2015; Writing Group et al., 2016). Moreover, a vast majority (87%) of all strokes are ischemic which are caused by blood clots formed inside the blood vessels and block blood flow to the brain (Haast, Gustafson, & Kiliaan, 2012; Mozaffarian et al., 2015; Y. Zhang et al., 2008). This ultimately leads to a rapid loss of brain function and a slow cell death caused by lack of oxygen to the cells, programmed cell death (apoptosis), free radical formation and potentially an uncontrollable cell death (necrosis), all contributing to detrimental effects to the brain tissue (Kalogeris et al., 2012; Northington, Chavez-Valdez, & Martin, 2011). In other situations the strokes can be hemorrhagic which are characterized by a severe burst of the vessel resulting in the blood being poured out into the brain (Jordan & Hillis, 2007). The approximate expenditure in the United States associated with stroke are in the billions of dollars annually, which includes costs associated with health care services and medications which are needed to reduce the amount of injury and to improve recovery from the ischemic episode (Mozaffarian et al., 2015; Writing Group et al., 2016). Moreover, stroke is leading cause of disabilities which adds on to the health

31

care costs long-term (Mozaffarian et al., 2015; Writing Group et al., 2016). Taken together, stroke is a global issue, there is a great need to treat stroke and the current therapies are limiting (ie. narrow therapeutic therapies) and some cases ineffective and only appropriate to certain population demographics (Mozaffarian et al., 2015; Writing Group et al., 2016). The aim of this study to find a novel treatment for stroke patients that suffer ischemic strokes but this study could be theoretically expanded to test whether the treatment could be used in post-hemorrhagic conditions.

1.9 NMDAR-mediated excitotoxicity and its relationship with an ischemic stroke event

Neurodegeneration following stroke, cardiac arrest, or profound hypotension is a frequent and devastating phenomenon that is a major cause of morbidity and mortality in North America (Collins, 1986; DeGracia, Neumar, White, & Krause, 1996; Gladstone et al., 2002; S. A. Lipton & Rosenberg, 1994). Ischemic stroke is caused by a reduction in blood flow to the brain (Kristian & Siesjo, 1998; Uchino et al., 2016). This results in a depletion of oxygen and produces impaired energy metabolism and depolarization of cells that leads to excessive release and buildup of extracellular glutamate, which consequently leads to a buildup of Ca²⁺ ions in the intracellular space, elevated lactate levels, acidosis, formation of free radicals/nitrogencontaining species and if the disruption is severe enough, predominate cell death occurs which can effect nearby cells and causes a "domino" effect (Kristian & Siesjo, 1998; Rossi, Brady, & Mohr, 2007; Uchino et al., 2016). The mechanisms mediating this neurodegeneration likely involve multiple events; however there is increasing evidence that ischemic injury to neurons is caused, at least in part, by overactivation of glutamate receptors, particularly the NMDAR subtype (Hara & Snyder, 2007; Lai et al., 2014; Mark et al., 2001).

Both *in-vitro* and *in-vivo* studies have consistently showed that overactivation of NMDARs is the initial stage leading to neuronal injury following stroke or traumatic brain injury (TBI) (Arundine & Tymianski, 2004; J. M. Lee et al., 1999; S. A. Lipton & Rosenberg, 1994; Mattson, 1997). These NMDAR become overactivated by the high levels of extracellular glutamate that get released to the synaptic space and their concentration within depend on location and timing (Mark et al., 2001). These high levels are observed in the ischemic areas but they also remain high in the core region and are sustained well after few hours after an ischemic insult. On the other hand in the penumbra region where the damage is not too severe, the glutamate concentration would decrease down to normal levels in about 1 hour after the ischemic insult (Baron, Yamauchi, Fujioka, & Endres, 2014; Ceulemans et al., 2010; Kiewert, Mdzinarishvili, Hartmann, Bickel, & Klein, 2010). Basal extracellular glutamate concentrations is about 0.5-5 µM detected by microdialysis coupled with high-performance liquid chromatography (HPLC) but when substantial neuronal excitotoxic injury occurs, extracellular glutamate can rise to detrimental toxic levels of $30-80 \ \mu M$ and can remain at its peak for up to 3 hours, causing distraterous results for the cell via a massive amounts of Ca²⁺ influx (Kanthan, Shuaib, Griebel, & Miyashita, 1995; Kiewert et al., 2010). At these levels NMDARs would be obviously overactivated since EC_{50} of glutamate towards NMDARs are within the low 2-5µM range (Erreger et al., 2007). Consequently, the massive amounts of glutamate in the extracellular compartments overactivate NMDARs in adjacent and nearby neurons, triggering apoptotic cell death signaling pathways (P. Lipton, 1999). It has been shown in stroke patients and in animal stroke models that the consequence of this is that the cortical, CA1 hippocampal and cerebellar neurons would be particularly vulnerable to this ischemic insult via the massive release of glutamate and these neurons can degenerate 3-7 days after ischemia insult as the result of process called delayed neuronal death which NMDAR seem to play an important role to initiating this process (Abe et al., 1995; Bramlett & Dietrich, 2004; DeGracia et al., 1996; S. A. Lipton & Rosenberg, 1994; Petito, Feldmann, Pulsinelli, & Plum, 1987; Ribary & Lichtensteiger, 1989).

1.10 The evolution of an ischemic infract damaged area

To keep the brain functioning normally it needs an uninterrupted supply of oxygen and glucose delivered through blood flow but interruption of the cerebral blood supply can and frequently causes irreversible brain damage (Iadecola & Anrather, 2011a; Rink & Khanna, 2011). This damaged area can be evaluated by quantifying and measuring the total volume of the infract area, portraying the degree of the damaged brain tissue which can be visualized by magnetic resonance imaging (MRI), computed tomography (CT) or staining the infract versus undamaged areas (Goldlust, Paczynski, He, Hsu, & Goldberg, 1996; Popp, Jaenisch, Witte, & Frahm, 2009; Tatlisumak, 2002). Surrounded around the ischemic area, there are two critical well-defined zones that are vulnerable to injury; core ischemic zone and the ischemic penumbra zone (Y. Li, Chopp, Jiang, Zhang, & Zaloga, 1995). Interestingly, it has been found that neurons are more afflicted to this injury than glia and vascular cells and become quickly compromised and eventually become dysfunctional and gradually die when exposed to a stressful stimulus or hypoxia-ischemic conditions (Iadecola & Anrather, 2011a, 2011b). An ischemic stroke can be produced by occlusion of the middle cerebral artery (MCAo), the most common type of stroke in patients and consequently it's the most commonly used in the animal models (Iadecola & Anrather, 2011a; Rink & Khanna, 2011). In this type of stroke the injury is swifter and more

pronounced in the center of the ischemic core characterized by the relatively lower blood flow (< 10-25%)(Iadecola & Anrather, 2011a, 2011b; Rink & Khanna, 2011).

In the periphery of the ischemic region called the ischemic penumbra, neuronal injury evolves at a much lower rate because blood flow coming from nearby vascular areas (collateral flow) keeps cerebral perfusion above a certain level/ceiling that would stop cells from dying instantaneously, providing sufficient oxygen and glucose levels (Kunz & Iadecola, 2009; P. Lipton, 1999). Initially, the infract is manifested in the core ischemic zone and then progresses into the ischemic penumbra, where ~30 minutes of ischemia causes a diffused amount of cell death in the core, whereas increasing the ischemia to ~60 minutes would cause a more pronounced injury to the core due to this longer stroke-ischemic event (J. M. Lee, Grabb, Zipfel, & Choi, 2000; S. Liu, Levine, & Winn, 2010; Popp et al., 2009). In a severe cases where the ischemia lasts for an extended period of more than 6 hours, the infract is quite prominently extended into the penumbra and consequently reaches a dire situation in which the damage is not salvageable and it underscores the importance of getting a treatment early (Heiss, 2012; Hossmann, 2009; P. Lipton, 1999; S. Liu et al., 2010).

The progression of the ischemic brain infract has been classified and divided into three different levels which are defined by different pro-death mechanisms and based on timing from the onset of stroke: acute (~0-7 days), subacute (~1-3 weeks) and chronic (~late stage > 3 weeks) (Allen, Hasso, Handwerker, & Farid, 2012; Birenbaum, Bancroft, & Felsberg, 2011; Hossmann, 2006). The acute stage can be further divided into two phases: early hyperacute (0-6 hours) and late hyperacute (6-24 hours) where the early hyperacute is the stage in which therapies are administered and the ultimate goal is to achieve a therapy that can be given in the late hyperacute

stage (Allen et al., 2012). In the acute phase the ischemic damage is initially produced by depletion of adenosine triphosphate (ATP) and a development of an uncontrollable depolarization of cell membranes called anoxic depolarization (R. R. Liu & Murphy, 2009; Nedergaard & Hansen, 1993), which occurs 1-3 minutes after the stroke onset (Mongin, 2007; Welsh, Marcy, & Sims, 1991). This causes a spreading depolarization which is characterized by neuronal edema, dendritic spines distortion, an alteration of the slow electrical activity and suppressed brain electrical activity (Dreier, 2011). In the subacute stage, molecular cell injury occurs and consequently the infract moves from the ischemia core (not salvageable region) and extends to the ischemia penumbra which usually is manifested 4-6 hours after the onset of stroke (Allen et al., 2012; Q. Wang, Tang, & Yenari, 2007; Woodruff et al., 2011). Depending on the salvageable and theoretically targetable to stop the pro-death signaling pathways that are being activated (Allen et al., 2012; Q. Wang, Tang, & Yenari, 2007; Woodruff et al., 2011).

The main mechanism underlying this transition is the peri-infract spreading depression or depolarizations (Dreier, 2011; Lauritzen et al., 2011; Pettigrew et al., 1996) and myriad of cellular death mechanisms caused by excitotoxicity induced by glutamate (Jia, Njapo, Rastogi, & Hedna, 2015; Lucas & Newhouse, 1957), formation free radical/nitrogen-containing species that cause cytotoxicity (Eliasson et al., 1999; Pacher et al., 2007), Ca²⁺-activated proteases and calpains (Machado et al., 2015; Vosler, Brennan, & Chen, 2008), failure and dysfunction has been identified for the mitochondria, and endoplasmic reticulum (ER) (Kalogeris et al., 2012; Ouyang & Giffard, 2012; Paschen & Frandsen, 2001), which are all causal factors for the significant cell death pathology. The late phase or chronic stage which would be the most severe

in terms of pathology and in this will encompass brain edema and inflammation and usually difficult to treat and may lead to death (Jin, Yang, & Li, 2010; Kalogeris et al., 2012).

In the first stage, the ischemic core undergoes major mechanisms of cell death associated with energy failure due to the lack of oxygen and glucose and therefore neurons cannot produce enough ATP necessary to energize the ionic pumps that maintain the ionic gradient across the membrane, mainly the Na⁺–K⁺ ATPase (Nedergaard & Hansen, 1993). Subsequently, massive Na⁺ and Ca²⁺ cytoplasmic buildup leads to neuronal swelling (cytotoxic edema) with damaged organelles, deteriorated membranes/nucleus and eventual uncontrollable cell death (necrosis) (Elmore, 2007; Fink & Cookson, 2005). In the initial stages, unlike the ischemic core, the flow reduction in the ischemic penumbra is not enough to cause energy failure and hence the neurons stay alive for a prolonged period of time after the insult and thus the neurons in these regions are characterized as being salvageable (J. M. Lee et al., 2000). Nevertheless, these afflicted neurons can be stressed and susceptible to other harmful stimuli or pathogenic events as a consequence of their compromised weakened state caused by the original insult (P. Lipton, 1999).

Since, an infract evolves over an extended period of time (slow cell-death) after the blood flow has been reinstated or reperfusion, it was noticed that the advancement of the ischemia is not only be exclusively attributed by the failure of the energy deficient ATP pumps in the cells and therefore has led to the idea that other mechanisms are involved for the resulting delayed cell death that is occurring after the insult (Kalogeris et al., 2012). These mechanisms responsible for the ischemic damage has been blamed on a plethora of cellular and molecular events elicited by the sudden lack of blood flow and even after a situation where the blood has been reperfused (Iadecola & Anrather, 2011a; Kalogeris et al., 2012). The primary step and the trigger for these

cellular and molecular events has been blamed on the massive amount of extracellular buildup of glutamate which has been portrayed as being the main causative factor for the death observed in the ischemic penumbra regions (Iadecola & Anrather, 2011a; Moskowitz, Lo, & Iadecola, 2010; Xing, Arai, Lo, & Hommel, 2012). These pathways lead to a controlled cell death (apoptosis) or even uncontrollable cell death (necrosis) depending on the severity of the stroke insult and the metabolic conditions in which the neurons are in (Iadecola & Anrather, 2011a, 2011b; H. Liu et al., 2012; Xing et al., 2012). The cells that are injured or in the process of dying are also a contributor to inflammation because they expel danger signaling proteins which can trigger mechanisms associated with the immune system (Iadecola & Anrather, 2011a, 2011b; Rock & Kono, 2008). It is known that blocking glutamate receptors with antagonists, particularly NMDARs, produces a significant protective outcomes against ischemic injuries in several animal models when given in the first hour of the onset of stroke (Hoyte, Barber, Buchan, & Hill, 2004; J. M. Lee et al., 2000; Minnerup, Sutherland, Buchan, & Kleinschnitz, 2012). This blockade of NMDARs showed that the protective effects is attributed to the blockade of Ca^{2+} influx through these receptors and this strongly supports the idea that the death was correlated to the surplus of Ca²⁺ in the cells (Hoyte et al., 2004; Moha Ou Maati et al., 2013; Yu, Wu, & Wang, 2015).

The initial abnormal release of extracellular glutamate overactivates the NMDARs which leads to cytoplasmic buildup of Ca^{2+} , which in turn activates Ca^{2+} -dependent enzymes, including the proteases, caspases and calpains, formation of free radicals, synthesis and function of arachidonic acid metabolites and enzymes producing species such as NO (Dong et al., 2009; Iadecola & Anrather, 2011a, 2011b; Weber, 2012). As a result of this observation there is a high degree of evidence that increased amount of intracellular Ca^{2+} is known to be a trigger of the

downstream pro-death signaling pathways (Chung, Ryu, Kim, & Yoon, 2015; Fink & Cookson, 2005). Hippocampal CA1 cells are known to have a high expression levels of functional NMDAR protein and that under an ischemic event the intracellular amounts of Ca²⁺ elevates to from 0.1 μ M to cytotoxic levels of ~30-60 μ M (Kristian & Siesjo, 1998). At these detrimental concentrations of Ca²⁺, the calpains will be activated and lead to unregulated proteolysis into fragments, which can in turn breakdown many different proteins, including axonal cytoskeleton proteins such as α -spectrin, microtuble-associated protein (MAP), tau, and neurofilament proteins (NFP) as well as components of the myelin such as myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) (Kampfl et al., 1997; Saatman, Creed, & Raghupathi, 2010; Vosler et al., 2008). The consequence for the degradation of the cytoskeleton and plasma membranes has been cell death which can be initially blamed for the excess amounts of intracellular Ca²⁺ necessary to activate these degradative components of the cell (ie. calpain-mediated cleavage) (Elmore, 2007; Kampfl et al., 1997; Vosler et al., 2008).

Another detrimental effect of the massive buildup of intracellular Ca^{2+} is mitochondriamediated oxidative stress and this in turn can elicit the gating of a high-conductance pore in the inner mitochondrial membrane called the mitochondrial permeability transition (MPT) (Kristian & Siesjo, 1998; Webster, 2012). The MPT causes a collapse of the electrochemical potential for H⁺, thereby stopping or diminished ATP production, effecting the electron transport system, swelling of the mitochondria and subsequent production of reactive oxygen species (ROS) such as superoxide radical (O_2^{-}), superoxide anion (O_2^{-}), peroxide (O_2^{-2}), hydroxyl ion (OH⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (OH⁻), and singlet oxygen (1O₂) (G. Cheng et al., 2012; Kristian & Siesjo, 1998; M. P. Murphy, 2009). This process would lead to dire cellular consequences which include mitochondrial swelling and a breach of the outer mitochondrial membrane followed by a release death factors and certain necrosis of the cell (G. Cheng et al., 2012). The ROS will provide the swelling capabilities and help to breakdown of the outer membranes, releasing pro-apoptotic molecules such as apoptosis inducing factor (AIF), endonuclease G (Endo G), and cytochrome c, which can trigger apoptotic cell death (Cai, Yang, & Jones, 1998; Gogvadze, Orrenius, & Zhivotovsky, 2006; Kalogeris, Bao, & Korthuis, 2014). The formation and release of ROS is greatly elevated during an ischemic event due to the overactivation of the arachidonic acid metabolizing enzymes cyclooxygenase and lipoxygenase and their effect on the electron transport system (Kalogeris et al., 2014; Kiritoshi et al., 2003; Rink & Khanna, 2011). Furthermore, ROS radicals which are known to be extremely reactive that can further interact with nitric oxide (NO) which has been shown to increase after an ischemic event, can give rise to superoxide anion peroxynitrite (ONOO⁻) or other reactive nitrogen species that are deemed extremely toxic due to their ability to cause oxidative damage to cells and to the endoplasmic reticulum (ER) and also functional disturbances of the mitochondria, attacking proteins/lipids and damaging DNA especially in the ischemic core (Forstermann & Sessa, 2012; P. K. Liu, Robertson, & Valadka, 2002). The most disastrous implication of this interaction is the superoxide anion with cellular constituents such as 3-nitrotyrosine in proteins and base modifications of DNA (P. K. Liu et al., 2002).

As mentioned, the dysfunction of the mitochondria and its role in cell death is quite pronounced and well-documented after a brain injury but has also lead to the potential detrimental effects and dysfunction of the ER as well (P. K. Liu et al., 2002). It is quite evident that the dysfunction of the ER also plays a causal factor to cell death through ROS formation and this has implications of diminishing intracellular Ca^{2+} stores and therefore altering the Ca^{2+} homeostasis inside the cell (Bodalia, Li, & Jackson, 2013; Kristian & Siesjo, 1998). As free radicals are formed in the brain during ischemic injury they can play a detrimental role in secondary injury processes (dysfunction of the ER and mitochondria) therefore in light of this, drugs have been developed to coordinate with these free radical/nitrogen-containing species and prevent them from building up and causing harm (Shirley, Ord, & Work, 2014; Slemmer, Shacka, Sweeney, & Weber, 2008). In animal studies, these drugs have shown a neuroprotective positive outcomes against ischemic stroke and but unfortunately human clinical trials using free radical trapping drugs failed due to a lack of efficacy (Green, 2008; Kimelberg, 2008; C. X. Wang & Shuaib, 2007).

In the late phase (chronic), brain swelling and inflammation are the two main factors which contribute to the ischemic injury (Jin et al., 2010; Unterberg, Stover, Kress, & Kiening, 2004). Neuronal swelling has been reported to start within 30 minutes of a MCAo, predominantly near capillaries and may last for up to one day after reperfusion (Garcia et al., 1993). Brain swelling post-stroke is caused by the increased intracellular osmolality due to the entry of water in the intracellular space and the breakdown of blood-brain barrier (BBB) which can allow entry of blood related elements into the brain tissue and increasing the chances for more cell damage (Fernandez-Lopez et al., 2012; Hossmann, 2006; Liang, Bhatta, Gerzanich, & Simard, 2007). More recently, Rungta et al. have discovered a new mechanism for cytotoxic edema and have successfully identified a new target that is responsible for neuronal swelling (Rungta et al., 2015). They have showed that neuronal swelling depends on Na⁺ and Cl⁻ influx tu is autonomous from Ca²⁺ flux into the cell and that the swelling after Na⁺ and Cl⁻ influx causes Ca²⁺-independent neuronal death (Rungta et al., 2015). The protein target responsible for

this observation was the ion exchanger SLC26A11 which was identified by combination of knockdown experiments and the use of specific antagonists to block potential channels that have or could be implicated to the increased swelling found in neurons (Rungta et al., 2015). It was shown that the knockdown or the blockade for this ion exchanger SLC26A11 caused a significant attenuation of neuronal swelling and identified a new paradigm shift for stroke-induced edema (Rungta et al., 2015).

Inflammation is the one of the main culprits to the damage that can contribute strongly to the slow progression ischemic damages, especially the increased levels of pro-inflammatory cytokines, IL-1 β , IL-6, TNF- α and MMP-9 which are the important mediators for inflammation response following ischemic insults and can also be released by microglia through the production of ROS via NADPH oxidase (Ceulemans et al., 2010; Doll, Barr, & Simpkins, 2014; Jin et al., 2010). IL-1 β and TNF- α are known to be pro-inflammatory that appear to exacerbate injury where TNF- α is arguably one of the most important initiators of neuro-inflammation which mediate the apoptotic death signal via the induction of cell death paths such as caspase-8 activation pathways and caspase-3 activation (Pei, You, & Fu, 2015; L. Wang, Du, & Wang, 2008). Overall, the information stated above, the evolution of ischemic stroke depend on multistep mechanisms from the first few minutes of an anoxic depolarization of neurons and the progression to excitotoxic conditions shortly after that lead to massive amounts of intracellular Ca²⁺ followed by activation of calpains and formation of ROS which leads to inflammation and edema (Seidenstein, Barone, & Lytton, 2015; Xing et al., 2012). All of these mechanisms depend on the severity of insult and depend on time after the original insult (Seidenstein, Barone, & Lytton, 2015; Xing et al., 2012). The brain injury starts from the core region within 1-3 minutes

of the ischemia event and evolve to the penumbra in a matter of 60 minutes from early towards the late hyperacute stages (Moskowitz et al., 2010; Woodruff et al., 2011). The progression and the development of this damage afflicted to the tissues can continue even after 7 days and has implications after months even after the blood has been reinstated because of the "domino" effect from one neuron effects nearby neurons in a time dependent fashion (Woodruff et al., 2011). These slow progression and development of the ischemic injury are extremely complex and complicated and they involve countless of different pathways, it is almost impossible to identify which domino is being affected (activated/deactivated) at any given time and therefore in this work we have moved away from conventional-thinking of targeting a specific domino (pro-death signaling protein) and tried to shift this paradigm to enhance the pro-survival signaling pathway which will be independent of what pro-death signaling protein is being activated at any given time which in turn potentially improves narrow therapeutic windows (Majid, 2014). In this dissertation we are going to explore a neuroprotective therapy that will try to block the progression and try to save cells for ultimate death by enhancing the cell's own pro-survival capabilities which we hope can override the activation of the pro-death signaling cascade incurred after stroke.

1.11 Current treatments for ischemic stroke

Current treatments options for ischemic strokes have been limited to either restoring the blood flow by reperfusion after the onset of stroke through a vascular-based therapy or by blocking or interrupting the signaling pathways that lead to ischemic cell death through a neuroprotection strategy (Woodruff et al., 2011). The most commonly used therapy for the treatment of ischemic stroke has been to quickly reinstate the blood flow to the brain using a
vascular-based therapy which will restore the levels of glucose and oxygen back to the cells (Woodruff et al., 2011). The only clinically approved drug for this type of therapy has been the intravenous injection of tissue plasminogen activator (tPA), which helps to reinstate blood flow by dissolving the blood clot causing the stroke (Gravanis & Tsirka, 2008).

Nevertheless, the administration of tPA is restrictive because the treatment is only effective and more importantly safe when it's given within the first 3 hours of the stroke onset and a recent study has shown that it may be effective and safe when given at 4.5 hours after symptom onset in "selected" patients which further limits its use (N. T. Cheng & Kim, 2015; Gravanis & Tsirka, 2008; Hacke et al., 1998). Given, that the drug needs to be administered at the hospital and the average time for patients to arrive at the hospital could be anywhere close to 4-6 hours, tPA treatment is sadly often not a clinically reasonable choice for many stroke patients (McCulloch, 1991; Prass & Dirnagl, 1998; Scatton, 1994). Another inhibiting feature to this type of medication is that stroke patients are required to receive a computed tomography (CT) or magnetic resonance imaging (MRI) test to ascertain that the stroke is not hemorrhagic (caused by a tear in the artery's wall that produces bleeding into the brain) before tPA treatment can be given as this administration causes severe side effects and/or exacerbate the bleeding and potentially death (Green & Shuaib, 2006; Miller, Simpson, & Silver, 2011). Diffusion weighted imaging (DWI) is also a normally used MRI sequence for the evaluation of an acute ischaemic stroke and it is sensitive enough to detect small and early infarcts whereas the traditional T1 and T2-weighted imaging MRI sequences detect infract after 16 and 8 hours after onset of stroke respectively (Allen et al., 2012; van Everdingen, van der Grond, Kappelle, Ramos, & Mali, 1998). A frequent complication of an acute ischemic stroke is a hemorrhagic transformation

(HT) which does not apparent in the first 12 hours (hyperacute stage) after stroke induction but rather commonly found in the acute stage (24-48 hours) and consistently found 4 days after the stroke onset and therefore HT limits the use of tPA treatment because the therapeutic window of tPA is only 3-4 hours after stroke onset (Allen et al., 2012; J. Zhang, Yang, Sun, & Xing, 2014). Interestingly, the damaging effects of tPA could also include its interaction with the GluN1 subunit of NMDARs, leading to a potentiation of NMDAR-mediated signaling and consequential intercellular influx of Ca^{2+} and exacerbation of the pro-death signaling pathways which is a similar consequence to activating NMDARs via massive glutamate release (Adibhatla & Hatcher, 2008; Chevilley et al., 2015; Gaberel et al., 2013; Lopez-Atalaya et al., 2008).

Overall, the wide array of pitfalls concerning the use of tPA has led to its administration to only a handful (2-5%) of stroke patients due to the restrictive use and its and narrow therapeutic windows (Green & Shuaib, 2006; Micieli, Marcheselli, & Tosi, 2009). Another therapeutic strategy for the pathogenesis of stroke has been to block or interfere cell death signaling pathways triggered downstream during stroke (Broughton, Reutens, & Sobey, 2009; Shu et al., 2014). While molecular and experimental animal studies have convincingly revealed that overactivation of NMDARs is the initial step leading to neuronal injury following insults of stroke and traumatic brain injuries (Arundine & Tymianski, 2004; J. M. Lee et al., 1999; S. A. Lipton & Rosenberg, 1994; Y. Liu et al., 2007; Mattson, 1997), many large-scale clinical trials have failed to find the needed efficacy of NMDAR antagonists in reducing brain injuries (Gladstone et al., 2002; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002; J. M. Lee et al., 1999). The precise reasons underlying the apparent inconsistency between basic research results and human clinical trials remain largely unknown and elusive, although several explanations have been proposed. These include:

1) difficulty to use antagonists at therapeutically effective doses required for neuroprotection due to side effects,

2) inability to administer the drugs within neuroprotective windows due to time required for patient transport and diagnosis,

3) inability to use the treatments at protective doses due to potential blocking of normal brain function and neuronal survival,

4) poor experimental designs, and

5) heterogeneity in the patient population (Albensi et al., 2004; Corbett & Nurse, 1998; Gladstone et al., 2002; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002; Prass & Dirnagl, 1998). While 4 and 5 factors require better designed clinical trials, we focused our research on finding ways to lessen the first three factors.

One reason why experimental neuroprotection has yet to translate from animals to human stroke patients could be that unlike humans, rodent brains have very little white matter which has been shown to less susceptible to brain injury and this may lead to the possibility that different mechanisms of stroke injury-related pathophysiology and neuroprotection (Carmichael, 2005; Mattson, Duan, Pedersen, & Culmsee, 2001; Sozmen, Hinman, & Carmichael, 2012; Woodruff et al., 2011). Moreover, the models used in many studies have been mostly been rodents and thus have some limitations which include differences in resistance to swelling, a limited region of white matter, differences in inflammatory cascades and thus would be quite difficult to mirror the conditions witnessed in a human (Carmichael, 2005; Casals et al., 2011; Chesselet & Carmichael, 2012; Macrae, 2011; Sozmen et al., 2012). Moreover, the conditions observed in

stroke victims since many patients for instance are older-aged individuals and could also have other secondary afflictions like cardiovascular diseases that could affect the efficacy of the treatments or more importantly may affect the different mechanisms associated to ischemic injury especially when comparing it to a rodent model for stroke (Carmichael, 2005; Casals et al., 2011; Chesselet & Carmichael, 2012; Macrae, 2011; Sozmen et al., 2012).

The biggest hurdle at this point would be that treatments need to be given within the first hour of stroke onset, whereas the clinical trials usually consist of patient inclusion times that are a lot longer than 1 hour and closer to 4-6 hours for patient admission especially if people do not live close to hospitals or live in major cities (N. T. Cheng & Kim, 2015; Ford, 2008). Undoubtedly, there are major challenges in obtaining positive therapeutic outcomes from rodent models of stroke and translating them to humans but may suggest that these disparities between them highlights the possibility to use of a higher-order gyrencephalic non-human primates (such as cynomolgus macaques) (Cook, Teves, & Tymianski, 2012) which share genetic, anatomical and behavioral resemblances to humans and may constitute similar cellular mechanisms to relation after a stroke insults (Cook, Teves, & Tymianski, 2012; Courtine et al., 2007; Enard, Depaulis, & Roest Crollius, 2010; Ford, 2008). One possibility for the disparity between animal models to human positive therapeutic outcomes, as discussed previously, may arise from the different NMDAR subtypes which have opposing roles in promoting cell survival and cell death therefore, we hypothesize that inhibition of NMDAR-mediated cell survival actions by some of the NMDAR blockers used in these previous clinical trials may be an important contributing factor to the confounding results in relation to their ability to block normal brain function and neuronal survival which are extremely important to avoid.

1.12 Computer-aided drug discovery (CADD)

Given the long and expensive process involved in the development of a therapeutic drug, there is a mounting interest to augment computational capabilities to the drug discovery process including the drug design, optimization and development (Kapetanovic, 2008). In light of this, computer-aided or *in-silico* design has been used extensively to assist hit identification (initial active compounds), hit-to-lead selection, pharmacological optimization (absorption, distribution, metabolism, excretion and toxicity (ADMET) profile) and to enhance brain-blood-barrier (BBB) penetration for CNS pilot drugs (Kapetanovic 2008).

In particular, CADD can facilitate and expedite the identification of hit compounds by filtering large databases into a more practical chemical sets which can then be tested experimentally and biologically validated (Kapetanovic 2008). CADD is also used to guide the design of the initial hit compounds discovered either from *in-silico* or via high-throughput screens (Sliwoski, Kothiwale, Meiler, & Lowe, 2014). The process involves adding or removing groups from the original hit compound to enhance its interactions with the surrounding residues of a particular site of the protein and to ultimately create new chemical entities. Furthermore, computers can also assist with the identification of structure-activity-relationships (SARs) which facilitates the understanding on how to further improve lead compounds in order to generate the best possible chemical entities for clinical testing (Kapetanovic, 2008; Sliwoski et al., 2014). With the recent advancement in structural biology, in particular, the 3D crystal structures of proteins, computers can facilitate the prediction of the biological actions of proteins and their interacting compounds.

CADD can be divided into two main methods known as 1) ligand-based drug design usually when a pharmacophore is identified, (a 3D spatial arrangement of chemical features important for biological activity) and 2) structure-based drug design (target-drug docking), where a hit compound is identified based on the predicted interactions of the ligand to a protein pocket (Sliwoski et al., 2014). Ligand-based approach is used in cases where there is no previous structural information of protein and therefore depends entirely on the bioactivity profile of known drugs (usually need modest dataset) and usually includes some form of similarity searching based on ligands known to be active or/and ligand pharmacophore identification (known feature that is known to constitute the activity of the drug) (C. H. Lee, Huang, & Juan, 2011; Sliwoski et al., 2014). In this dissertation we use a structure-based drug design approach, where the 3-D structure (X-ray crystallography, NMR) or in this case a generated homology model (a comparative model created from an already known homologues structure of another protein) of the NMDAR protein are combined with a chemical library to screen against specific sites on NMDARs (Kapetanovic, 2008; Sliwoski et al., 2014).

1.13 Rationales, hypotheses and specific aims

As already noted, despite a large body of well documented evidence for a critical role of NMDAR-mediated excitotoxicity in brain damage following stroke and brain trauma, clinical trials of NMDAR antagonists as neuroprotectants have not been proven successful (Gladstone et al., 2002; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002; J. M. Lee et al., 1999). Thus, novel NMDAR-based stroke therapies are urgently needed. Based on our previous findings that activation of GluN2A and GluN2B-containing NMDARs promote neuronal survival and death,

respectively, and that NMDAR antagonists, even GluN2B specific ones, are limited as effective post-stroke therapeutics due to their narrow therapeutic window, may in part explain some of the failure of NMDAR antagonists in clinical trials (Y. Liu et al., 2007). Based on our studies, the selected activation of GluN2A-containing NMDARs may have several advantages over previous NMDAR antagonism-based stroke therapies. Firstly, we would predict this approach possesses a wider therapeutic window than GluN2B-containing receptor blockade. In fact, there may be no appreciable therapeutic window limitation as it protects neurons against brain damage by promoting neuronal survival, rather than blocking the activation of a death signal initiated by the stroke insult. Second, it is important to note that GluN2A-containing receptor activation is likely to be not only effective against NMDAR-mediated cell death (primary neuronal injuries), but also to guard against non-NMDAR-mediated cell death (secondary neuronal injuries) (Papadia et al., 2008). Increasing evidence supports the notion that some non-NMDAR-mediated mechanisms, while secondary to NMDAR activation, may contribute significantly to brain damage, particularly following severe stroke-mediated insults (M. Aarts et al., 2003; Xiong et al., 2004). Another potential advantage is that the enhanced activity of GluN2A-containing NMDARs promotes the induction of long-term potentiation (LTP) processes, which potentially increase synaptic strength and might be used to treat pathological synaptic states by inducing synaptic plasticity to ameliorate conditions arising from disrupted synaptic drive, such as in stroke conditions. These points lead to our working hypothesis that the *selective enhancement of* GluN2A-containing receptors, rather than a generic blockade of all NMDAR subtypes, is a more effective post-stroke neuroprotective therapy. In this regard, there is an urgent need for the development of highly specific positive modulators for the GluN2A-containing NMDAR subtypes.

The primary goal of this thesis is to validate and extend these predictions with the following specific research aims:

- Development of potent and selective positive allosteric modulators (PAMs) for GluN1/GluN2A NMDARs using iterative synergies between *in-silico* methods of chemical genomics, chemical synthesis, biochemical and electrophysiological testing techniques.
- Identification of the novel modulation site in the GluN1/GluN2A complex and generation of structure-activity relationships (SAR) for compounds interacting at this site.
- Evaluation of the effects of GluN2A modulators towards synaptic transmission and longterm potentiation in hippocampal slices
- Evaluation of the effects of the selective modulation of GluN1/GluN2A on downstream cell-survival signaling (CREB phosphorylation) and protection of neurons against NMDA-mediated excitotoxicity and H₂O₂ oxidative stress.
- Evaluation of the selectivity profile of a lead compound against GluN1/GluN2A, GluN1/GluN2B, AMPA, GABA-A and hERG potassium channels.
- 6) Evaluation of the preclinical profile of a lead PAM including brain-blood permeability, dose-concentration response, and *in-vivo* half-life.

- 7) Investigate whether a PAM can promote CREB phosphorylation *in-vivo*.
- 8) Investigate whether a PAM can reduce infract volume as well as rescue neurological deficits caused by ischemic insults in a rodent MCAo stroke model.

The positive allosteric modulators (PAMs) identified here are predicted to have numerous advantages over other therapeutic options. First, the PAMs will be small organic molecules, a more desirable therapeutic option as this relates to its superior oral bioavailability, pharmacological stability and the ease of synthesis. Moreover, treatments could potentially be given orally (and perhaps even at home) compared to other traditional treatments that need to be administered intravenously in hospitals. Another, advantage to this approach is that it is predicted to have reduced side effects as it should not inhibit downstream signaling pathways but rather promote a natural trigger to protect cells from various forms of death including excitotoxicity and oxidative stress. Taken together, this work will provide a novel putative therapeutic treatment for ischemic stroke and other pathological diseases associated with cell death.

Chapter 2: Methods and methodology

2.1 Homology model

Currently, there is no crystal structure of the GluN1/GluN2A N-terminal domain (NTD). Therefore we created a homology model based on the crystal structure of the homomeric GluN1/GluN2B NMDAR. This homology model of the rat NMDAR N-terminal extracellular domain (NTD) of the GluN1/GluN2A receptor was constructed using the X-ray structure of the analogous GluN1/GluN2B N-terminal domain (PDB code: 3QEK) (Webb & Sali, 2014a, 2014b)(Karakas et al., 2011). Although the GluN1/GluN2A receptor is a tetrameric receptor, this homology model was based on one dimer consisting of 1 subunit of GluN1 and 1 subunit of GluN2A. The NTDs of GluN2A and GluN2B show 72% sequence identity and 82% homology in the sequence alignment. The GluN1/GluN2A interface was subjected to multiple rounds of side chain optimization and energy minimization using Modeller to alleviate any strain introduced by our homology model (Webb & Sali, 2014a, 2014b).

2.2 Chemical library

The identification of quality hit compounds is one of the key steps in a drug development program. As high-throughput screening (HTS) is the main method to find hit and lead compounds, it is clear that well designed screening collections are more likely to yield tractable molecular entities (Davis, Keeling, Steele, Tomkinson, & Tinker, 2005; Hertzberg & Pope, 2000; Mander, 2000). Screening collections are now compiled using the principles of drug-like and lead-like chemical space. This is where compounds are filtered using various molecular descriptors, for example, according to Lipinski's rule of five (Lipinski, Lombardo, Dominy, & Feeney, 2001) and (Lipinski, 2000) or based on the findings of Oprea (Oprea, 2000; Oprea, Davis, Teague, & Leeson, 2001; Teague, Davis, Leeson, & Oprea, 1999). In the present study, we used Lipinski rule of three, specifically designed for developing CNS drugs. A subset of lead-like compounds was selected from the ZINC chemical library that provides a wide array of structures that can be purchased commercially. The lead-like subset has been filtered by incorporating molecular weight, lipophilicity and rotatable bond restrictions.

This subset was further filtered based on Lipinski's rule of three (Pajouhesh & Lenz, 2005) and three additional chemical properties which includes no carboxylic groups, no peptide bond-like structures, polar surface area between 60-80 A² and the compound possessed at least 1 nitrogen, 1 oxygen and 1 aromatic ring. The rationale for exclusion of carboxylic acid groups was their polarity consisting of charged functional groups detrimental for penetrating the BBB. Moreover, all candidate compounds were filtered based on whether they incorporated chemical motifs or known toxicophores. This exclusion process was based on moieties deemed "undersirable" for assay formats used in high-throughput screening campaigns (Axerio-Cilies, Castaneda, Mirza, & Reynisson, 2009).

These toxic functional groups excluded from the database were electrophilic reactive moieties (leaving or hydrolysable groups), heteroatom-heteoratom single bonds, suspected cytotoxic groups, tight-binding, metal chelating moieties, redox/thiol reactive species, common substructures of promiscuous non-specific inhibitors and moieties that are known to cause toxicity issues by DNA intercalation (Axerio-Cilies et al., 2009). The resulting database was finalized by adding hydrogens along with removal of minor components (salts), de-pronating

strong acids and protonating strong bases. Finally, this database was then energy minimized to attain the three-dimensional structure of the compound at the lowest energy state. The finalized optimized structures were then ready for the docking process against the interstice interface of GluN1/GluN2A heterodimer NTD.

2.3 Virtual Screening (docking) for selective modulators for GluN1/GluN2A-containing NMDARs

Using a previously described consensus-based *in-silico* methodology, (Axerio-Cilies et al., 2011; Lack et al., 2011) we conducted a virtual screen of ~200,000 purchasable chemical substances pre-filtered (Axerio-Cilies et al., 2009; Pajouhesh & Lenz, 2005) from the lead-like ZINC chemical library (Irwin & Shoichet, 2005) to identify specific binders that may be capable of positive allosteric modulation (PAM) of GluN1/GluN2A-containing NMDARs. The results from each stage of this multiparametric approach were compiled, and the compounds were ranked using a consensus scoring procedure. The ~10,000 highest ranked compounds were visualized, and 200 initial candidates, predicted to have a high potential for binding to the GluN1/GluN2A interface, were selected for empirical testing.

2.4 Analog search

To provide confirmation that the hit compounds are positive allosteric modulators for the GluN1/GluN2A NMDARs, an analog search was conducted to obtain a large pool of chemicals from which to generate structure-activity-relationships (SAR) between the ligand and protein. An analog search was implemented to identify structurally similar compounds that are commercially available from chemical suppliers. This approach is based on the notion that similar compounds

should have similar biological properties. In this process, the active compound (Npam02) was used as a template/query to search against a database of chemicals which will hopefully generate a subclass of compounds with enhanced activity. Molecular fingerprint-based similarity is a commonly used method for similarity searching. The fingerprint is defined as a 'bit string' where each bit position accounts for the presence or absence of a given feature in a molecule (C. Williams, 2006). If the feature is present in a molecule the bit is set to '1' and if the feature is not present, it is set to '0' forming a distinctive and unique fingerprint profile for each chemical structure. The similarity between two molecules is identified by comparing bit strings of molecules and quantified as Tanimoto coefficient (Tc) (Bajusz, Racz, & Heberger, 2015). This process generated ~50 compounds available from a chemical supplier. These compounds were combined with the chemically synthesized compounds designed *in-silico* to generate a list of compounds that could be tested by whole-cell voltage clamp recordings in cortical neurons to generate SARs to ultimately identify a lead compound that would be selective for the GluN1/GluN2A, potent, soluble, non-toxic, stable and permeable to the blood-brain barrier (BBB).

2.5 Chemicals

N-methyl-D-aspartate (NMDA), D-2-amino-5-phosphonovaleric acid (APV), α-amino-3hydroxyl-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 Chemical synthesis

PART I: 2-hydroxy-3-ethoxybenzaldehyde (1.0 g, 5.2 mmol) was dissolved in acetic acid (20 mL) and N-chlorosuccinimide (NCS) or N-bromosuccinimide (1.4 g, 11 mmol) was added all at once. The reaction mixture was stirred overnight at 80°C, and then cooled to room temperature. Water and CH_2Cl_2 were then added, the phases were separated and the water phase was further extracted with CH_2Cl_2 , dried over MgSO₄ and evaporated under vacuo. The crude product was purified by flash chromatography (CH_2Cl_2 /hexane) to afford the pure product (1.2 g, 89 %) as a yellow solid. (see appendix 1 for NMR and mass spectroscopy)

PART II: Dissolve equimolar amounts of hydrazine (0.30mmol) and aldehyde (0.30mmol) in THF (0.5 M solution). Add 2 equivalents MgSO₄ and heat to reflux for 1 h. The product may precipitate. If not, the reaction was checked by TLC or NMR to ascertain consumption of the aldehyde. If the product precipitated, it was recovered by filtration and washed with water (2x2ml) to remove residual MgSO₄. If the product did not precipitate the reaction mixture was diluted with water until the product precipitated. The mixture was acidified to pH 3 with dilute HCl for the removal of residual hydrazine and consequently filtered and if necessary it was recrystallized. The compound is a high-melting solid (mp 210-212 °C). Extraction was avoided. If no precipitation was observed, the solvent was evaporated in vacuo and the compound was purified by flash column chromatography. (see appendix 1 for NMR and mass spectroscopy).

2.7 Determination of compound purity

Compound identity and purity were confirmed by LC–MS/MS. Briefly, an Acquity ultraperformance liquid chromatograph (UPLC) with a 2.1 mm × 100 mm BEH, 1.7 μ M, C18 column coupled to a photodiode array (PDA) detector in line with a Quattro Premier XE (Waters, Milford, MA) was used with water and acetonitrile containing 0.1% formic acid as mobile phases. A 5–95% acetonitrile gradient from 0.2 to 10.0 min was used, and 95% was maintained for 2 min followed by re-equilibration to starting conditions for a total run time of 15.0 min. The MS was run at unit resolution with 3 kV capillary, 120 and 300 °C source and desolvation temperatures, 50 and 1000 L/h cone and desolvation N₂ gas flows, and Ar collision gas set to 7.4–3 mbar. On the basis of the full range of the diode array absorbance (210–800 nm), the relative purity (area-under-the-curve (AUC) of Npam43 versus AUC of all the peaks detected) was calculated. All compounds described had a purity of > 90– 95%. HPLC-MS/MS Npam43-T_R = 5.02 min.

2.8 Buffers and media

Phosphate buffered saline (PBS, pH 7.4) contains 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.76 mM KH₂PO₄. Cell lysis buffer contained 0.5% Triton X-100, 0.5% 11 deoxycholic acid, and 1× protease and phosphatase inhibitor cocktail (Thermo Scientific, 78442) in sterile PBS. 4× sample buffer contains 50% Glycerol, 125 mM pH 6.8 TrisHCl, 4% SDS, 0.08% bromophenol blue, and 5% β -mercaptoethanol. Neuron culture media contained 2% B-27 supplement (Invitrogen, 17504-044) and 0.5 mM GlutaMax supplement (Invitrogen, 35050-061) in Neurobasal Media (Invitrogen, 21103-049).

2.9 Plasmids

Cells were transfected with a combination of pcDNA3-CMV expression vectors, each of which expressed one of the rat recombinant (GluN2A_{WT}, GluN1_{WT}, GluN2B_{WT}) subunits. An enhanced green fluorescent protein (GFP) pcDNA3-GFP was co-transfected to facilitate microscopic visualization. GluN2A_{A108G}, GluN2A_{P79A}, GluN2A_{P178G}, GluN2A_{Q111A}, GluN2A_{F114Y}, GluN2A_{F114S}, GluN2A_{F177S}, GluN2A_{I176Y}, GluN2A_{M111I}, GluN1_{R115E}, GluN1_{L135Q} plasmids were constructed by site-directed mutagenesis from either GluN1_{WT} and GluN2A_{WT} using PFU DNA polymerase. The sequences of all plasmids were confirmed by automated DNA sequencing.

2.10 HEK293 cell culture and plasmid transfection

Human Embryonic Kidney 293 (*HEK293* cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). When *HEK293* cells achieved 90% confluence, plasmids of either (GluN1 & GluN2A & eGFP) or (GluN1 & GluN2B & eGFP) were co-transfected into cells using Lipofectamine 2000 (Invitrogen, 11668019) according to manufacture's instruction. *HEK293* cells were then maintained in the 37°C incubator with 95% O_2 and 5% CO_2 for 48 hours before being used in experiments. The transfection ratio with these NMDAR subunit combinations were all 1:1 (GluN1/GluN2A or GluN1/GluN2B). pcDNA3-GFP was also co-transfected along with the NMDAR subunits as a transfected marker, in order to facilitate the visualization of the transfected cells during electrophysiological experiments. 10-12 hours after transfection, cells were re-plated on glass coverslips and after 20-24 hours transfection cells were ready to be used for whole-cell patch-clamp recordings.

2.11 Electrophysiology

Whole cell patch-clamp recordings were performed under voltage-clamp mode using an Axopatch 200B or 1D patch-clamp amplifier (Molecular Devices). Whole-cell currents were recorded at a holding potential of -60 mV unless indicated elsewhere, and signals were filtered at 2 kHz, digitized at 10 kHz (Digidata 1322A). Recording pipettes (3-5 M Ω) were filled with the intracellular solution that contained (mM): CsCl 140, HEPES 10, Mg-ATP 4, QX-314 5, pH 7.20; osmolarity, 290-295 mOsm. BAPTA (10 mM) was added in the intracellular solution (otherwise specified). The coverslips were continuously superfused with the extracellular solution containing (mM): NaCl 140, KCl 5.4, HEPES 10, CaCl₂ 1.3, glucose 20, pH 7.4; osmolarity, 305-315 mOsm. NMDA, GABA or AMPA induced currents were either applied by NMDA, GABA, AMPA either through perfusion fast-step (Warner Instruments). With perfusion fast-step system, NMDA, GABA, AMPA application was achieved by using a two-square barrel glass tubing and depending on age of the cultured neurons, CNQX (10 µM) and TTX (0.5 µM) were added in the extracellular solution to minimize the activation of ionotropic glutamate receptors and voltage-gated sodium channels, respectively. All experiments were performed at room temperature. Recordings from at least six HEK293 cells/neurons were performed for all active compounds. Data were pooled among HEK293 cells or primary neurons and composite dose-response data were fitted by the equation Percentage Response = $100 \times \text{Relative Efficacy}/[1]$ + $(EC_{50}/Concentration)^{nH}$], where EC_{50} is the concentration of agonist that produces a halfmaximal response, relative efficacy is the response at maximally effective concentration relative to the maximal response of glutamate, and $n_{\rm H}$ is the Hill slope.

2.12 Slice recordings

6-8 week old C57/B16 mice underwent cervical dislocation followed by decapitation. The brain was immediately transferred to an ice cold NMDG-based cutting solution consisting of: (in mM): 120 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 25 NaHCO₃, 1.0 CaCl₂, 7.0 MgCl₂, 2.4 Na-pyruvate, 1.3 Na-ascorbate, 20 D-glucose with pH adjusted to 7.35 using HCl acid (unless stated, all chemicals and drugs were purchased from Sigma or BioShop, Canada). The hippocampus was dissected out and transverse hippocampal slices (400 μ m) were obtained using a manual tissue chopper (Stoelting, Wood Dale, IL, USA). Slices revered in a heated (30°C) incubating chamber for 1 hr which contained ACSF composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgSO_{4.7}H₂O, 2 CaCl₂, 26 NaHCO₃ and 15 D-glucose which was bubbled continuously with carbogen (95%O₂/5%CO₂) (pH to 7.3). After 30 additional minutes at room temperature, slices were transferred to a submerged recording chamber and were perfused continuously with carbogenated ACSF (2-3 ml/min). Whole-cell recordings of CA1 pyramidal neurons were performed using the "blind" method with a MultiClamp 700B amplifier. EPSCs (excitatory post synaptic currents) were elicited by stimulating the SC pathway. For isolation of NMDAR currents, cells were voltage clamped at +40 mV. Recording pipettes were filled with solution containing (in mM): 122.5 Cs-methanesulfonate, 17.5 CsCl, 2 MgCl2, 10 EGTA, 10 HEPES, 4 ATP (K), and 5 QX-314, with pH adjusted to 7.2 by CsOH. Bicuculline methiodide (10 µM; Abcam) to block GABA receptor-mediated inhibitory synaptic currents and CNQX(10 µM; Abcam) to block AMPAR mediated currents were used to further isolate NMDAR currents. To

67

specifically isolate GluN2A and GluN2B components of NMDAR currents, NVP or ifenprodril (IF) were added to inhibit these receptors respectively. Confirmation that residual synaptic current was conducted by NMDARs was attained through application of APV at towards the end of experiments. EPSCs were recorded and analyzed using WinLTP. Statistical analyses were completed using GraphPad InStat. An ANOVA comparing NMDAR currents in reponse to various drug cocktails with Tukey's posthoc test were conducted to determine differences between treatments. Statistical significance was set at p < 0.05 with n = number of cells. Data are presented as mean \pm SEM. For extracellular recordings (fEPSPs) slicing conditions were similar to the whole-cell preparation. A stimulating electrode was positioned in SC pathway with the recording electrode positioned in stratum radiatum in CA1. Recordings were acquired and analyzed using WinLTP. The initial slope of the fEPSP was measured to quantify synaptic strength (Johnston and Wu, 1995). Student's t-test was used for statistical comparisons of mean fEPSP slopes between groups. All values shown are mean \pm SEM, with n = number of slices.

2.13 Primary culture of cortical neurons

Dissociated cultures of rat cortical neurons were prepared from 18 day-old Sprague Dawley rat embryos as described previously (Mielke & Wang, 2005). To obtain mixed cortical cultures enriched with neurons, uridine (10μ M) and 5-Fluor-2'-deoxyuridine (10μ M) were added to the culture medium at 3 day *in-vitro* (DIV) and maintained for 48 hours to inhibit non-neuronal cell proliferation and consequently the cultures were changed back to the normal culture medium. Mature neurons (11-14 DIV) were used for experiments. Mouse cortical cultures were prepped using embryos at 18 day postcoitum from litters resulting from heterozygote GluN2A+/- or GluN2B+/- matings (Kutsuwada et al., 1996; Sakimura et al.,

1995). To attain homozygous and wild-type (WT) littermate control neuronal cultures, cortical cells from individual embryos were plated independently. Genotyping was performed as described previously (Thomas, Miller, & Westbrook, 2006; Tovar & Westbrook, 1999) using tail samples collected from each embryo. Electrophysiological recordings were performed on these cortical cultures which were stimulated with NMDA (10 μ M) and glycine (2 μ M). Electrophysiological recordings protocol is described in section 2.11 using Mg²⁺-free extracellular solution (ECS) containing the following (in mM): 25 HEPES acid, 140 NaCl, 33 glucose, 5.4 KCl, and 1.3 CaCl₂, with pH 7.35 and osmolarity 320–330 mOsm. Specific blockade of GluN2A NMDA receptors was achieved by treatment with NVP-AAM077 (0.2-0.4 μ M) and GluN2B NMDA receptors were blocked ifenprodril (3 μ M) or Ro 25-6981 (0.3 μ M).

2.14 Data analysis

Values are expressed as mean \pm SEM (n = number of experiments). A two-tailed Student's test was used for statistical analysis and *p* values less than 0.05 were considered statistically significant. Dose–response curves were created by fitting data to Hill equation: I = Imax/[1+(EC₅₀/[A])ⁿ], where I is the current, Imax is the maximum current, [A] is a given concentration of agonist, n is the Hill coefficient.

2.15 Site-directed mutagenesis

The site-directed mutagenesis of GluN1 or GluN2A subunits were performed by using the QuikChange method (Stratagene) according to the manufacturing's directions. All mutant clones were confirmed by DNA sequencing. Wild-type or mutant subunits were transfected in *HEK293 cells* and subjected to electrophysiology examinations. Cells were transfected with a combination of pcDNA3-CMV expression vectors, each of which expressed one of the rat recombinant (GluN2A_{WT}, GluN1_{WT}) subunits. An enhanced green fluorescent protein (eGFP) pcDNA3-GFP was co-transfected to facilitate microscopic visualization. GluN2A_{A108G}, GluN2A_{P79A}, GluN2A_{P178G}, GluN2A_{Q111A}, GluN2A_{F114Y}, GluN2A_{F114S}, GluN2A_{F177S}, GluN2A_{I176Y}, GluN2A_{M111I}, GluN1_{R115E}, GluN1_{L135Q} plasmids were constructed by site-directed mutagenesis from either GluN1_{WT} and GluN2A_{WT} using PFU DNA polymerase. The sequences of all plasmids were confirmed by automated DNA sequencing.

2.16 Neuronal culture

Cultured hippocampal neurons were prepared from the brains of D18 fetal Wister rats. Tissues were digested with a 0.25% trypsin solution (Invitrogen) for 25 min at 37 °C, and then mechanically dissociated using a fire-polished Pasteur pipette. Next, the cell suspension was centrifuged at $2500 \times \text{g}$ for 50 s and the cell pellets re-suspended in DMEM containing 10% Fetal Bovine Serum (FBS; Sigma-Aldrich). Cells were seeded on poly-D-lysine-coated 24-well coverslips at a density of 2.5×10^5 cells/well. Cultures were maintained in a humidified incubator with 5% CO2 at 37 °C. After 24 hrs, plating medium was changed to Neurobasal medium supplemented with B-27 supplement and L-glutamine and the media changed twice weekly

thereafter. Cultured neurons were used for electrophysiological recordings 10-14 days after plating.

2.17 Measurement of Ca²⁺ in rat cortical cultures using a Ca²⁺-sensitive dye

Rat neurons isolated from the entire cortex were plated onto poly-d-lysine (PDL)-coated 96-well plates. After 12-14 days in culture, the level of intracellular Ca²⁺ was assayed using the Fluo-4. No Wash Ca²⁺ assay kit according to the manufacturer's protocol (Thermo Fisher Scientific). In brief, the neuronal culture medium was removed and replaced with a Ca²⁺ assay buffer (CAB) containing 1× HBSS, 20 mM HEPES, 2.5 mM probenacid, and Fluo4-NW dye mix (pH 7.4; Thermo Fisher Scientific). The cells were then incubated for 45 minutes at 37°C for dye loading and then 15 minutes at room temperature. To isolate Ca²⁺ signals mediated by NMDARs, NMDA was used as an agonist (10µM), and 2µM glycine were added. The antagonist NVP-AAM007 was used to block the GluN1/GluN2A NMDARs. The calcium fluorescence measurement was performed at 25°C after 60 seconds of recording with a FLEXStation II benchtop scanning fluorometer (Molecular Devices); then, the NMDAR agonist (NMDA (10µM)/glycine (2µM) was added at 180 seconds. Fluorescence plate reading continued for a total of 30mins with use of an excitation of 485 nM, an emission of 538 nM, and a cutoff of 530 nM. The data were recorded using SoftMax Pro software (Molecular Devices).

2.18 Slice preparation for probing pCREB

Male mice (6- to 10-week-old) underwent cervical dislocation after which brains were rapidly removed and placed in ice-cold slicing solution consisting of (in mM): 120 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 25 NaHCO₃, 1.0 CaCl₂, 7.0 MgCl₂, 2.4 Na-pyruvate, 1.3 Na-ascorbate, 20 Dglucose, with pH adjusted to 7.35 using HCl acid (unless stated, all chemicals and drugs were purchased from Sigma or Bioshop, Canada). The hippocampus was dissected out of each hemisphere and sliced in the transverse plane (400 µm thickness) using a manual tissue chopper (Stoelting). Slices were allowed to recover in a heated (30°C) incubating chamber for 1 hr which contained ACSF composed of (in mM): 124 NaCl, 3KCl, 1.25 NaH₂PO₄, 1 MgSO₄ 7H₂O, 2 CaCl₂, 26 NaHCO₃ and 15 D-glucose which was bubbled continuously with carbogen (95%O₂/5%CO₂) to maintain the pH at 7.3. Slices recovered for an additional 30 min at room temperature prior to beginning the treatments and transferred into 6 well plate while bubbling continuously drugs (Npam43 or BiC or NVP-AAM007) were administered to the slices and incubated for 30 minutes and then transferred to an Eppendorf for homogenization and consequent immunoblotting to probe for pCREB/CREB protein levels.

2.19 NMDA-induced excitotoxicity and H₂O₂-induced cytotoxicity

Primary cultures of mature cortical/hippocampal neurons (12–14 DIV) were used in this study. Preliminary tests showed that both cortical and hippocampal neurons reveal very similar results in response to NMDA challenge. Specifically, hippocampal neurons were used in the immunocytochemical experiments, as it is easier to distinguish them from glia cells, whereas cortical neurons were used to provide sufficient material for biochemical experiments. Immediately before NMDA treatment, half of the conditioned medium was taken out and saved

for further use. Neurons were stimulated with 75 μ m NMDA through bath application, along with other drug treatments as specified in each individual experiment. After 90 min incubation with NMDA, neurons were washed once with fresh neural basal medium, and then returned to the previously saved conditional medium. Cortical cultures were treated with Npam43, and assessed for NMDA-induced excitotoxicity after 20-24 hrs by measuring lactate dehydrogenase (LDH) release. Briefly, cells were treated with 75 μ M NMDA for 1.5 h or 600 μ M H₂O₂ for 1 h after which, neurons were washed once with fresh neural basal medium and the media was exchanged with conditional medium. LDH release was measured using an in vitro toxicology assay kit obtained from Sigma-Aldrich (no. TOX-7). The cell death rate was expressed as a ratio (%) between the absorbance of the treated group and that of the control group.To show selectivity of Npam43 for GluN1/GluN2A NMDARs, neurons were treated with the GluN1/GluN2B selective antagonist, ifenprodril (3 μ M), or the GluN1/GluN2A selective anatagonists, NVP-AAM077 (0.2 μ M) and TCN-201 (10 μ M).

2.20 Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that can convert nicotinamide adenine dinucleotide (NAD) into NADH (the reduced form). LDH is released from cells into culture medium when the plasma membrane integrity is compromised. Therefore, the amount of released LDH represents the degree of cell death. In this study, the extracellular LDH level was measured using an *in vitro* toxicology assay kit obtained from Sigma-Aldrich (no. TOX-7). The basis of this LDH assay is as follows: (1) LDH reduces NAD into NADH, (2) the resulting NADH is then used in the stoichiometric conversion of a tetrazolium dye, and (3) the resulting colored compound is measured by a spectrophotometric microplate reader at a wavelength of 490 nm. The cell death rate was expressed as a ratio (%) between the absorbance of the treated group and that of the control group.

2.21 Immunoblotting

Brain tissues or cultured cells were lysed on ice in the lysis buffer and then the solution was centrifuged at 14,000 rpm for 10 min at 4°C. Next, the supernatant was collected and protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, 23227). Equal amount of protein samples were mixed with 4 times sample buffer, boiled at 100°C for 5 min, and seperated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to Immobilon-PTM polyvynilidene fluoride (PVDF) membranes (Bio-Rad, 162-0177). The membranes were blocked with 5% non-fat milk in Trisbuffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibody. After washing 3 X 5min in TBST, protein was visualized in the Bio-Rad Imager using ECL Western blotting substrate (Pierce, 32016). For the detection of phospho-CREB, samples prepared in the same day were used. The polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) was incubated with primary antibody against phospho-CREB (Ser133) (Cell Signaling Technology, Beverly, MA). For the detection of total CREB, the same polyvinylidene difluoride membrane was stripped and then reprobed with primary antibody against total CREB (Cell Signaling Technology). The band density of each protein was quantified by the Bio-Rad Quantity One software and the relative optical density was analyzed relative to loading total-CREB on the same membrane.

2.22 Formulation

The pharmaceutical formulation of Npam43 was prepared in combination of DMSO, cremophor EL and saline. Npam43 was initially dissolved in DMSO (1-2% v:v) and then mixed with a combination of cremophor EL (2-3% v:v) and saline. This formulation (DMSO, cremphor El, saline) was used for all experiments *in vivo* and used in control animals.

2.23 Reagents

Phosphate buffer solution was prepared using $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.12H_2O$, the pH of which was adjusted by changing the molar ratio of $NaH_2PO_4.2H_2O$ to $Na_2HPO_4.12H_2O$. Other chemicals used were of analytical grade or better quality and Milli-Q ultrapure water (> 18 MU cm) was used throughout the experiments.

2.24 Instrumentation and chromatographic conditions

Npam43 was isolated from the CSF and serum matrix using high performance liquid chromatography and quantified via electrochemical detection. The system consisted of an ESA 582 pump (Bedford, MA), a pulse damper (Scientific Systems Inc., State College, PA), a Rheodyne Inert manual injector (model 9125i, 20 μ L injection loop; Rohnert Park, CA), a Tosoh Bioscience Super ODS TSK column (2 μ m particle, 2 mm × 10 mm; Montgomeryville, PA), and an Antec Leyden Intro Electrochemical detector with VT-03 flow cell with a Ag/AgCl reference electrode ($V_{applied} = +800$ mV; Leyden, The Netherlands). The mobile phase was a 20 mM phosphate buffer–acetonitrile (80: 20, v/v) mixture, pH 7.0, flowed through the system at 0.1mL/min. The column was maintained at 40°C throughout the analysis and the injection volume was 8uL. To our knowledge, this type of mobile phase can be well used here for the

75

HPLC-ECD detection for both Npam43 and Npam50, due to the good compromise of both the HPLC-separation performance and the ECD-detection preference. Prior to use, the mobile was filtered through a 0.22 mm membrane and degassed using a vacuum pump and maintained under helium purging during experimental testing. EZChrome Elite software (Scientific Software, Pleasanton, CA) was used to acquire and analyze chromatographic data.

2.25 Sample preparation

Samples were prepared by mixing aliquots (50:50) of the specimen with acetonitrile. The samples were mixed, allowed to rest at ambient temperature for 10 min and centrifuged at 5000g for 5 min. Eight microliters of the supernatant was injected.

2.26 Cerebrospinal fluid (CSF), serum extraction and HPLC-ECD analysis

Cerebrospinal fluid (CSF) and serum samples generated from the *in-vivo* studies were thawed, and 8 µl was transferred to individual Eppendorf tubes. The internal standard (IS) of 2 µl of 0.5 µg/ml Npam50 was then added, followed by 22 µl of acetonitrile, after which samples were vortexed for 5–10 s and centrifuged for 5 min at 20,000 × g to sediment precipitated protein. The clarified supernatant was transferred to HPLC vials for analysis. Standards were prepared in a similar fashion using blank rat CSF and serum. Optima grade (Fisher Scientific) solvents and 18 MΩ water (Millipore) were used for sample preparation and subsequent HPLC-ECD analysis. Calibration standards ranged from 0.1-50µM (6 points, CSF equivalent level) with $R^2 > 0.99$. The detection limit was > 0.8µM of Npam43. Comparisons of pre- and post-spiked serum with neat standards indicated a suppression of about 10% and extraction efficiencies of 95%. Any samples out of the calibration range were diluted 10-fold for reanalysis.

2.27 Cerebral ischemia

All animals used in the study were housed, cared for, and used experimentally in accordance with the protocols approved by the Ethical Committee for Animal Research at China Medical University Hospital, Taiwan. Adult male Sprague Dawley rats weighting ~ 200 g were anesthetized, and the middle cerebral artery (MCA) was exposed by making a craniotomy window (2 mm in diameter) 1 mm rostral to the anterior junction of the zygoma and the squamosal bone. The exposed MCA was ligated with a square knot using a 10-0 nylon suture. Next, the bilateral common carotid arteries (CCAs) were clamped with nontraumatic arterial clips. Successful surgery was confirmed by a marked drop in regional cerebral blood flow, monitored by a laser Doppler flowmeter (PF-5010, Periflux system; Perimed AB). Moreover, the core body temperature was monitored with a thermometer probe (Hewlett-Packard Model 21090A probe), and maintained at $37.0 \pm 0.5^{\circ}$ C with a heating pad. Both blood pressure and blood gas levels were also monitored during the experiment. After 90 min ischemia, the suture and clips were removed to allow instant reperfusion. Experimental rats were subdivided into two main groups: several groups to receive different doses of Npam43 (0.1, 0.5, 1, 2.5mg/kg) or saline/vehicle (DMSO/cremophor EL/saline) via femoral vein injection. The bolus of Npam43 and saline/vehicle was administered at 3.5 h after stroke onset. To achieve the optimal outcome, another two doses of Npam43 were administered on the second and third days, respectively. Rats were then allowed to recover for different periods of time until additional experiments.

2.28 Magnetic resonance image

The rats were anesthetized, with body temperature maintained at $37.0 \pm 0.5^{\circ}$ C with a heating pad during imaging. The T2-weighted spin-echo imaging sequence (T2WI) was performed by the 3.0 T General Electric imaging system (R4, GE) with the following parameters: repetition time, 4000 ms; echo time, 105 ms; 6–8 contiguous coronal slices with each 2 mm thick. At this stage of stroke development (7 d post-ischemia), brain infarct manifests as high signal (bright white) on the magnetic resonance image (MRI) images. The non-infarct areas were drawn manually from slice to slice and the volumes were measured with Voxtool analysis software (General Electric). The infarct size was quantified by subtracting the non-infarct volume of the ischemic hemisphere from the total volume of the contralateral hemisphere.

2.29 Neurological behavioral tests

In order to assess functional recovery of neural circuits damaged by the ischemic insult we assessed three typical locomotor activity (sensorimotor) deficit modalities, including 1) vertical activity (the total number of beam interruptions that occurred in the vertical sensor), 2) number of vertical movements (number of animal rears) and 3) vertical movement time (the amount of time, in seconds, the animal rears) using the VersaMax Animal Activity Monitor (Accuscan Instruments). The monitor has 16 horizontal *x-y* and eight vertical *z* infrared sensors spaced 2.5 cm apart. The vertical sensors were situated 10 cm from the floor of the chamber. An ischemic rat was placed into the recording chamber during the 'dark' phase of the day/night cycle, and vertical movement time (seconds) was automatically recorded by computer over a 2 hours period. The total length of its vertical movement represents the recovery of locomotor circuits injured by ischemic stroke.

Chapter 3: Structure-based modeling, target site identification and drug screening on GluN1/GluN2A NMDARs

3.1 Introduction

Using the sophisticated drug discovery process described above, involving computerbased receptor modeling of the GluN1/GluN2A homomeric NMDAR structure in conjunction with the resolved GluN1/GluN2B crystal structure. This allowed us to probe the receptor to identify regions that are most likely to cause conformational changes. We hypothesized that for a small molecule to cause a conformational change, it would most likely need to bind between two subunits or an interface. Numerous reports have already shown that interfaces are critical regions that can be exploited for positive or negative modulation of receptors (Axerio-Cilies et al., 2012; Cossins & Lawson, 2015; Fischer, Rossmann, & Hyvonen, 2015; Nero, Morton, Holien, Wielens, & Parker, 2014).

The region that was our main focus was extracellular region of the GluN1/GluN2A was of particular interest as it is the region where neurotransmitters bind. We reasoned that modulation sites on the receptor could come from the interfaces from the ligand-binding domain (LBD) or the N-terminal domain (NTD). Among these two regions, the interface of the LBD has been already targeted by a selective GluN1/GluN2A negative allosteric modulator (NAM) by TCN201 (Hansen et al., 2012). Interestingly, the NAM is highly dependent on the concentration of glycine, where increasing concentrations of glycine can cause TCN201 to lose significant affinity and vice versa (Hansen et al., 2012). This negative influence of glycine to the NAM was

observed as low as 3µM and at 300µM glycine the NAM activity was completely lost (Hansen, Ogden et al., 2012). This phenomenon deterred the idea to target the LBD due to the fact that under ischemic conditions the glycine concentrations have been reported to be greater than 100µM. In light of this, we postulated that targeting the NTD would be a wiser alternative to avoid possible dependency issues with the agonists. Moreover, there is mounting evidence that N-terminal domain (NTD) can cause drastic conformational changes and that these movements can be transmitted into the LBD layer, we reasoned that targeting the NTD in the interface would be viable approach to cause a positive modulation effect on the GluN1/GluN2A NMDARs. We scoured the NTD interfaces for possible druggable binding sites that could accommodate a small molecule and that could potentially discriminate between the two subtypes (GluN2A vs. GluN2B).

The structural model of the GluN1/GluN2A revealed a druggable interstice between the GluN1 and GluN2A interface in the NTD. We used this region as our leading target site and consequently exposed to an *in-silico* virtual screen using a pre-filtered chemical library (described in methods). The virtual screen (docking) of ~200K purchasable chemical substances was implemented against the identified site. A previously described consensus-based *in-silico* methodology (described in methods) yielded ~200 compounds that were predicted to have a high potential for binding to the GluN1/GluN2A interface NTD. Schematic representation of the drug discovery pipeline used for the screening process and hit selection is provided in Figure 3.1. These 200 compounds were then initially biologically validated in GluN1/GluN2A or GluN1/GluN2B transiently transfected human embryonic kidney cells (*HEK293* cells) which allows us to isolate the receptor in a non-neuronal system and functionally characterize the

modulation effects of these two subtypes individually. All compounds were tested in both systems using $10\mu M$ of glutamate and $2\mu M$ glycine to activate the NMDAR via a whole-cell voltage-clamp configuration recordings.

3.2 Identification of a druggable interface between the GluN1 and GluN2A subunits in the N-terminal domain (NTD)

The allosteric binding site was identified through several bioinformatic and X-ray crystallographic observations. The goal was to identify a region that was sufficiently different between GluN2A and GluN2B based on protein primary sequence alignment and structural superimposition. Efforts were focused on the N-terminal domain (NTD) since this domain has been previously shown to be important for inducing conformational changes of the clamshell-like structure (Axerio-Cilies et al., 2011; Axerio-Cilies et al., 2012; Zoraghi et al., 2011). Moreover, our previous experience indicated that targeting protein-protein interfaces can be beneficial for allosteric regulation (Lack et al., 2011); (Pajouhesh & Lenz, 2005). Therefore, we elected to target the interface between GluN1 and GluN2A to identify an allosteric modulation (positive/negative) site. Regions near the agonist binding sites also were thought to increase the possibility of observing an allosteric effect while also increasing the chances of inducing modulation dependent on the concentration of glycine/glutamate. Under stroke conditions drug effects may decrease as agonist concentration increases, ie. TCN-201.

Taking all this into consideration, we believed that targeting a region not in close proximity to the agonist binding sites would reduce the possibility of modulation dependent on the glycine/glutamate concentration. Consequently, a homology model of the GluN1/GluN2A NTD was based on the resolved crystal structure of the GluN1/GluN2B NTD dimer. Figure 3.2a shows the cartoon representation of the full GluN1/GluN2A NMDAR structure illustrating the extracellular domains of the NTD (light blue & light purple) and the ligand-binding domain (LTD) (orange & red). Based on this model, we focused on the interface of GluN1 and GluN2A at the upper-lobes (R1) of the clamshell NTD shown in (Figure 3.2a), which was defined by an interstice that could accommodate a small molecule drug (yellow region) (Figure 3.2b). Figure 3.2c shows the homology model of the NTD of the heterodimer of GluN1/GluN2A and the identified crevice target region shown in green spheres. This particular region was then used to screen ~200,000 compounds that could potentially positively modulate GluN2A-containing NMDARs.



Figure 3.1: *In-silico* pipeline developed to identify potential GluN2A-containing NMDARs binders from the pre-filtered ZINC database

Initially started from 200K compounds from the prefiltered ZINC database and virtual screening reduced the amount of possible active ("hit") compounds down to 10K compounds. 200 compounds were then manually selected based on their structural characteristics (BBB penetration and ease of synthesis), binding pose and score.



Figure 3.2 Targeting the dimer interface of the homomeric GluN1/GluN2A NMDARs in the N-terminal domain (NTD)

(a) Cartoon representation of homomeric GluN1/GluN2A NMDARs, color coded by their respective domains. The N-terminal domains (NTD) are comprised of two light blue clamshell lobes depicting the GluN1 subunit and the two purple clamshell lobes belonging to the GluN2A subunits. The ligand-binding domains (LTD) are composed of two clamshell lobes as well, 84 where orange is showing GluN1 subunits defined by the binding site of glycine/D-serine and the red clamshell lobes comes from the GluN2A subunit characterized by the glutamate binding site. (b) Yellow wedge/ball in the NTD depicts the region we defined as being targetable by small molecules and projected to be mediating a positive modulation effect. This site was modelled and virtually screened using a library of small molecules. (c) Ribbon representation of the homology model dimer interface between GluN1 and GluN2A in which the virtual screen was implemented. Green spheres represent the amino acids that outline the binding site. The ribbon representation of the homology modeling of a GluN1/GluN2A NMDAR built from the resolved crystal structure of GluN1/GluN2B N-terminal domain (PDB: 3qek). Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311)(Pettersen et al., 2004).

3.3 Initial drug screening using GluN1/GluN2A or GluN1/GluN2B cDNA transiently transfected in human embryonic kidney (*HEK293*) cells and tested by whole-cell patch clamp recordings

A series of ~200 compounds were evaluated for positive allosteric modulation at recombinant GluN1/GluN2A and GluN1/GluN2B NMDA receptors at a test concentration of 100 μ M. At this concentration, compounds producing less than 20% enhancement of the glutamate response were not investigated further. All other active compounds were tested on a minimum of six transiently transfected *HEK293* cells expressing GluN1/GluN2A or GluN1/GluN2B for each subunit combination. A variety of structures were evaluated for their
ability to modulate NMDA receptor responses evoked by 10µM L-glutamate and 2µM glycine. GluN1/GluN2A or GluN1/GluN2A receptors were expressed in *HEK293* cells, and receptor activity was determined by whole-cell patch electrophysiological recordings.

From the compounds purchased, 12 compounds showed a marginable enhancement in potentiation for the GluN1/GluN2A subunit (Figure 3.3a) and at the same time a comparable inhibition for the GluN1/GluN2B subunit (Figure 3.3b) in the presence of 10µM glutamate and 2µM glycine in HEK293 cells. The first hit compound that was identified in the screen (Npam01) showed a significant potentiation of GluN1/GluN2A NMDARs (Figure 3.3a) and at the same time was able to inhibit the GluN2B-currents in transiently transfected *HEK293* cells (Figure 3.3b). The representative traces for the currents of the control and the drug condition in HEK293 cells expressing GluN1/GluN2A and GluN1/GluN2B are shown in Figure 3.3c, d respectively. The potentiation effects were measured by normalizing the area under curve (AUC) with the glutamate/glycine control. Moreover, one compound in particular Npam02 at 100µM showed an increase at ~40% compared to controls in HEK293 cells expressing GluN1/GluN2A (Figure 3.4a) but showed no significant inhibition for the GluN1/GluN2B combination (Figure 3.4b). Interestingly, a close analog (Npam04) of Npam02 showed a dual effect on the two subtypes where it was able to positively modulate GluN1/GluN2A (Figure 3.4a) and inhibit GluN1/GluN2B NMDARs (Figure 3.4b) at the same time. The only structural difference between Npam02 and Npam04 was an extra methyl group on Npam02's aryl group. It was speculated that the methyl group on Npam02 could conceivably contribute to its diminished binding effect towards the GluN1/GluN2B-NMDARs. Due to their homologues structural

features and differential effects on the two NMDA subtypes, we used these two compounds as probes to further study their effects *in-vitro* (Figure 3.5).

Whole-cell patch clamp recordings were performed to measure glutamate evoked currents with chloride-based pipette solutions at a holding potential of -60mV. To rule out the possibility that Npam02 itself may induce any currents in the GluN1/GluN2A or GluN1/GluN2B expressing HEK293 cells, Npam02 (100µM) was applied alone and no changes of inward or outward currents (Figure 3.4a, d). Co-application of Npam02 (100µM) with co-agonists modestly enhanced NMDA-mediated currents in HEK293 cells expressing GluN1/GluN2A receptors (100µM; n = 6; 38.85 ± 3.70 %; P < 0.001; and (200µM; n= 6; 71.69 ± 5.03 %; P < 0.001) (Figure 3.4b, d) compared to glutamate application alone, suggesting that Npam02 can act as a PAM (Figure 3.4a, d). The increased NMDAR currents could be completely blocked by the co-application of a selective GluN1/GluN2A antagonist of NVP-AAM007 (0.2µM) in the presence of both co-agonists, confirming that there were no secondary effects attributed to endogenous proteins in HEK293 cells (Figure 3.4a, d). In contrast, HEK293 cells expressing the GluN1/GluN2B combination in the presence of Npam02 (100µM) did not exhibit potentiation of NMDAR currents (Figure 3.4c, e). Similarly, Npam02 did not induce currents on its own and NMDAR currents attributed to the GluN1/GluN2B-receptors were successfully blocked by GluN2B specific antagonist ifenprodril (IF; 3µM) (Figure 3.4c, e). All other compounds were treated in a similar manner and tested at high concentrations (50µM or 100µM) in presence of glutamate and glycine. Since the aim of this project was to discover a selective potentiator for the GluN1/GluN2A combination, we focused our interest towards Npam02 which was characterized as being preferentially favors the GluN2A-containing NMDARs.



Figure 3.3 Potentiation and inhibition effects of the "hit" compounds of initial screen validated in *HEK293* cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs via whole-cell voltage clamp recordings

(a) Hit compounds that showed a positive potentiation effect towards GluN2A mediated currents. Quantification of the potentiation effect of initial screen preformed in HEK293 cells expressing GluN1/GluN2A NMDARs. Concentrations used are shown above and the positive potentiation effect varied from 10-120% above the glutamate control levels. The potentiation effect was normalized to the glutamate control (5µM). (b) Inhibition effects of the hit compounds in HEK293 cells expressing GluN1/GluN2B NMDARs. Except for Npam02, all the compounds shown above showed a negative modulation effect (inhibition) of the GluN2B mediated currents. Concentrations used are shown above and inhibition varied from 2-100% inhibition of the GluN2B currents. The negative (-) percent potentiation signifies inhibition. The potentiation effect was normalized to the glutamate control (5µM). (c) Representative traces from whole-cell electrophysiological recordings of Npam01 in transiently transfected HEK293 cells expressing GluN1/GluN2A. Npam02 alone induced no detectable currents, but instead potentiated glutamate currents that were sensitive to NVP-AAM007 (d) Representative traces from whole-cell electrophysiological recordings of Npam43 in transiently transfected *HEK293* cells expressing GluN1/GluN2B. Npam43 showed a significant inhibition of glutamate-induced GluN1/GluN2B currents and was sensitive to ifenprodril (IF).



Figure 3.4 Npam02 potentiated GluN1/GluN2A-mediated NMDAR currents via direct binding in *HEK293* cells transfected with GluN1/GluN2A subunits and did not change GluN1/GluN2B-mediated NMDAR currents

(a) Representative traces from whole-cell electrophysiological recordings of Npam02 in transiently transfected HEK293 cells expressing GluN1/GluN2A. Npam02 alone induced no detectable currents, but instead potentiated glutamate currents that were sensitive to NVP-AAM007 (b) Representative traces of Npam02 in transiently transfected HEK293 cells expressing GluN1/GluN2A. Npam02 showed an increased potentiation effect at a higher concentration of 200µM versus the 100µM concentration dose. (c) Representative traces from whole-cell electrophysiological recordings of Npam02 in transiently transfected HEK293 cells expressing GluN1/GluN2B. Npam02 showed no significant potentiation of glutamate-induced GluN1/GluN2B currents and was sensitive to ifenprodril (IF). (d) Quantification of the effect of Npam02 on GluN1/GluN2A NMDARs mediated currents. Npam02 (100µM) alone induced no detectable currents (100 μ M; n = 6; 0.07 ± 0.20 %), and showed a significant potentiation effect on GluN2A-mediated NMDAR currents (100 μ M; n = 6; 38.85 \pm 3.70 %; P < 0.001) and $(200\mu M; n = 6; 71.69 \pm 5.03 \%; P < 0.001)$ with the presence of glutamate (arbitrarily 100%). These currents were susceptible to the GluN2A antagonist of NVP (0.4 μ M; 0.05 ± 0.20%; P < 0.001; to glutamate control) where currents were completely blocked. (e) Quantification of the effect of Npam02 on GluN1/GluN2B NMDARs mediated currents. Npam02 (100µM) alone induced no detectable currents (100μ M; n = 6; 0.08 ± 0.15 %), and showed no significant potentiation of GluN2B-mediated NMDAR currents (100 μ M; n = 6; 96.7 ± 3.9 %; P > 0.05; compared to glutamate control) with the presence of glutamate (arbitrarily 100%). These currents

were susceptible to the GluN2B antagonist of IF ($3\mu M$; n = 6; 0.05 \pm 0.14 %; P < 0.001; to glutamate control) where currents were completely blocked.



Figure 3.5 Structural differences between Npam04 and Npam02

The arrow highlights the only structural difference between Npam04 and Npam02. Based on the whole-cell voltage clamp recordings in *HEK293* cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs, the methylated Npam02 shows selectivity towards GluN1/GluN2A and the de-methylated version Npam04 showed potentiation effects on GluN1/GluN2A NMDARs but also inhibition effects with GluN1/GluN2B NMDARs.

Chapter 4: Hit identification and characterization of Npam02 as a selective GluN1/GluN2A positive allosteric modulator (PAM) that targets the Nterminal domain (NTD)

4.1 Introduction

In order to develop an effective treatment for excitotoxic/ischemic neuronal injuries, the drug would need to be effective in an *in vitro* model that contains neuronal cells which express both subtypes GluN1/GluN2A and GluN1/GluN2B NMDARs. Cortical and CA1 hippocampal brain regions are particularly vulnerable to ischemic insults and express both subtypes of NMDARs (J. M. Lee et al., 2000; H. Zhu et al., 2012). Thus, we elected to validate whether Npam02 could potentiate GluN2A-containing NMDARs in 12-14 days old cultured cortical neuronal preparations with NMDA (5μ M) and glycine (2μ M), while confirming that it had no effects on GluN2B-containing NMDARs as observed previously in transiently transfected *HEK293* cells. The selectivity profile for Npam02 was evaluated using either GluN2A antagonist (NVP-AA00M77) or GluN2B antagonist (ifenprodril) to isolate the GluN2A and GluN2B NMDAR currents via whole-cell voltage clamp recordings. Moreover, to further ascertain whether Npam02 had effects on GluN2B-containing NMDARs, we evaluated Npam02 in GluN2B-knockout cortical neurons in the presence and absence of GluN2A and GluN2B antagonists.

Once Npam02 potentiation and selectivity had been verified in wild-type and GluN2Blacking cortical neurons, it then be used as a starting point for further chemical modification to enhance its target affinity and drug-like properties. Before this step, we thought it would be beneficial to identify the chemical features responsible for Npam02 selectivity. Conserving these features in subsequent lead compounds would facilitate the rational drug design process to maintain selectivity towards the GluN2A subtype. The identification for these molecular species can be established by comparing different closely related analogs for Npam02 and generating structure-activity relationships (SAR), thereby identifying the key chemical features on the molecule that mediate potency or selectivity and thus guide the drug design process.

Another key component in this drug design process is to validate whether Npam02 does indeed bind to the interface between GluN1 and GluN2A subunits of the NTD. Validation of the binding site helps to ascertain the key residues within the pocket interacting directly to Npam02 while also aiding in the design of new leads. To ascertain whether Npam02 binds to the indicated interface, two single-point mutations within the NTD interface of the GluN2A subunit were modified (Phe₁₇₇Ser and Gln₁₁₁Ala). It was expected from the docked pose of Npam02 in the site that these two mutations would cause a significant reduction on the binding of the ligand.

Through an iterative process of drug screening and sequential electrophysiological testing, we discovered Npam02, which is a positive allosteric modulator that preferentially binds to the GluN2A NMDARs in *HEK293* cells. To ascertain whether Npam02 does indeed bind to the NTD as predicted by the modelling, we conducted two single-point mutations that were deemed important for direct interactions to the ligand (Phe₁₇₇Ser and Gln₁₁₁Ala). Based on the docked position of Npam02, it was expected that these two mutations would significantly reduce the binding of the ligand, given that the Phe₁₇₇Ser would disrupt some of the hydrophobic

contacts with the phenyl ring of Npam02 and the $Gln_{111}Ala$ would diminish hydrogen-bonding associated with the hydroxyl group of the ligand. As Npam02 cannot compete with glutamate at the glutamate agonist binding sites located in the S1 domain of the GluN2 subunit, we further deduced that Npam02 is not competitive toward glycine or glutamate. The validation of the binding site is crucial to ascertain the key residues within the pocket that are interacting directly to Npam02 and can also aid in the design of the new leads.

Since Npam02 has shown a noticeable selectivity toward GluN1/GluN2A over GluN1/GluN2B NMDARs in *HEK293* cells, we were also interested to evaluate early in the drug discovery process whether Npam02 had any major off-target effects with respect to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and GABA receptors (GABAR). Whole-cell cortical neurons were tested using AMPA (20µM) and GABA (1µM) to isolate the current responses corresponding to AMPAR and GABAR respectively.

4.2 Evaluation of the potentiation effect of Npam02 in mature cortical wild-type and GluN2B-lacking neurons using whole-cell voltage clamp recordings

To test whether these compounds could potentiate NMDARs in mature 14-18 day old cortical neurons at a time when GluN2A receptors are highly expressed, we used NVP-AAM007 (a selective GluN1/GluN2A antagonist at lower concentrations) to block the GluN1/GluN2A-containing receptors. As shown in Figure 4.1a, bath application of Npam02 (100 μ M) modestly modulated NMDAR currents in the presence of co-agonists NMDA (5uM) and glycine (2 μ M) in hippocampal neurons (n = 6, 43.04 ± 6.55 %; P < 0.001; compared to NMDA control group.

Consistent with our *HEK293* cell data, Npam02 was not able to induce inward or outward currents on its own (Figure 4.1a). Furthermore, application of APV (50µM) blocked all currents indicating that the potentiation was mediated through NMDARs and not through secondary effects attributed to other endogenous proteins. On the other hand, the potentiation effect of Npam02 was able to be blocked by the presence of NVP (0.2μ M; n = 6, 0.06 ± 1.36 %; P > 0.05), suggesting that the potentiation effect came from GluN2A-containing NMDARs (Figure 4.1b).

Npam01 and Npam04 were also tested in a similar fashion but unlike Npam02, had no observable potentiation or depression effect of the NMDAR-currents (Figure 4.1c). This suggested that the potentiation effect of Npam01 and Npam04 in neurons, previously observed in HEK293 cells, could be potentially masked by the inhibition effect on GluN1/GluN2Bcontaining NMDARs in neurons causing a zero net change in NMDAR currents. To further assess whether the potentiation effect on Npam02 was selective towards GluN1/GluN2Acontaining NMDARs, Npam02 was tested in neurons lacking the GluN2B subunit. Coapplication of NVP-AAM007 (0.2µM) and NMDA (20µM) in the conditional GluN2B-knockout neurons, almost completely blocked the NMDAR-currents, (n = 4; 90.73 \pm 1.11%; P < 0.001) (Figure 4.2a, b), suggesting that the residual currents came from the GluN2B. Bath application of Npam02 (100µM) had a modest positive modulation effect on NMDAR currents in the presence of co-agonists NMDA (20μ M) and glycine (2μ M) in neurons from GluN2B-lacking mice (n = 4; 44.72 \pm 4.29 %; P < 0.001) (Figure 4.2a, b) compared to NMDA control group. This was consistent with observations in HEK293 cells expressing GluN1/GluN2A-NMDARs and the potentiation effect observed in wild-type neurons.

Furthermore, to isolate the pure GluN2A-component even further, co-application of the GluN2B-antagonist ifenprodril (IF) with Npam02 resulted in a potentiation effect similar to that observed previously (n = 4; 42.22 ± 4.03 %; P < 0.001) (Figure 4.2a, b) compared to NMDA control group. Finally, GluN2B-knockout cortical neurons were used to determine whether Npam02 is selective for the GluN1/GluN2A-containing NMDARs. Selective GluN2A antagonists were used to block the GluN2A mediated currents and confirm whether the compound discriminated between the two subtypes. Bath application of two co-agonists with GluN2A-NMDAR antagonist NVP-AAM007 (0.2µM) and Npam02 prevented the potentiation of NMDAR currents by Npam02 (100 μ M; n = 4; 88.87 ± 3.45 % P > 0.001) (Figure 4.2a, b) compared to NMDA control. This suggests that the residual GluN2B-NMDAR current was not potentiated and is consistent with the idea that Npam02 is a PAM for the GluN2A-containing NMDARs. Npam01 and Npam04 were also tested in the cortical GluN2B-lacking neurons but did not show any significant change in the NMDAR mediated currents and therefore were not tested further due to a lack of efficacy in wild-type and GluN2B-knockout cortical neurons. Taken together with preferential binding towards the GluN1/GluN2A-containing NMDARs, at this point Npam02 was considered to be the first lead compound and therefore was analyzed further to evaluate its selectivity for GluN2A-containing NMDARs and other ionotropic transmembrane receptor and ligand-gated ion receptors. The binding site of Npam02 was also validated through single-point mutations thereby verifying that the ligand could bind directly to the GluN1/GluN2A NMDARs.



Figure 4.1 Npam02 enhanced neuronal NMDAR function in cultured hippocampal neurons and the potentiation effect was blocked by GluN2A antagonist

(a) Representative traces from whole-cell electrophysiological recordings of Npam02 which showed it can potentiate NMDA-induced currents in cultured hippocampal neurons. Npam43 alone induced no detectable currents, but instead potentiated NMDA currents that were sensitive to APV. (b) Representative traces from whole-cell electrophysiological recordings of Npam02 which showed it can potentiate NMDA-induced currents that were sensitive to NVP as well. (c)

Quantification of the effect of Npam02 in NMDA mediated currents. Npam02 (100 μ M) alone induced no detectable currents (100 μ M; n = 6; 0.08 ± 0.15 %), and showed a significant potentiation effect on GluN2A-mediated NMDAR currents (100 μ M; n = 6, 43.04 ± 6.55 %; P < 0.001; with the presence of NMDA (arbitrarily 100%). These currents were susceptible to the GluN2A antagonist of NVP (0.2 μ M; n = 4; 51.1 ± 2.45 % P < 0.001) where the currents were not significantly changed with respect to NVP alone (0.2 μ M, n = 4; 52.0 ± 2.67 % P < 0.001). Npam01 and Npam03 showed no significant change in NMDAR mediated currents.



Figure 4.2 Npam02 application in genetic deletion of GluN2B subunit cortical culture neurons potentiates GluN2A NMDARs mediated currents

Cultured mouse cortical neurons from GluN2B–/– embryos were treated with NMDA (20μ M plus 2μ M glycine). (a) Representative traces from whole-cell electrophysiological recordings of Npam02 which showed it can potentiate NMDA-currents that were sensitive to NVP-AAM0077 but were not blocked by GluN2B antagonist. (b) Quantification of the effect of Npam02 in NMDA mediated currents in GluN2B knock out mice cortical neurons. Npam02 showed a significant potentiation effect on GluN2A-mediated NMDAR currents (100μ M; n = 4; 44.72 ± 4.29 %; P < 0.001) with the presence of NMDA (arbitrarily 100%). In the presence of GluN2B antagonist-IF, Npam02 comparatively potentiated GluN2A NMDAR-mediated currents

 $(3\mu M; n = 4; 42.22 \pm 4.03 \%; P < 0.001)$ with the presence of NMDA (arbitrarily 100%). Coapplication of NVP-AAM007 (0.2µM) and NMDA (20µM) in the conditional GluN2B-knockout neurons, almost completely blocked the NMDAR-currents, (n = 4; 90.73 ± 1.11 %; P < 0.001), suggesting the residual currents came from the GluN2B. Bath application of two co-agonists with NVP-AAM007 (0.2µM) and Npam02 prevented the potentiation of NMDAR currents by Npam02 (100µM; n = 4; 88.87 ± 3.45 % P < 0.001) compared to NMDA control, suggesting that the residual GluN2B-NMDAR current was not potentiated and consistent with the idea that Npam02 is a PAM for the GluN2A-containing NMDARs. Npam01 and Npam04 did not show a significant potentiation effect in GluN2B knock-out mice cortical cultures.

4.3 Npam02 had no visible modulation effects on AMPAR and GABAR-mediated currents in mature cortical neurons

Next, we evaluated the selectivity profile for Npam02 on two other key ionotropic receptors of the central nervous system (CNS). An early analysis of the selectivity for the lead compound was important to evaluate potential off-target issues. Consequently, we tested whether Npam02 was able to elicit a similar potentiation or depression effect on other similar receptor; α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor AMPAR, which are known to be a non-NMDA type ionotropic transmembrane receptor for glutamate that mediates fast synaptic transmission in the central nervous system (CNS) and GABA receptors which are the principal inhibitory ligand-gated ion channel receptor. Whole-cell patch clamp recording was conducted in cultured hippocampal neurons. The tests revealed that Npam02 had no observable effects on AMPAR (Figure 4.3a, c) and GABAR (Figure 4.3b, c) mediated currents, suggesting it was a

good starting point as a lead and could be potentially used as a probe for modify further to optimize its potency towards GluN1/GluN2A-containing NMDARs.



Figure 4.3 Npam02 does not affect current responses induced by AMPA and GABA in neuronal cultures

(a) Npam02 did not modulate current responses induced by AMPA in neuronal cultures. (b) Npam02 did not modulate or decrease GABA currents in neuronal cultures (c) Quantification of the effects of Npam02 towards current responses for GABA (100 μ M; n = 6; 0.07 ± 4.5 %; P > 0.05 compared to GABA control) and AMPA (100 μ M; n = 6; 0.04 ± 1.53 %; P > 0.05; compared to AMPA control). Two-tailed Student's t-test ***P < 0.001; bars represent relative mean values ± SEM.; n represents individual experiments from at least 3 separate primary cultures.

4.4 Npam02 binding position in the interface site of the GluN1/GluN2A

As mentioned before, Npam02 was designated as the initial hit compound due to its marginal potentiation effect on GluN1/GluN2A-contianing receptors in *HEK293* cells expressing GluN1/GluN2A, wild-type cortical neurons and GluN2B-lacking cortical neurons. More importantly, it was selected as our lead due to its preferential selectivity toward GluN1/GluN2A-containing NMDARs. This selectivity was augmented by the fact that the compound also showed no effects on GABAR and AMPAR. The downside of Npam02 was its marginal potentiation effect on GluN1/GluN2A using high concentrations of the ligand. Therefore, an iterative process was performed to alter the chemical structure in order to enhance its potency towards the GluN2A-containing NMDARs. Initially, the lead optimization stage was guided by the binding pose of Npam02 in the pocket which was used as a template to design analogs that could interact more favorably with its surrounding residues. To rationally design a drug that would have an

enhanced potency relative to Npam02, one must first identify and understand how Npam02 binds within the pocket and then determine key interactions and subsequently modify them to enhance those interactions. Initially, it was observed that the dimer interface was largely surrounded by hydrophobic residues such as (GluN2A_{F115}, GluN2A_{M112}, GluN2A_{P79}, GluN1_{F113}, GluN1_{Y109}, GluN1_{L135}, GluN2A_{F177}, GluN2A_{P178}) which are spread all over the pocket and around the ligand. In fact, the Npam02 ligand is situated in hydrophobic cage depicted by GluN2A_{F115}, GluN2A_{P79}, GluN1_{F113} and GluN1_{Y109}. This cage is characterized by residues that consist of ring structures in their side chains. This feature was of particular interest because ideally a ring from a ligand within this cage could potentially anchor itself within this area via strong hydrophobic interactions mediated by two Π systems (aryl-aryl). Npam02 appears to conform to this possibility, as its aryl ring (positions 1-6 in Figure. 4.4) is situated within this area and is hydrophobically linked with all the aforementioned residues which surround Npam02's aryl ring (positions 1-6 in Figure 4.4). More specifically, for interactions between two Π systems (arylaryl) from the GluN2A_{F115} and the aryl ring from Npam02 (positions 1-6 in Figure. 4.4), the Tshaped edge-to-face conformation interacts strongly and appears to be energetically attractive and favorable. A similar effect was seen in the opposite side of ligand where the other aryl ring (positions 11-16 in Figure 4.4) interacted strongly with a GluN1_{L135}, GluN2A_{F177}, and $GluN2A_{P178}$. $GluN1_{L135}$ seems it could interact directly with the second aryl ring of Npam02 via an edge-to-face interaction and the other residues from GluN2A interact hydrophobically as well but most likely to a lesser extent due to their distance further away from the ligand. Conversely, the GluN2A₀₁₁₁ shows a polar hydrogen bond accepting interaction with the proton of the hydroxyl group (position 18 in Figure 4.4) of Npam02.



Figure 4.4 2D chemical structure of Npam02

The blue numbers correlate to different atom types and will be discussed in the text.

4.5 Site-directed mutagenesis of the predicted binding site in the N-terminal domain (NTD)

To validate whether the drug is binding to the dimer interface between GluN1 and GluN2A, we performed a site-directed mutagenesis of the N-terminal domain (NTD) pocket. Using the docking pose as a template we selected two residues which were deemed crucial for direct interaction with the drug. Extensive induced-fit docking suggested that Npam02 can occupy and exploit the space between the upper-lobe (R1) of the NTD (Figure 4.5a). We therefore tested whether potentiation of the GluN1/GluN2A-containing NMDAR by Npam02 is altered by mutations at residues that surround the pocket interface by choosing side chains for substitutions that occlude the interactions for the compound but do not otherwise perturb the overall protein structure. Docked model of Npam02 in the predicted modulation site showed that two residues GluN2A(Gln₁₁₁) and GluN2A(F₁₇₇) (Figure 4.5b) directly interact with Npam02, where the GluN2A(Gln₁₁₁) makes a hydrogen-bond with the hydroxyl group (position 18 in 105).

Figure 4.4) and GluN2A(F₁₇₇) may elicit hydrophobic interactions towards the aromatic ring (positions 11-16 in Figure 4.4). Indeed, a noticeable reduction of the degree of modulation was achieved through both mutations compared to the modulation effect observed to the wild-type GluN1/GluN2A receptor. The modulation effect of Npam02 (100µM) in the wild-type neurons was (n = 7, 41.75 \pm 2.62 %) (Figure 4.5c) significantly reduced when GluN2A was mutated from Gln₁₁₁Ala (n = 7, 30.69 \pm 1.60 %, P > 0.05) (Figure 4.5c) and reduced further with the Phe₁₇₇Ser mutation (n = 7, 22.86 \pm 1.12 %, P > 0.01) (Figure 4.5c). This incorporation of these two mutations and the subsequent reduction of the modulation effect of Npam02, suggests that Npam02 could bind to this interstice interface site.





GluN2A-NTD - GluN1-NTD - Pose-Npam02
Critical residues-mutated GluN2A-NTDs

С

GluN1/mutated GluN2A



Figure 4.5 GluN2A F₁₇₇ and Q₁₁₁ form the Npam02 binding pocket between the GluN1 and GluN2A interface of NMDAR receptors in the NTD

(a) Binding pose of Npam02 predicted by docking in the interstice interface between the GluN1 (light blue) and GluN2A (purple) subunits. Bold amino acids were characterized as being amino acids that defined the dimer cleft in the NTD. (b) Binding pose of Npam02 predicted by docking in the interstice interface between the GluN1 (light blue) and GluN2A (purple) subunits. Pink amino acids (Gln₁₁₁Ala and Phe₁₇₇Ser) were picked to demonstrate whether Npam02 could bind to the interface. (c) The normalized potentiation by Npam02 on the substituted GluN2A subunit. The normalized potentiation by Npam02 (100µM) in the wildtype form of GluN2A (n = 6; 41.7 \pm 5.7 %) was significantly reduced in the GluN2A mutation of Q₁₁₁A(100 µM; *n* = 6; 30.7 \pm 4.8%; P < 0.05) and F₁₇₇S (100µM; n = 6, 22.9 \pm 2.9 %; P < 0.01) Two-tailed Student's t-test, *P < 0.05 **P < 0.01; bars represent relative mean values \pm SEM..; *n* represents individual experiments from at least 3 separate transiently transfected cell lines (as described in section 4.4).

4.6 Structure-activity relationships (SARs) of Npam02 and its closely related analogs to identify chemical features that are responsible for selectivity

One of the key procedures in the drug discovery process is to gain insight about the structural features that can be used to determine the SARs or can be correlated with its selectivity. Feature selection can be accomplished by visual inspection (qualitative selection by a human), by data mining or by molecular mining. A qualitative visual inspection of all the 2D structures of the first round compounds (shown Appendix Table 1) revealed a structural feature

that appears to dictate whether the potentiation effect is preferentially toward GluN1/GluN2A or GluN1/GluN2B receptors. Table 4.1 shows the structures of the first round and whether their modulation effect was more dominantly toward GluN2A or GluN2Bs (Ratio GluN2A:GluN2B). The structural feature that was of particular interest was the substituent in the ortho-position of the aryl ring (positions 1-6 in Figure 4.4). The compounds that contained this moiety showed a preferential effect on GluN2A receptors (Npam02, Npam75, Npam30, and Npam66) whereas all other compounds that lacked this moiety were either more dominant for GluN2B NMDARs or equally effected. Since Table 4.1 represent a rather small dataset this observation could only be considered as a hypothesis at this point to be confirmed by additional electrophysiological tests with other similar compounds, to determine whether the ortho hydrophobic substituent actually played a role in the selectivity. According, a similarity-based virtual screening was performed against the compounds shown in Table 4.1 in order to build up SARs within this subclass of compounds.



Name	R1	R2	Ratio GluN2A : GluN2B	Name	R1	R2	Ratio GluN2A : GluN2B
Npam01	Č → - E		Equal	Npam75	HO HO CI	Br	GluN2A
Npam02	HO CH3	CH3	GluN2A	Npam64	B He →		GluN2B
Npam04	e ₽	CH3	GluN2B	Npam68	но	HO	GluN2B



Table 4.1 Hit compounds identified in the first screen that showed a positive modulation of the GluN1/GluN2A NMDARs or/and inhibition effect of GluN1/GluN2B NMDARs The table highlights if the modulation effect was more dominantly affecting GluN2A-containing NMDARs or GluN2B-containing NMDARs at a single concentration of 100µM (Ratio GluN2A:GluN2B). Chapter 5: Hit optimization of Npam02 using a rational drug design approach and development of SARs to select a potent positive allosteric GluN2A-type modulator

5.1 Introduction

Structure guided methods are an integral feature of drug discovery program for known 3D structure of potential drug binding sites. Since there is no known crystal structure for the GluN1/GluN2A NTD, a homology model of GluN1/GluN2A was constructed based on the GluN1/GluN2B resolved crystal structure (Karakas et al., 2011). We used this model as a virtual screen for large collections of chemical compounds to identify potential selective modulators for the GluN2A-containing NMDARs. Recognizing, that the probability of finding a potent, selective, and stable compound would be extremely difficult via a virtual screen, it was used to discover compounds that are marginally active and could serve as chemical probes rather than drug-like prototypes. These less sophisticated leads would ultimately need to be custom-made so that they are specifically tailored for the particular target site on the GluN1/GluN2A structure. This has numerous advantages including having full control over the discovery and development of a wide variety chemical entities specifically designed to have a superior potency, selectivity, toxicity and stability profiles. Conversely, a serendipitous approach of screening through chemical libraries leads to low hit rates (few biologically active compounds from screening) and the quality of leads are poor with respect to their pharmacology. In light of this, since we knew that GluN1/GluN2A structure is highly homologues to the GluN1/GluN2B complex and other ionotropic receptors like AMPA, we believed the discovery of a selective allosteric modulator for the GluN1/GluN2A combination would be best achieved through a rational drug design approach, where the chemical leads would have to be fine-tuned specifically for the proposed modulation site in Figure 4.5a. To this end, we used the modelled docked pose of Npam02 (initial hit compound) as a chemical probe to investigate further potential improvements that can be made to the scaffold backbone structure. Two different mutations from the GluN2 subunit (Phe₁₇₇ & Q₁₁₁A) (Figure 4.5b,c) suggested that the binding site is located in the NTD between the interface of GluN1 and GluN2 subunits as predicted originally from the modeling.

The design of the compounds was based on chemical expertise, whereby different functional groups are added or removed to the backbone of Npam02 in order to generate a large group of homologues compounds that may ultimately show enhanced potency. Therefore the aim was to gain insight in the SARs between the NTD interstice interface pocket and the ligands. This was then used to organize the groups of compounds and to provide a cost-effective data development plans. Once a SAR has been established within a subclass of compounds, the compounds can be ranked based on their potency and pharmacological profiles to ultimately choose the best possible candidate that would be stable, potent, selective, and non-toxic for your target. We used this technique, where drugs were initially structurally designed based on the docked pose of Npam02 in order to generate a list of compounds that could be either chemically synthesized or purchased from a chemical supplier. A handful of these compounds were then tested electrophysiologically to determine whether an ortho-substituent in positions 1-6 in Figure 4.4 could be exploited to discriminate between the two NMDAR subtypes in HEK293 cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs. All synthesized and purchased compounds were then tested using whole-cell patch recording to determine their potentiation effects towards NMDARs in cortical neurons. Sequentially, the EC_{50} potency values were determined for the most active compounds and this generated an adequate SAR profile that lead to a potent, selective, stable, BBB permeable, non-toxic allosteric modulator for GluN2A-containing NMDARs.

5.2 Rational drug design to discover selective positive allosteric modulators of GluN2Acontaining NMDARs

The binding pose was predicted by docking sites where Npam02 bound to the interface in a conformation that was flat and elongated. The ortho-substituted ring (positions 1-6 in Figure 4.4) was located in a region that was entirely defined by hydrophobic residues and the other more polar hydroxylated ring (positions 11-16 in Figure 4.4) was located in a region that was defined by a mixture of hydrophilic and hydrophobic residues (Figure 4.4). This pose was reasonable and other conformations would not have made sense especially when the space in the interface was fairly narrow and the compound itself was not very flexible. After the evaluation process of the docked pose for Npam02, we were able to use the 3D modeled structure of the ligand and the protein depicted in Figure 14a to hypothesize possible chemical groups that could be added to the scaffold. The first obvious structural arrangement that was proposed was to increase the interactions with Phe₁₇₇ and Pro₁₇₈ which were in close proximity to methoxy group (positions 19-20 in Figure 4.4). To achieve this, we speculated that the replacement of the methoxy moiety with an ethoxylated version of Npam02 would be suitable to facilitate the hydrophobic interactions to the aforementioned residues. Other possible alterations that were proposed were the addition of two hydrophobic substituents in positions 14 and 15 in Figure 4.4.

These two functional groups were the obvious chooses to enhance the hydrophobic interactions with Ile_{335} of the GluN2 subunit and Phe_{113} of the GluN1 subunit just below the ring of positions 11-16 in Figure 4.4. Due to the ease in chemical synthesis, we proposed to 1 or 2 chlorine or bromine functional groups to these empty slots.

Lastly, replacement of the methyl group in positions 19-20 in Figure 4.4 with bromine or a chlorine was contemplated to increase the hydrophobic nature of ring (positions 1-6 in Figure 4.4). This was proposed because this particular ring lies in a hydrophobic "cage" depicted by four hydrophobic residues (GluN1-Phe₁₁₃, GluN2-Phe₁₁₅, GluN1-Tyr₁₀₉ and NR2-Pro₈₆) and therefore such an alteration is predicted to enhance the van-der-Waals contacts toward these residues and should still maintain the selectivity profile of the drug. This alteration may also prove the hypothesis that ortho-substituent in ring (positions 1-6 in Figure 4.4) will still constitute a selective modulation effect towards GluN1/GluN2A. To prove more decisively whether the hypothesis is true, we postulated that compounds that did not have this hydrophobic ortho-substituent would lose their selectivity. Initially, due to the high structural homology to GluN1/GluN2B NMDARs, the selectivity of the compounds was a major focus given the anticipated difficulty in achieving discrimination between the two receptor subtypes. In light of this, compounds were purchased or chemically synthesized to prove that an intact ortho-moiety could elicit selectivity towards GluN1/GluN2A NMDARs. Once this was established, other chemical alterations could be proposed based on the binding pose of Npam02 in the NTD of GluN1/GluN2A. We sought to enhance the activity of the lead compounds and generate possible SARs, which will help to design further compounds that will ultimately be chemically/metabolic stable, non-toxic, potent and still maintain its selectivity.

5.3 Chemical synthesis of Npam compounds

The chemical synthesis for the Npam compound were attained by a 2 step synthesis which initially involved the synthesis of the appropriate aldehyde and subsequently reacting the corresponding aldehyde with the appropriate hydrazine via a SN₂ mechanism. More specifically, the aldehyde was combined with N-chlorosuccinimide in the presence of acetic acid and that resulted in the corresponding substituted benzaldehyde. Next, the corresponding aldehyde was mixed with the hydrazine in the presence of THF to obtain the final product. Other Npam compounds were synthesized using a 1 step synthesis approach of reacting the purchasable aldehyde and hydrazine reagents and combine them using basic conditions of THF and refluxed to give the final product. Proton Nuclear magnetic resonance ¹H-NMR and electrospray mass spectrometry (ESI-MS) was performed to validate the compound's structure and purity. The ¹H-NMR and ESI-MS for compounds that were synthesized are located in the Appendix 1 section of chemical synthesis.

5.4 Does the ortho-substituent on ring R1 (positions 21 in Figure 4.14) elicit selectivity towards GluN1/GluN2A containing NMDARs

To prove that the ortho-substitution played a role in the selectivity profile of the drug, a hand-full of compounds were either purchased or rationally chemically synthesized with the idea that the compounds that did not have the ortho-moiety would bind to GluN1/GluN2B NMDARs and the ortho-containing compounds would show a preferential effect toward GluN1/GluN2As. In light of this, we used transiently transfected *HEK293* cells expressing either GluN1/GluN2A

or GluN1/GluN2B NMDARs and tested by whole-cell voltage patch clamp recordings to see whether these two groups (ortho-containing versus ortho-absent) compounds were controlling the subtype selectivity profile of the drugs. The results shown in Figure 5.1a demonstrate that the all the compounds had a significant modulation effect on GluN1/GluN2A NMDARs. Conversely, in Figure 5.1a, there was a clear distinction between the compounds that were classified as ortho-containing versus ortho-absent. Npam01, Npam04, Npam59 were characterized as being ortho-absent drugs and showed significant modulation effects on GluN1/GluN2B NMDARs as well. On the other hand, Npam02, Napm58, Npam72, and Npam43 were categorized as ortho-containing compounds and were deemed to be selective for GluN1/GluN2A NMDARs as shown in Figure 5.1b. This strongly suggests that the ortho-substituent plays an important role in the selectivity of the compound, where the presence of the ortho functionality prefers GluN1/GluN2A NMDARs and the absence of this moiety does not discriminate between the two subtypes.



Figure 5.1 Modulation effects of different analogs for Npam02 in *HEK293* cells expressing GluN1/GluN2A and Glu1/GluN2B NMDARs

(a) Quantification of the potentiation effect of different analogs for Npam02 on GluN1/GluN2A NMDARs mediated currents in *HEK293* cells. Npam59 (10 μ M, n = 6, 387 ± 0.12 %; P < 0.001; compared to glutamate control) and Npam43 (10 μ M; n = 6; 326 ± 0.15 %; P > 0.001; compared to glutamate control) significantly potentiated (5 μ M) glutamate-induced currents. (b) Quantification of the potentiation effect of different analogs for Npam02 on GluN1/GluN2B NMDARs mediated currents in *HEK293* cells. Npam59 (10 μ M, n = 6, 217 ± 0.10 %; P < 0.001; normalized to glutamate control) significantly potentiated GluN1/GluN2B NMDARs currents whereas Npam43 (10 μ M; n = 6; 1.2 ± 0.26 %, P > 0.05; normalized to glutamate control) did

not show any modulation effects in transiently transfected *HEK293* cells expressing GluN1/GluN2B NMDARs.

5.5 Structure-activity relationships (SAR) for the lead family of compounds

SAR is based on the idea that structurally similar compounds have a similar activity. The analysis of SAR within a homologous set of compounds enables the determination of the chemical groups responsible for evoking a target biological effect. This identification of these structural features allows the modification of the effect or the potency of the bioactive compound by changing its chemical structure. Deciphering the SAR within a subclass of compounds will give insight into what modifications are needed to enhance the biological effect (potency) of the compound. This becomes very important especially in the last stages of the lead optimization process where the drug maybe potent but may have a poor half-life or high metabolism rate. At this stage, a clear understanding of the SARs helped to pinpoint the areas on the chemical structure that could be modified to enhance the metabolic stability and kept the potency of the drug intact. Ultimately, the aim of the SARs is to create a dataset of compounds that have a wide range of activities and this will help to ascertain the structural characteristics that are deemed vital for potency and others that maybe detrimental to the binding. Computational methods of drug design can help to visualize the relationship between the ligand and protein and therefore can guide the chemical synthesis for the refinement of the chemical structures called lead optimization. In this study, we used a step-by-step approach to create a subclass of compounds that bind to the NMDARs by using the three initial hit compounds as probes.

All the compounds that were chemically synthesized or purchased were tested at $(5\mu M)$ in cortical primary neurons via whole-cell voltage clamp recordings to evaluate their ability to potentiate NMDAR currents in the presence of NMDA ($5\mu M$) and glycine ($2\mu M$). The sole purpose of this experiment was to study the SAR between this subclass of compounds against NMDAR activity and to discover possible chemical alterations that will improve the potency and pharmacological profile of the leads. Initially, Npam02 was characterized as good starting point due to its unusual selectivity toward GluN1/GluN2A receptors. A key interaction that was identified very early on was the ortho-methyl group (positions 1-6 in Figure 4.4) which seemed to play a crucial role in Npam02's selectivity. Npam02's potentiation for GluN1/GluN2A NMDARs was (n = 6, $36.9 \pm 3.8 \%$, P < 0.001) and a subtle change was observed for GluN1/GluN2B NMDARs (n = 6, -4.82 ± 2.8 %, P > 0.05). Npam04, a close analog of Npam02, shows that the absence of this functional group will affect the discrimination between GluN2A and GluN2B- receptors (n = 6, 33.7 \pm 4.8 %, P < 0.001) vs. (n = 6, -54.6 \pm 5.5 %, P < 0.001) respectively. It was discovered that ortho-substituent could be bromine, chlorine or a methyl group which all facilitated the interaction with the nearby Tyr_{109} and Phe_{115} (Figure 4.5a) (Figure 10).

Other larger substituents like a methoxy group of R2 ring (Figure 5.2) (Npam53 in Appendix Table 2) showed reduced binding suggesting that the space around this area was limited (n = 4, 110.2 \pm 4.7 %, P > 0.001) versus the potentiation effect for Npam43 (n = 6, 363.5 \pm 27.3 %, P < 0.001). Moreover, a polar substituent in this position such as an amino group (NH₂) (Npam21 in Appendix Table 2) also greatly diminished the modulation affect to (n = 4, 37.3 \pm 4.6 %, P < 0.001), suggesting that the nearby Tyr₁₀₉ and Phe₁₁₅ will elicit a polar-

hydrophobic mismatch between the amino group and the hydrophobic rings on Tyr₁₀₉ and Phe₁₁₅. Another type of interaction that was crucial for binding was the hydroxyl group of the R1 ring (Figure 5.2) (position 18 in Figure 4.4) which makes an important H-bond towards Gln₁₁₀. Methylation of the hydroxyl group of ring R2 (Figure 5.2) exhibited by Npam51 (n = 4, 33.4 \pm 6.4 %, P < 0.001) or moving the hydroxyl group to the para position of ring (positions 11-16 in Figure 4.4) (Npam27 in Appendix Table 2) (n = 4, -0.278 \pm 5.6 %, P > 0.5) or Npam40 (n = 4, 42.5 \pm 3.8 %, P < 0.001) abolished or greatly diminished the modulation down to approximately 0-40% instead of 200-400% when the hydroxyl (position 18 in Figure 4.14) is in the orthoposition of (positions 1-6 in Figure 4.4) and is unbound (Npam43) (n = 6, 363.5 \pm 27.3 %, P < 0.001). This strongly suggests that this particular H-bond was clearly needed for favorable potent binding. This was further collaborated by the mutational study, where Gln₁₁₀ was mutated to alanine (Ala) and this also significantly reduced the potentiation which suggested its importance towards ligand binding.

Another key relationship between drug and receptor was that increasing the size of the meta-methoxy group of ring R1 (Figure 5.2) (positions 19-20 in Figure 4.4) to a meta-ethoxy group enhanced the potentiation effect of the drug, as demonstrated by methoxlated-Npam20 (n = 5, 131.9 \pm 23.2 %, P > 0.001) versus the ethoxylated-Npam04 (n = 4, 292.1 \pm 44.7 %, P < 0.001). This could be explained by the fact that the ethoxy group was long enough to reach two adjacent residues Pro₁₇₆ and Phe₁₇₇, which are capable of interacting through strong hydrophobic contacts (Figure 4.5). Another area of interest within the pocket was the Phe₁₁₃ from GluN1 and Ile₃₃₅ from the GluN2 subunit located just below the phenyl ring R1 (Figure 5.2) (positions 11-16 in Figure 4.4). The absence of hydrophobic substitute on the phenyl ring in positions 14 and 15
in Figure 4.4 led to poor contacts with the aforementioned residues. This was evident for Npam42 and Npam31 (Appendix Table 2) where the lack of hydrophobic groups lead to a marginal modulation effect for NMDARs (n = 4, 43.0 ± 6.6 %, P > 0.001) and (n = 4, 15.1 ± 8.8 %, P > 0.001) respectively. From the modeling indicated that chemical modification was needed to facilitate the interaction with the two residues. The SAR analysis showed that the addition of one chlorine atom in position 15 in Figure 4.4 greatly bolstered the modulation effect on NMDARs as shown for Npam18 (n = 4, 213.6 ± 43.6 %, P < 0.001). Similarly, the addition of a bromine atom in the same position also increased the potentiation of NMDARs in cultured neurons (n = 4, 163.6 ± 13.9 %, P < 0.001).

Other modifications such as an allyl or NO₂ onto this position of the phenyl ring R1 (Figure 5.17) were not beneficial as the potentiation effect drastically reduced in Npam03 (n = 4, 97.6 \pm 6.0, P < 0.001) and Npam24 (n = 4, 61.6 \pm 8.2 %, P < 0.001). Moreover, the addition of two chlorines on positions 14 and 15 in Figure 4.4 showed a pronounced increase in potentiation and the effect was more impactful than the addition one chlorine alone to position 15 in Figure 4.4. The dichloirnated-Npam43 showed a potentiation effect of (n = 6, 363.5 \pm 27.3 %, P > 0.001) and dibrominated-Npam10 showed a slightly lower modulation effect of (n = 4, 206.3 \pm 33.63 %, P > 0.001). Nevertheless, the double addition of two small hydrophobic atoms at these positions greatly enhanced the potentiation effect for NMDARs, where position 15 in Figure 4.4 most likely contributed to hydrophobic interactions toward Ile₃₃₅ from GluN2 subunit and position 14 in Figure 4.4 engages with Phe₁₁₃ from GluN1 subunit. The next observation that was identified was the addition of a hydrophobic group in the para-position of the R2 ring (Figure 5.2) (position 3 in Figure 4.4). The incorporation of methyl in the para-position of R2 ring

(Figure 5.2) also showed an enhancement in modulation, showing a (n = 4, 466.6 \pm 38 %, P < 0.001) increase in Npam46 and a (n = 4, 443.3 \pm 83.4 %, P < 0.001) surge in Npam44. Similarly, a methyl group in meta-position of R2 group (Figure 5.2a) (position 2 in Figure 4.4) also showed an augmentation of the potentiation effect of the NMDAR currents. Npam50 (n = 5, 441.1 \pm 36.5 %, P > 0.001) and Npam49 (n = 6, 530.1 \pm 114 %, P < 0.001) showed a greater affinity than their sister compound Npam43, suggesting that the incorporation of a methyl in the meta or para-positions of the R2 ring (Figure 5.2a) was a sufficient to boost the modulation. This could be attributed to the increase of hydrophobicity of the ring and boosting the interactions in the hydrophobic cage that surrounds the methylated-R2 ring (positions 1-6 in Figure 4.4) (Figure 5.2a) depicted by (Tyr₁₀₉, Phe₁₁₅, Phe₁₁₃ and Pro₈₆).

Addition of larger groups to the para-position of R2 ring (Figure 5.2a) were not beneficial and suggested this area might be confined and therefore such groups may clash with nearby residues. This was evident in Npam23 (n = 4, 87.0 \pm 6.5 %, P < 0.001) and in the case of Npam25 (n = 4, -14.32 \pm 2.2 %, P > 0.5) it lead to a slight inhibition effect of the NMDAR currents. One other noticeable pattern was the modification of the actual R1 ring (positions 1-6 in Figure 4.4) (Figure 5.2b) itself, which was converted to a heterocycle. A nitrogen within the ring in the meta-position (position 3 in Figure 4.4) (Npam06) showed a marked reduction to (n = 4, 201.2 \pm 14.6 %, P < 0.001) compared to the parent compound Npam32 (n = 4, 374.6 \pm 19.1 %, P < 0.001). This suggested that the heterocycle version lost significant amount of hydrophobicity, thus diminishing the interactions with hydrophobic cage region. A more pronounced decline in the modulation was observed when the heterocycle nitrogen was placed in the para-position of the ring where the potentiation was almost completely lost (n = 4, 30.3 \pm 2.9 %, P < 0.001). More delicate alterations were also performed to study the SAR effects of the linker between the R2 ring (positions 1-6 in Figure 4.4) and the other ring R1 (positions 11-16 in Figure 4.4). The first alteration to the linker was to remove the carbonyl group in position 18 in Figure 4.4, which allowed us to understand whether it contributed to important interactions to the binding site. The removal of the carbonyl was detrimental to the modulation effect as it was reduced down to (n = 4, 105.9 \pm 12.6%, P < 0.001) when compared to Npam43 (n = 6, 363.5 \pm 27.3 %, P > 0.001). This suggested that the carbonyl may offer substantial beneficial effects that could be explained by the possible interaction to the nearby Gln₁₁₀ or to the overall stability of the compound. Moreover, to possibility understand whether the linker could be longer, we incorporated an extra carbon in between the R2 ring (Figure 5.2) (positions 1-6 in Figure 4.4) and the carbonyl in position 18 in Figure 4.4. This addition greatly reduced the effects and suggested that such an addition will endow flexibility into the compound and this attribute may not be beneficial as entropy of binding increases and this in turn is energetically expensive.

Lastly, the addition of a methyl group in position 8 in Figure 4.4, also reduced the potentiation effect (n = $175.5 \pm 25.9 \%$, P < 0.001) but this decrease was surprisingly not as significant as expected and this addition may help to increase the metabolic stability of the compound in the future. The proton attached to nitrogen in position 8 in Figure 4.4 is vulnerable to enzymatic hydrolysis and the replacement of the proton to a non-hydrolysable methyl will ultimately block this process from occurring. Overall, the SAR analysis not only allowed us to understand binding interactions that might be occurring in the binding site but also helped to identify weaknesses and strengths of the compounds and to ultimately guide the lead

optimization stage even further to achieve the best possible compound that would be selective for the GluN1/GluN2A-containing NMDARs, potent, non-toxic, and metabolically stable. Considering, all the data obtained from this SAR analysis, there are several modifications that could be done to improve the potency even further and this information will be extremely important for future chemical alterations to improve pharmacokinetic parameters of the leads.



Figure 5.2 Potentiation effects of different chemically synthesized R1 and R2 ring modifications based on the structure of Npam02 in cortical neurons

(a) Quantification of the potentiation effect of chemically synthesized R1 ring groups on NMDARs mediated currents in cortical neurons. Potentiation effect ranged from 0-363 % normalized to NMDA control. Compounds were tested at 10 μ M. Npam43 showed the best potentiation effect (10 μ M, n = 6, 363 ± 3.65 %; P < 0.001; normalized to NMDA control) significantly potentiated NMDAR-mediated currents. (b) Quantification of the potentiation effect of chemically synthesized R1 ring groups on NMDARs mediated currents in cortical neurons. Potentiation effect ranged from 21-464 % normalized to NMDA control. Compounds were tested at 10 μ M. Npam44 showed the best potentiation effect (10 μ M, n = 6, 465 ± 7.65 %; P < 0.001; normalized to NMDA control) significantly potentiated NMDAR-mediated NMDAR-mediated currents. Numbers on the scaffold represent different atom types.

5.6 Selection of a lead compound

At the early stage of this drug discovery process, several chemical compounds were synthesized or purchased and assayed for potency with cortical primary neurons and *HEK293* cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs. Initial screening of a modest number of selected compounds was used to construct a SAR. Based on this model, all the compounds that were tested at 5μ M that showed a greater than 200% increase in potentiation of NMDAR currents in the presence of NMDA and glycine were shortlisted. These compounds were further tested to determine their EC₅₀ against NMDAR activity shown in Figure 5.3. To assess the selectivity profile for these analogs we originally selected three compounds that showed an enhanced potency in neurons and that their pharmacological profile was favorable towards stability, toxicity and possess chemical features that would make it more likely to pass the brain-blood barrier (BBB). Three compounds (Npam43, Npam49, and Npam50) were chosen from the list based on several considerations which included its $logP_{oct}$, EC₅₀, solubility in water and finally its chemical structure, particularly whether ring R2 (Figure 5.2) incorporated a substituent, which was deemed important to occlude the effect on GluN2B-containing NMDARs. From our previous data obtained from neurons and transiently transfected *HEK293* cells we chose Npam43 as our lead candidate, mainly due to its more desirable pharmacological profile with respect to its superior ability to dissolve in water as compared to Npam49/50. This becomes extremely important in *in-vivo* experiments where the desolvation of the drug needs to be at least partially soluble in saline.



Figure 5.3 Chemically synthesized analogs of Npam43 enhanced neuronal NMDAR function in cultured hippocampal neurons

0.07

1.15

0.19

0.01

Npam86

1.02

(a) Dose-response relationship of chemically synthesized analogs of Npam43 which strongly potentiated NMDA currents. (b) Table summarizing the EC_{50} , $logEC_{50}$ and hill-slope coefficients for the most potent analogs of Npam43.

Chapter 6: Identification and characterization of Npam43 as a selective and potent PAM for the GluN2A-containing NMDARs

6.1 Introduction

Our previous results demonstrated that Npam02 and its analogs positively modulated GluN2A-mediated currents by physically acting at allosteric sites in both recombinant and endogenous GluN2A-containing NMDARs. Once a lead compound has been identified and shows promising results, it would ultimately need to be scrutinized in depth to ascertain its validity in a number of biological tests to demonstrate its efficacy *in-vitro*, *ex-vivo* and *in-vivo*. Npam43 was selected as our lead compound for sequential in depth biological testing to evaluate its potential as a lead compound for the GluN2A-contianing NMDARs based on the criteria outlined in section 5.6. Initially, Npam43 was tested in NMDAR-mediated currents in transfected *HEK293* cells expressing either GluN2A or GluN2B NMDARs.

Our previous results show that the initial hit compound potentially bound to the predicted interstice interface site of the NTD between GluN1 and GluN2A subunits. As, the structure has been greatly modified a more sophisticated mutational study was implemented to confirm the binding site is still in the interstice interface site of the NTD. Therefore, we first investigated the efficacy of Npam43 in primary cortical neurons to ascertain whether the potentiation effect was robust and was selective toward GluN2A-containing NMDARs. A similar experiment was conducted to show the modulation effect could be observed in hippocampal slices and that the enhanced potentiation was mediated by GluN2A-containing NMDARs.

The role of NMDARs in the induction of long-term potentiation (LTP) is well established but the question of which subunits are involved is still very controversial. However, many reports have supported the idea that the induction of LTP is mediated via synaptic GluN2A NMDARs (L. Liu et al., 2004). As such, we chose to test the effect of Npam43 on LTP in hippocampal slices in order to determine whether the selective potentiation of GluN2Acontaining can affect LTP processes using high-frequency stimulation. As activation of GluN2Acontaining NMDARs has been shown to facilitate synaptic plasticity, we wanted to demonstrate whether LTP was altered by Npam43.

6.2 Npam43 selectively potentiates GluN1/GluN2A-containing NMDAR-mediated currents in transiently transfected *HEK293* cells

Npam43 (10µM) was initially evaluated for PAM using recombinant GluN1/GluN2A and GluN1/GluN2B NMDA receptors. Whole-cell patch clamp recordings were performed to measure NMDA evoked currents with chloride-based pipette solutions at a holding potential of - 60mV. To rule out the possibility that Npam43 itself may induce any currents in the GluN1/GluN2A or GluN1/GluN2B expressing *HEK293* cells, Npam43 (10µM) was applied alone and caused no observable change of inward or outward currents (Figure 6.1a). In order to prevent receptor desensitization by high concentrations of the modulator, we induced inward NMDAR currents through co-application of two co-agonists: L-glutamate (5µM) and glycine (2µM). Co-application of Npam43 (10µM) with co-agonists dramatically enhanced NMDA-mediated currents in *HEK293* cells expressing GluN1/GluN2A receptors (10 µM; n = 6; 326 ± 0.15 %; P > 0.001; compared to glutamate application alone Figure 6.1b), consistent with

Npam43 acting as a PAM. The increased NMDAR currents were abolished by co-application of a selective GluN1/GluN2A antagonist NVP-AAM077 (0.2µM) in the presence of both coagonists, confirming that there were no secondary effects attributable to endogenous proteins in HEK293 cells (Figure 6.1a). In contrast, HEK293 cells expressing the GluN1/GluN2B subtype in the presence Npam43 (10 μ M) did not exhibit potentiation of NMDAR currents (Figure 6.1c). Similarly, Npam43 did not induce currents on its own and NMDAR currents attributed to the GluN1/GluN2B-receptors were successfully blocked by GluN2B specific antagonist ifenprodril (IF; 3µM) (Figure 6.1d). The enhancement of GluN1/GluN2A NMDAR-mediated currents in HEK293 cells showed a dose dependent relationship in the presence of Npam43 that reached saturation at ~350% relative to the co-agonist alone baseline. The EC_{50} was pEC50 of -0.614 \pm 0.05 μ M (0.24 \pm 0.05 μ M) with a hill-slope of 0.95 \pm 0.03 (Figure 6.2a). In the presence of Npam43 no dose-dependent enhancement of currents was observed from GluN2B-containing NMDARs recordings in (Figure 6.2a). Moreover, Npam43 (1µM) shifted the L-glutamate dose response curve to the left and changed the pEC₅₀ of glutamate to $0.683 \pm 0.03 \mu M$ (4.82 ± 0.69 μ M) to -0.0152 \pm 0.07 μ M (0.97 \pm 0.30 μ M) without affecting the Hill coefficient (control, 1.036) \pm 0.05; Npam43 treatment, 1.286 \pm 0.237) (n = 6) indicating that Npam43 may affect glutamate binding affinity on NMDARs (Figure 6.2b). Notably, Npam43 had a greater potentiation effect on currents induced by low-doses rather than high-doses of glutamate, and exhibited lower potentiation effect when glutamate reached saturation concentrations. The 2D structural representation of transoid E isomer Npam43 is shown in Figure 6.3a.



Figure 6.1 Npam43 potentiated GluN1/GluN2A-mediated NMDAR currents via direct binding in *HEK293* cells transiently transfected with GluN1/GluN2A subunits

(a) Representative traces from whole-cell electrophysiological recordings of Npam43 in transiently transfected *HEK293* cells expressing GluN1/GluN2A. Npam43 alone induced no detectable currents, but instead potentiated glutamate currents that were sensitive to NVP-AAM007. (b) Quantification of the effect of Npam43 potentiation of GluN1/GluN2A NMDAR mediated currents. Npam43 itself did not influence the GluN1/GluN2A NMDAR currents (10 μ M; n = 6; 1.6 \pm 0.6 %). Npam43 significantly potentiated (5 μ M) glutamate-induced currents (10 μ M; n = 6; 326 \pm 0.15 %; P > 0.001; compared to glutamate control) and these currents were

completely blocked by a GluN2A antagonist NVP-AAM007 (0.2 μ M; n = 6; 1.8 \pm 0.2 %; P < 0.001; compared to glutamate control group). (c) Representative traces from whole-cell electrophysiological recordings of Npam43 in transiently transfected *HEK293* cells expressing GluN1/GluN2B. Npam43 showed no significant potentiation of glutamate-induced GluN1/GluN2B currents and was sensitive to ifenprodril (IF). (d) Quantification of the effect of Npam43 on GluN1/GluN2B NMDARs mediated currents. Npam43 (10 μ M) alone induced no detectable currents (10 μ M; n = 6; 1.2 \pm 0.26 %), and showed no significant potentiation of GluN2B-mediated NMDAR currents (10 μ M; n = 6; 98.6 \pm 5.0 %; P > 0.05; compared to glutamate control) with the presence of glutamate (arbitrarily 100%). These currents were susceptible to the GluN2B antagonist of IF (3 μ M; n = 6; 1.1 \pm 0.22 %; P < 0.001; to glutamate control) where the currents were completely blocked.



Figure 6.2 Npam43 dose response curve and glutamate dose response curve in the presence of Npam43

(a) Dose-response curve of Npam43–induced potentiation on 5 μ M glutamate-induced currents in GluN1/GluN2A or GluN1/GluN2B NMDARs. The potency of Npam43 on GluN1/GluN2A NMDARs was pEC₅₀ of -0.614 ± 0.05 μ M (0.24 ± 0.05 μ M) and a hill-slope of 0.95 ± 0.03. Npam43 did not significantly potentiate GluN1/GluN2B NMDARs. (b) Npam43 (1 μ M) shifted the L-glutamate dose response curve left and changed the pEC₅₀ of glutamate to 0.683 ± 0.03 μ M (4.82 ± 0.69 μ M) to -0.0152 ± 0.07 μ M (0.97 ± 0.30 μ M) without affecting the Hill-slope coefficient (control, 1.036 ± 0.05; Npam43 treatment, 1.286 ± 0.237) (*n* = 6).



Figure 6.3 Npam43's chemical structure and its binding to the NMDAR

(a) 2D chemical structure of Npam43 and its respective atom enumeration (b) Cartoon representation of homomeric GluN1/GluN2A NMDARs, colour coded by their respective domains and the predicted binding site of Npam43 in the NTD. Glutamate control group was arbitrarily set as 100%; Two-tailed Student's t-test ***P < 0.001; bars represent relative mean values \pm SEM. normalized to the NMDA control (white bar, arbitrarily set as 1). *n* represents individual experiments from at least 3 separate primary cultures.

6.3 Identification of critical amino acid residues at the GluN1/GluN2A interface in the Nterminal domain (NTD) required for formation of the Npam43 binding pocket

Extensive induced-fit docking suggested that Npam43 can occupy and exploit the space between the upper-lobe (R1) of the NTD (Figure 6.3b). We therefore tested whether potentiation of the GluN1/GluN2A-containing NMDAR by Npam43 is altered by mutations at residues that surround the pocket interface by choosing side chains for substitutions that occlude the interactions with the compound but do not otherwise perturb the overall protein structure. Primary sequence of GluN1 and GluN2A subunits is shown in Figure 6.4a and bolded residues were defined as being required for the site pocket based on the GluN1/GluN2A 3D model 136 (Figure 6.4b). Docked model of Npam43 in the predicted modulation site showed that GluN1-(Leu₁₃₅) (Figure 6.4b), which forms a bend just before beta-strand 5, may play a role in interacting with the ligand particularly towards the aromatic ring (positions 11-16 in Figure 6.3a) and the ethoxy functional group (positions 23-24 in Figure 6.3a). Indeed, a pronounced reduction of the degree of modulation was achieved with GluN1-(Leu₁₃₅Gln), which significantly decreased positive modulation by more than 70% (n = 6; -77.5 \pm 2.12 %; P < 0.001; normalized to Npam43 response in wild-type GluN1/GluN2A), suggesting that this amino acid is crucial for hydrophobic contacts with the compound (Figure 6.4c). The model also showed that GluN2A- (Gln_{111}) in α -helix 80 made a key H-bond interaction with Npam43 (Figure 6.4b) and thus when Gln₁₁₁ was mutated to GluN2A-(Gln₁₁₁Ala) a decrease in potentiation was also observed (Figure 6.4d), consistent with a role for this region in mediating binding of the ligand (n = 6; -38.4 ± 0.78%, P < 0.001; normalized to Npam43 response in wild-type GluN1/GluN2A). Furthermore, GluN2A-(Phe₁₁₅Ser) mutation located in α -helix 80 reduced positive modulation by (n = 6; -67.4 \pm 11.8 %; P < 0.001; normalized to Npam43 response in wild-type GluN1/GluN2A), which suggests that hydrophobic character in this position was essential (Figure 6.4d). Figure 23a shows that GluN1-(Leu₁₃₅Gln) and GluN2A-(Phe₁₁₅Ser) mutations both independently and together were not a direct reflection of a change in the overall protein structure but rather effected ligand binding. This was demonstrated by the absence of a shift in dose-response of Lglutamate from these two mutations compared with wild-type GluN1/GluN2A.

Another 8 mutations, within the defined pocket, summarized in Table 6.1 and shown in Figure 6.4d, also showed significant reduction in potentiation which ranged from (10-62%), strongly suggesting that the ligand binding occurs at this junction in the upper-lobe (R1) of the NTD. Four negative control mutations GluN1-(Arg₁₁₅Glu), GluN2A-(Met₁₁₂Ile), GluN2A-

(Ile₁₇₆Tyr) and GluN2A-(Ala₁₀₈Gly), which were defined as residues within the binding pocket that did not contribute to ligand binding were also mutated to further demonstrate the accuracy of the model binding site (Figure 6.4d). There were no single point mutation Table 6.2 that was able to abolish the binding of Npam43 so therefore a double mutation; one from GluN1 (Leu₁₃₅Gln) and one from GluN2A (Phe₁₁₅Ser) was constructed and tested to see whether residual positive modulation could be further diminished. The double mutation of the NTD, which individually impair the interaction, further reduced binding of Npam43 as reflected by the additive reduction in potentiation (n = 6; -93.9 \pm 10.3 %; P < 0.001; normalized to Npam43 response in wild-type GluN1/GluN2A) (Figure 6.4e: Figure 6.4f highlights these two point mutations, their relative positions and their strong influential interactions in the binding site). To determine whether this decrease was due to Npam43 binding or to de-sensitivity of the receptor, the dose-response relationship to glutamate activation was investigated with the double mutation with respect to the wild-type. Figure 6.5a shows the absence of a significant shift in glutamate dose-response for the double mutation compared to GluN1/GluN2A wild-type receptor, suggesting that the mutation did not affect protein function. To evaluate the accuracy of the structural model used for GluN1/GluN2A NTD, a correlation plot was performed between the relative potentiation of Npam43 observed from the mutated forms of GluN1 or GluN2A in HEK293 cells and the binding energy was predicted from the docking analysis (Figure 6.5b). There was a strong correlation $R^2=0.93$ between the observed potentiation and the predicted binding energy suggesting that the model was accurate enough to model the compound inside this binding pocket (Figure. 6.5b).



Figure 6.4 GluN1 L₁₃₅ or GluN2A F₁₇₇, P₇₈, F₁₁₄, Q₁₁₁, and P₁₇₈ form the Npam43 binding pocket between the GluN1 and GluN2A interface of NMDAR receptors in the NTD

(a) The primary sequence of GluN1 and GluN2A. Bold amino acids were characterized as being amino acids that defined the dimer cleft in the NTD. (b) Simulated docking pose of Npam43 in the interface between the GluN1 (light blue) and GluN2A (purple) subunits in the NTD, showing specific amino acids that define this cleft (c) The normalized potentiation by Npam43 on the

substituted GluN1 subunit. The normalized potentiation by Npam43 (10µM) in the wildtype form of GluN1 (n = 6; 209 \pm 6.7 %) was dramatically reduced in the GluN1 mutation of L₁₃₅Q $(10\mu M; n = 7; 47.1 \pm 2.8\%; P < 0.001)$ but not affected by the R₁₁₅E mutation ($n = 4; 2.55 \pm 3.6$ %; P > 0.05) (d) the normalized potentiation by Npam43 on the mutated form of the GluN2A subunit. The normalized potentiation by Npam43 (10uM) was significantly reduced by $F_{177}S$ (*n* = 5 ; 79.9±2.6 % P < 0.001); P₇₈A (n = 5; 99.3 ± 2.8%; P < 0.001), F₁₁₄S (n = 6; 68.1 ± 14.1 %; P < 0.001), $F_{114}Y$ (*n* = 6, 100±4.2 %; P < 0.001), $P_{178}G$ (*n* = 4; 95.5 ± 3.8 %; P < 0.001), and $Q_{111}A$ (*n* = 4; 129±6.7 %; P < 0.01), but no change from $I_{176}Y$ (*n* = 3, 189±3.8 %; P > 0.05), $M_{115}I$ (*n* = 3, 255±19 %; P > 0.05), and $A_{108}G$ (*n* = 3; 210±9.0 %; P > 0.05) mutations. Twotailed Student's t-test (e) The normalized potentiation by Npam43 on the double substituted GluN1 and GluN2A mutations. Npam43 (10µM) potentiation was almost completely abolished by the double mutation of $L_{135}Q$ from GluN1 and the $F_{114}S$ from the GluN2A subunit (n = 5; 12.7 ± 1.8 %; P < 0.001). (f) Simulated docking pose of Npam43 in the binding site, highlighting the strong ligand interactions (shown in brown) with L₁₃₅ and F₁₁₄ amino acids (shown in pink) in the opposite sides of the binding site and underscoring their influence to ligand binding. Twotailed Student's t-test, ***P < 0.001; bars represent relative mean values \pm SEM.; *n* represents individual experiments from at least 3 separate transiently transfected cell lines. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).(Pettersen et al., 2004)

	Relative potentiation	Interaction: Direct/Type	Net Difference potentiation	Ν
GluN1 ^{WT} /GluN2A ^{WT}	$209\pm6.7\%$			6
GluN1/GluN2AQ111A	129±6.7%	Yes/H-bond	$-38.4\pm0.78\%$	4
GluN1/GluN2A ^{F115Y}	100±4.2%	Yes/Hyd-C	$-52.2 \pm 0.52\%$	6
GluN1/GluN2A ^{F177S}	79.9±2.6%	Yes/Hyd-C	$-61.7 \pm 0.03\%$	5
GluN1/GluN2A ^{1176Y}	189±3.8%	No/-	$-10.1 \pm 0.12\%$	3
GluN1/GluN2A ^{M1111}	255±19%	Yes/Hyd-C	$+22.2 \pm 0.94\%$	3
GluN1/GluN2A ^{P178G}	$95.5 \pm 3.8\%$	Yes/Hyd-C	$-54.3 \pm 0.42\%$	4
GluN1/GluN2A ^{P79A}	$99.3\pm2.8\%$	Yes/Hyd-C	$-52.5 \pm 0.20\%$	5
GluN1/GluN2A ^{F115S}	$68.1 \pm 14.1\%$	Yes/Hyd-C	$-67.4 \pm 11.8\%$	6
GluN1/GluN2A ^{A108G}	210±9.0%	No/-	$+0.48\pm0.01\%$	3
GluN1 ^{R115E} /GluN2A	255±3.5%	No/-	$+22.0 \pm 0.40\%$	4
GluN1 ^{L135Q} /GluN2A	$47.1 \pm 2.8\%$	Yes/Hyd-C	$-77.5 \pm 2.12\%$	7
GluN1 ^{L135Q} /GluN2A ^{F115S}	12.7±1.8%	Yes,Yes/ Hyd -C, Hyd-C	$-93.9 \pm 10.3\%$	5

Table 6.1 Npam43-induced potentiation of the GluN1 or GluN2A mutants

The list includes all GluN1 or GluN2A mutants in the NTD that were tested. Npam43-induced potentiation effect was tested by measuring the chloride currents induced by 10μ M Glutamate and 2μ M of glycine with or without 10μ M Npam43 in transfected *HEK293* cells. Mutants were selected on the basis of which amino acids were directly interacting with the Npam43 guided by the docked-model of Npam43 and the GluN1/GluN2A NTD structure. Major interactions observed included a combination of hydrophobic interactions (Hyd-C) or hydrogen bonding (H-Bond). The percent decrease denoted (-) or increase denoted (+) in potentiation compared with the wild-type GluN1/GluN2A receptor were calculated for each corresponding mutation. Three

"negative control" mutations were also tested as they were classified as mutations that were in the binding site but did not play a role in direct binding to the compound.



Figure 6.5 Dose-response of glutamate in *HEK293* cells expressing wild-type GluN1/GluN2A, mutant GluN1 (L₁₃₅Q) and mutant GluN1 (L₁₃₅Q)/GluN2A (F₁₁₄S)

(a) There was no significant difference between the dose-response of glutamate in wild-type GluN1/GluN2A with an EC₅₀ of (n = 6; 3.162 ± 0.5 µM; P > 0.05) and hill-slope of (n = 6; 1.136 ± 0.08). The EC₅₀ mutant forms of GluN1 (L₁₃₅Q) was (n = 6; 4.337 ± 0.8 µM; P > 0.05) and the hill-slope was (n = 6; 1.068 ± 0.07). As for the double mutation of GluN1 (L₁₃₅Q)/GluN2A (F₁₁₄S) the EC₅₀ was (n = 6; 3.689 ± 0.4 µM; P > 0.05) and the corresponding hill-slope was (n = 6; 1.24 ± 0.04). This suggested that the mutations did not affect GluN1/GluN2A protein function. Dose-dependent curves are normalized by the maximal current response and arbitrarily set as 100%; Two-tailed Student's t-test ***P < 0.001; bars represent relative mean values ± 142

s.e.m.; *n* represents individual experiments from at least 3 separate primary cultures. (b) There was a strong correlation ($R^2 = 0.93$) between the binding energies of Npam43 from mutant GluN1/GluN2A forms *in-silico* and the actual relative potentiation observed in electrophysiological recordings of these mutations on the NTD of GluN1/GluN2A. The correlation was affected by two deviations $L_{135}Q$ and $M_{115}I$ but are within acceptable limits. This gives us some indication of the accuracy of the model and binding site with respect to Npam43 action to the receptor.

6.4 Modulation of Npam43 on GluN1/GluN2A-containing NMDARs potentiates NMDAR-mediated currents in cultured rat hippocampal neurons

We next tested whether the positive modulation of GluN1/GluN2A NMDARs expressed *HEK293* cells could be maintained in cultured rat hippocampal neurons. Recordings were taken from cultured hippocampal neurons with a pipette filled with a Cl⁻ based intracellular recording solution under whole cell voltage-clamp recording configuration at a holding membrane potential of -60mV. As shown in Figure 6.6a, bath application of Npam43 (10µM) strongly positively modulated NMDAR currents in the presence of co-agonists NMDA (5µM) and glycine (2µM) in hippocampal (HP) neurons (n = 6; 322 ± 27 %; P < 0.001; compared to NMDA control group: Figure 6.6a). Consistent with our *HEK293* cell data, Npam43 was not able to induce inward or outward currents on its own (Figure 6.6a). Furthermore, application of APV (50µM) blocked all currents indicating that the potentiation was mediated through NMDARs and not through secondary effects attributed to other endogenous proteins (Figure 6.6a). A dose-response analysis revealed that Npam43 dose-dependently enhanced the NMDAR currents with

an EC₅₀ of 0.25 \pm 0.12 μ M and a hill-slope of 1.03 \pm 0.11. (Figure 6.6b) in the presence of NMDA and glycine and reaches a saturation point of 10 μ M (n = 6; 322 \pm 27 % above control level baseline). To determine whether Npam43 in cultured neurons is selective for the GluN1/GluN2A-containing NMDARs we applied selective GluN2A- or GluN2B-NMDAR antagonists to isolate the currents and confirm if the compound discriminated between the two subtypes. Bath application of NMDA (10µM) and glycine (2µM) with GluN2A-NMDAR antagonist NVP-AAM077 (0.2µM) revealed a ~50% reduction (n = 6; 46 ± 7.3 %; P < 0.001; to NMDA control group) of the total NMDAR currents (Fig. 6.7a). Subsequently, the application of NVP-AAM007 (0.2µM) with Npam43 (10µM) prevented the potentiation of NMDAR currents by Npam43 suggesting that the residual GluN2B-NMDAR current was not potentiated, consistent with the idea that Npam43 is a PAM for the GluN2A-containing NMDARs. Similarly, APV (50µM) blocked the Npam43-dependent potentiation effect (Figure 6.7a). Application of the GluN2B antagonist, ifenprodril (IF; 3µM) with NMDA (10µM) and glycine (2µM) reduced the total NMDAR currents by ~50% (n = 6; 57 ± 6.7 %; P < 0.001; to NMDA control group) (Figure 6.7b). Addition of Npam43 (10µM) resulted in potentiation of residual GluN2A mediated currents (3 μ M; 302 \pm 19 %, n = 6; P > 0.001; compared to IF alone group) (Figure 6.7b). This suggests that the antagonism of GluN2B fails to block potentiation effects of the drug, consistent with results obtained in HEK293 cells. Similarly, in the same patched cell, the total NMDAR currents in the presence of IF (3µM) and Npam43 (10µM) was again able to be abolished via administration of APV (50µM) (Figure 6.7b) suggesting that the potentiation effect was mediated through NMDARs. A summary of the effects of GluN2A and GluN2B antagonists on Npam43 modulation are shown in Figure 6.7c and demonstrate that the allosteric effect of Npam43 was significant and was mediated through GluN2A but not GluN2B-containing

NMDARs. To further examine the selectivity of Npam43 to other extracellular transmembrane proteins, we tested the compound for activity on GABARs and AMPARs. Npam43 (100 μ M) did not show any effects on currents evoked by AMPA (20 μ M) (Figure 6.8a, c) but slightly positively potentiated GABAR currents in the presence of GABA (1 μ M) (Figure 6.8b, c). The dose-response curve and EC₅₀ for Npam43 was found to comparable to the dose-response and EC₅₀ value in *HEK293* cells expressing GluN1/GluN2A. Additionally the NMDA dose-response curve was found to be pEC₅₀ = 1.484 ± 0.082 μ M or 30.5 ± 11.7 μ M with a hill-slope of 0.765 ± 0.10 (n = 6) in which in the presence of Npam43 (5 μ M) ,shifted left bringing the pEC₅₀ of NMDA to 0.44 ± 0.08 μ M or 2.10 ± 0.69 μ M with a hill-slope of 1.48 ± 0.19 (n = 6) (Figure 6.9). Taken together, these results demonstrated that Npam43 causes a positive modulation of endogenous GluN1/GluN2A-containing NMDARs in cultured rat hippocampal neurons which could be blocked by inhibiting GluN1/GluN2A but not GluN1/GluN2B containing NMDARs.



Figure 6.6 Npam43 dose-dependently enhanced neuronal NMDAR function in cultured hippocampal neurons

(a) Representative traces from whole-cell electrophysiological recordings of Npam43 which showed it can potentiate NMDA-induced currents in cultured hippocampal neurons. Npam43 alone induced no detectable currents, but instead potentiated NMDA currents that were sensitive to APV. (b) Dose-response relationship of Npam43 potentiation on neuronal NMDA currents. The potency of Npam43 in the presence of 5 μ M NMDA was pEC₅₀ of -0.604 \pm 0.05 μ M (0.25 \pm 0.12 μ M) and a hill-slope of 1.03 \pm 0.11. (c) Representative traces from whole-cell patch electrophysiology recordings showing the effect of Npam43 in the presence of GluN2A selective antagonist or NMDA blocker.



Figure 6.7 Npam43 targets specifically GluN2A-containing NMDARs in cultured hippocampal neurons

(a) Representative traces from whole-cell patch electrophysiology recordings showing the effect of Npam43 in the presence of GluN2A selective antagonist or NMDA blocker. (b) Representative traces from whole-cell patch electrophysiology recordings showing the effect of Npam43 in the presence of GluN2B selective antagonist or NMDA blocker. (c) The quantification of the effect of NMDA stimulation and Npam43 in the presence of GluN2Acontaining or GluN2B-containing NMDAR antagonist. Npam43 positively potentiated NMDAR currents to (10 μ M; n = 6; 322 \pm 27 %; P > 0.001; compared to NMDA control group). Both NVP-AAM007 (0.2 µM) and ifenprodril (IF; 3 µM) individually showed a similar reduction of NMDAR currents at ~50% (n = 6; 46 ± 7.3 %; P < 0.001; to NMDA control group) and (n = 6; 57 \pm 6.7 %; P < 0.001; to NMDA control group) respectively. Npam43 was able to potentiate NMDAR currents in the presence of IF (3 μ M; 302 ± 19 %, n = 6; P > 0.001; compared to IF alone group) but not NVP-AAM007 (0.2 μ M; 45 ± 3.8 %; P > 0.05; to NVP-AAM077 alone group) where the potentiation effect was completely blocked. NMDA-induced currents were completely blocked by NMDAR antagonist APV (50µM) in the presence of the Npam43/NVP-AAM007 combination (50µM; n = 6; 2.3 \pm 1.3 %; P < 0.001; compared to NMDA/NVP-AAM0077/Npam43 group) or the Npam43/IF combination (50 μ M; n = 6; 1.3 \pm 1.1 %; P < 0.001; to NMDA/IF/Npam43 group). *** P < 0.001, **P < 0.01 * P < 0.05; Post- Hoc LSD; bars represent relative mean values ± SEM. normalized to the NMDA control (red bar, arbitrarily set as 100%). *n* represents individual experiments from at least 3 separate primary cultures.



Figure 6.8 Npam43 does not affect current responses induced by AMPA but modestly enhanced current responses induced by GABA at higher concentrations in neuronal cultures

(a) Npam43 did not modulate current responses induced by AMPA in neuronal cultures. (b) Npam43 modestly positively modulated GABA currents in neuronal cultures, however this effect was 10 fold less than NMDA potentiation (c) Quantification of the effects of Npam43 towards current responses for NMDA (10 μ M; n = 6; 322 \pm 27 %; P > 0.001; compared to NMDA 149 control group), GABA (100 μ M; n = 6; 30.9 ± 7.5 %; P < 0.001 compared to GABA control) and AMPA (100 μ M; *n* = 6; 0.04 ± 1.53 %; P > 0.05; compared to AMPA control). Two-tailed Student's t-test ***P < 0.001; bars represent relative mean values ± SEM; *n* represents individual experiments from at least 3 separate primary cultures.



Figure 6.9 NMDA dose-dependent curve in the presence and absence of Npam43

The NMDA dose-response curve was found to be pEC₅₀ = $1.484 \pm 0.082 \mu$ M or $30.5 \pm 11.7 \mu$ M hill-slope 0.765 ± 0.10 (n = 6) and in the presence of Npam43 (5 μ M) the dose response curve shifted left bringing the pEC₅₀ of NMDA to $0.44 \pm 0.08 \mu$ M or $2.10 \pm 0.69 \mu$ M and a hill-slope of 1.48 ± 0.19 (n = 6). Two-tailed Student's t-test, ***P < 0.001; NMDA control group was arbitrarily set as 100%; bars represent relative mean values \pm SEM. normalized to the NMDA control (white bar, arbitrarily set as 1). n represents individual experiments from at least 3 separate primary cultures.

6.5 The GluN2A component of synaptic transmission is enhanced by Npam43

In order to assay the effects of Npam43 on NMDAR currents in neurons more directly, we pharmacologically isolated the GluN2A and GluN2B components of synaptic transmission; bath applied Npam43 and measured the subsequent level of potentiation of these respective NMDAR currents. CA1 hippocampal neurons were voltage clamped to +40mV in the presence of bicuculline (10µm) and CNQX (10µM) to isolate NMDAR currents. The residual currents were further isolated by bath application of either NVP (0.2µM) or IF (3µM) after which, Npam43 (10µM) was added while monitoring evoked GluN2A or GluN2B-mediated synaptic currents (Figure 6.10). Application of Npam43 significantly potentiated NMDAR responses in the presence of ifenprodril (IF; 3 µM) (Figure 6.10). ANOVA; $F = {}_{10.52}$, p < .001; Tukey's posthoc p < 0.05) Conversely, NVP-AAM007 (0.2 µM) prevented this potentiation which was not significantly different from DMSO treated controls but was significantly reduced relative to IF treated slices (p < 0.05) (Figure 6.28). Application of APV further reduced residual NMDAR currents in both NVP and IF treated slices. These data are consistent with a selective potentiation of the GluN2A component of NMDAR currents (Figure 6.10).



Figure 6.10 The GluN2A component of synaptic transmission is enhanced by Npam43 Selective potentiation of GluN2A-mediated currents in CA1 pyramidal cells. Isolated NMDAR currents were elicited by stimulating the SC fiber pathway in the presence of gabaergic (BIC; 10μ M) and AMPA receptor (CNQX; 10μ M) antagonist while holding cells at +40mV (shown in insets; red trace represents cumulative NMDAR currents). Following isolation of the NMDAR component of synaptic transmission was further isolated by applying either ifenprodril (IF; 3 μ M, GluN2B antagonist).or NVP-AAM007 (NVP; 0.2μ M, GluN2A antagonist) (show in insets as green traces). Npam43 (10μ M) was then added and the potentiation relative to total NMDAR currents was plotted (represented by blue traces in insets). An ANOVA comparing all groups was significant; *F* (10,52) = 151.1, *p* < 0.001. Tukeys posthoc analysis demonstrated that pharmacologically isolated GluN2A currents (left; white bars) were significantly potentiated following Npam43 application (*p* < 0.001 denoted by "*" on graph. There was no significant difference in cells treated with NVP and Npam43.(center, black bars; p > 0.05, NS = not

significant). Grey bars show DMSO controls which did not significantly reduce isolated NMDAR currents. AVP was applied at the end of experiments to confirm that residual currents were mediated by NMDARs (shown in insets as black traces).

6.6 The GluN2A component of LTP is enhanced by Npam43

As activation of GluN2A-containing NMDARs has been shown to facilitate synaptic plasticity, we next tested whether long-term potentiation (LTP) was altered by treatment with Npam43. Field excitatory synaptic potentials (fEPSPs) were elicited in acute mouse hippocampal slices by stimulating Schaeffer collateral fibers while recording extracellularly in stratum radiatum of CA1. Following a 20min baseline, slices were perfused with saline containing Npam43 (Figure 6.11a). Following 5min of exposure to Npam43 a single train of high frequency stimulation (1x100Hz, 1 sec duration) was applied, after which Npam43 was applied for an additional 10 min. Compared to vehicle (DMSO) controls, the magnitude of LTP during the 1st hour after stimulation was significantly enhanced in Npam43 treated slices (t = 7.261, p < .001; Figure 6.11b. However, LTP decayed to levels similar to DMSO treated slices levels after approximately 1 hr (Figure 6.11c). These data suggest that Npam43 facilitates the induction but not maintenance of LTP consistent with a potentiation of the GluN2A component of synaptic transmission. Collectively, these data suggest that Npam43 is capable of selectively bolstering GluN2A-mediated synaptic currents in addition to facilitating the induction of LTP in mouse hippocampus slices.



Figure 6.11 Npam43 facilitates the induction of LTP

(a) Prior to inducing LTP (1x100 Hz stimulation, 1 sec), Npam43 (10 μ M) was pre-applied for 5 min and an additional 10 min post tetanization. The magnitude of LTP was significantly enhanced for the first hour following pairing of stimulation with Npam43 application relative to DMSO treated controls (b) Npam43 treated slices 20-60 min post-stimulation LTP mean: 177.6 \pm 4.83%; DMSO treated 20-60 min post stimulation, 137.9 \pm 2.98%; unpaired t-test, t₇ = 7.021,

*p < 0.0001; n = 5 slices per group). (c) Differences were no longer significant when compared from 1-2hrs. Insets are representative sweeps taken during baseline and 20 min post stimulation (scale bar, 1 mV, 5 ms).

6.7 Npam43 specificity for the GluN1/GluN2A may come from subtle differences in the relative positions of amino acid residues from the interface

During our drug discovery process, over 12 initial hit compounds and several medicinal chemistry efforts identified afforded a class of compounds called hydrazides which have shown a range of potency profiles $(0.07-200 \mu M)$. This sufficiently provided a good overall activity map in relation to the structure of the drug but also suggested one of the determining factors for Npam43's selectivity for GluN1/GluN2A. One crucial moiety from Npam43's structure that elicits its selectivity toward GluN1/GluN2A was the bromine (Br) functional group in position 22 in Figure 6.3a; the absence of this group resulted in a loss in selectivity and binding to GluN1/GluN2B (Npam58 in Appendix Table 2). Moreover, addition of the ethoxy group depicted in positions 19, 23 & 24 (Figure. 6.3a) also played a role in determining selectivity as the presence of a smaller methoxy group reduced the selectivity profile. Overall, both functional groups were essential as their absence within the backbone structure incrementally increased discrimination between the two subtypes. According to the GluN1/GluN2A model, the binding conformation of Npam43 was found to be linear/flat and elongated along the heteromeric interface, enabling key interactions with both GluN1 and GluN2A (Figure 6.4b, f). The binding pose of Npam43 is depicted in Figure 6.4b, f. The top-ranking configuration for Npam43 favors the Br attachment facing the $Gln_{111}(GluN2A)$ and $Tyr_{109}(GluN1)$ but the possibility that the

bromo-phenyl ring can be flipped 180° cannot be excluded which would create configuration that finds Br facing the Phe₁₁₃(GluN1). Npam43's intrinsic properties and our previous results have demonstrated that these types of compounds are binding in such a way that the conformation was flat (planar). The phenyl hydroxyl group (OH) located on position 18 (Figure 6.3a) is oriented in such way it will undergo an intramolecular interaction with the sp³ nitrogen (N) in position 9 (Figure 6.3a) which forces the molecule to be planar. This is important to note as it says something about how it would bind in the pocket. Our previous experience in working with a similar subfamily of benzimidazoles, we have found that they bind in a planar fashion verified by co-crystallized structure of ligand and protein where the molecule was situated in an interface between two subunits and conformationally flat. Taken together all these results allow the prediction that the conformation of Npam43 in the interface would also conform to a planar geometry. To further understand why Npam43 is selective, Npam43 was docked into the crystal structure of GluN1/GluN2B. Modelling of Npam43 in the GluN1/GluN2B site suggested that the aforementioned Br group may clash with the nearby residues particularly Gln₁₁₀(GluN2B), Tyr₁₀₉(GluN1) or Phe₁₁₃(GluN1) and the ethoxy group would be in close contact with Leu₁₃₅(GluN1) or Pro₁₇₇(GluN2B), and Phe₁₇₈(GluN2B). Nevertheless, all of these binding residues are conserved in the GluN1/GluN2A versus the GluN1/GluN2B and thus highlights that there might be subtle differences in the relative positions of the amino acids in the binding site in the NTD. Numerous reports suggest that a distinction may be attributed to the fundamental difference in the mode of subunit association between GluN1/GluN2A and GluN1/GluN2B in the NTD (Karakas et al., 2011; Malherbe et al., 2003). This could cause sufficient differences in the spatial dimensions of the pocket and may allow Npam43 enough room to bind to GluN1/GluN2A. The docking of Npam43 to the homologues site of GluN1/GluN2B (which was

kept rigid) was conformationally restrictive especially in areas defined by the aforementioned residues which generated no convincing binding configuration that was devoid of steric clashes (Figure 6.12).



Figure 6.12 Npam43 docks into the NTD of GluN1/GluN2B; the analogous binding site of GluN1/GluN2A

Npam43 was docked into the NTD using the available crystal structure of (PDB: 3QEK). The docking analysis highlighted that the binding of Npam43 to GluN1 (shown in light blue) and GluN2B (orange) crevice in the NTD was afflicted with steric clashes (shown in pink). Binding poses of Npam43 in this site was not devoid of these clashes particularly coming from GluN1 (L₁₃₅), (Phe₁₁₃) and GluN2A (P₁₇₈), (Q₁₁₁) amino acids. The crystal structure was kept rigid to avoid uncertainty in the positions of the amino acids and Npam43 was docked under flexible conditions. Molecular graphics and analyses were performed with the UCSF Chimera package.
Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).(Pettersen et al., 2004)

6.8 Npam43 increases intracellular Ca²⁺ via GluN1/GluN2A-containing NMDARs

A cell-based Ca^{2+} influx assay using primary cultured rat neurons was used to determine if modulation of Npam43 could contribute to increased intracellular Ca^{2+} and whether this affect was mediated through GluN1/GluN2A-containing NMDARs. NMDA (10µM) and glycine (2µM) were added to cell cultures to activate NMDARs. Application of Npam43 to cultured neurons increased the Ca^{2+} influx fluorescence signal in a dose-dependent manner (Figure 6.13a). To determine whether this influx of Ca^{2+} was through GluN1/GluN2A-containing NMDARs, we co-applied GluN2A antagonist NVP-AAM0077 and observed marked reduction of Ca^{2+} influx in response to Npam43 suggesting that GluN2A receptors mediate the enhanced calcium influx (Figure 6.13b). Conversely, co-application of GluN2B antagonist IF with Npam43, showed a similar increase in the Ca^{2+} influx fluorescence signal compared with Npam43 with no antagonists, suggesting GluN2B-containing NMDARs were not involved in the surge in intracellular calcium (Figure 6.13b).



Figure 6.13 Npam43 increases Ca²⁺ influx in primary culture neurons in a dose-dependent manner

(a) Acute application of Npam43 in primary neurons stimulated by co-agonists NMDA (10µM) and glycine (2µM) increased calcium influx in a dose-dependent manner. The EC₅₀ of Npam43 in the calcium assay was (n = 6; 0.180 ± 0.06 µM) and the hill-slope was (n = 6; 1.026 ± 0.092). Curve represents relative mean values ± s.e.m.; n represents individual experiments from at least 3 separate primary cultures. (b) The addition of NMDA to the cell cultures increased the Ca²⁺ fluorescence signal ~3 times more than the control group (10µM; n = 10; 344.1 ± 8.70 %; P < 0.001; to control group). The application of Npam43 post NMDA treatment caused a ~ 7 fold increase in Ca²⁺ influx fluorescence signal compared to control levels (10µM; n = 6; 750.7 ± 62.2 %; P < 0.001; to control group) and a further ~2 fold increase from the NMDA alone. The pre-application of ifenprodil (IF) in the presence of Npam43 resulted in a similar increase of the calcium signal in response to Npam43 (10µM) (3µM; n = 7; 686.6 ± 25.7 %; P < 0.001; to control group) suggesting the blockade of GluN1/GluN2B was not sufficient to block the surge in Ca²⁺. Conversely, the pre-addition of the NVP-AAM077 had a marked reduction in Ca²⁺

influx in response to Npam43 (10 μ M) (0.2 μ M; n = 4; 191.2 ± 19.5 %; P < 0.001; to control group) and a ~1.8 fold decrease in the Ca²⁺ signal compared to the NMDA (10 μ M) alone treatment. *P < 0.001; *Post- Hoc* LSD; bars represent relative mean values ± s.e.m. normalized to the Control (black bar, arbitrarily set as 1). *n* represents individual experiments from at least 3 separate primary cultures.

Chapter 7: Npam43 activates GluN2A-NMDAR mediated cell survival signaling, and thereby protects cultured neurons *in-vitro* against neuronal death

7.1 Introduction

In this present study, we show that Npam43 acts on GluN2A-containing NMDARs but not GluN2B-containing NMDARs, to mediate positively modulate the receptor by Npam43. As noted in the introduction, previous findings from the Wang lab indicating that activation of GluN2A and GluN2B-containing NMDARs can promote neuronal survival and death, respectively (Y. Liu et al., 2007). Therefore we undertook this experiment to examine whether Npam43 can activate the pro-survival pathway. Cortical neurons were treated with Npam43 and harvested the cells to probe for pCREB; a major pro-survival marker (Walton et al., 1999). The aim was to demonstrate that Npam43's action on GluN2A-containing NMDARs, but not GluN2B-containing NMDARs, can potentiate CREB phosphorylation (pCREB), which in turn confers protection against neuronal death. pCREB is known to play a role in neuronal survival through activation or inhibition of substrates (Hardingham & Bading, 2002, 2010; Hardingham et al., 2002; Lonze & Ginty, 2002). Activated CREB promotes survival through phosphorylation of transcription factors forkhead/FOXO, NF-KB and mdm2 or through phosphorylation of Bcl-2 family members Bad and Bim (Hardingham & Bading, 2010; Hardingham et al., 2002; Lai et al., 2014; Lonze & Ginty, 2002). We predict that potentiation of the GluN2A-containing NMDARs activity can enhance pCREB and consequently reduce NMDA-dependent and independent neuronal death.

In order to ascertain whether Npam43 protects against neuronal death caused by an excitotoxic event, we used an *in-vitro* model whereby cortical neurons were exposed to NMDA stimulation to mimic cellular mechanisms that occur during stroke. This experiment was performed under high concentrations of NMDA (75uM) and with an exposure time of 1.5 h as it can produce a clear excitotoxicity-induced neuronal death. Previous reports suggest that NMDA treatment is able to induce necrosis and apoptotic cell death, depending on the strength of NMDA insults. Modest NMDA stimulation primarily induces apoptosis, whereas highconcentration of NMDA mainly causes necrosis (Nicotera, 2003). The intensity of the cell-death was done through a LDH assay, which quantifies the amount of LDH released from the damaged cells to the media. LDH is a soluble enzyme located in the cytoplasmic environment and gets released into the extracellular medium upon plasma membrane damage, a process that occurs during apoptosis and necrosis. Because of this, the amount of LDH released into the media is proportional to the extent to the cell death. The media was tested 20-24 hours after the NMDA stimulation via a LDH colorimetric assay which quantifies the LDH levels. The NMDA stimulation caused a significant cell death that was 2 times higher than the untreated control levels. To ascertain of this neurotoxicity was mediated through NMDARs, the positive control GluN2B antagonist (ifenprodril) was co-administered with NMDA. Ifenprodril robustly blocked completely the cell death associated with the NMDA insult, confirming cell death was mediated through NMDARs.

We also evaluated effects of Npam43 against other secondary effects of NMDAindependent oxidative stress. Oxidative stress results from an imbalance between the productions of reactive oxygen species (ROS) and the cell's capacity to neutralize them through its intrinsic antioxidant defenses (Papadia et al., 2008). Neurons are particularly vulnerable to oxidative damage because of their high levels of ROS production (through respiration and metabolism) and relatively low levels of certain antioxidant enzymes (Papadia et al., 2008). Oxidative damage builds up during normal aging and is associated in many pathogenesis of several neurodegenerative diseases, especially stroke (Uttara et al., 2009). Previous work has shown that synaptic NMDARs activity increases intrinsic antioxidant defenses by enhancing thioredoxin activity (Papadia et al., 2008). They further showed that this in turn helps to reduce the overoxidized thioredoxin-peroxiredoxin (Prx) system and stimulated resistance to oxidative stress (Papadia et al., 2008). Conversely, inhibition of NMDAR upregulated thioredoxin-interacting protein (Txnip), where it interacted with thioredoxin and promoted sensitivity to oxidative damage (Papadia et al., 2008). Synaptic activity also enhanced the Prx reactivating genes Sesn2 (sestrin 2) and Srxn1 (sulfiredoxin) which helped to boost the antioxidant defences (Papadia et al., 2008). Synaptic stimulation of NMDARs was crucial for enhancing antioxidant defenses whereas chronic bath activation of all (synaptic and extrasynaptic) NMDARs prompted no antioxidative outcomes (Papadia et al., 2008). It was postulated that synaptic NMDAR activity may play a role in the progression of pathological processes associated with oxidative damage (Papadia et al., 2008). In light of this, unlike GluN2B and GluN2A antagonists which show no protective effects against H_2O_2 cytotoxicity, we expected that a potentiation on synaptic NMDARs, which are mostly GluN2A-containing NMDARs, can boost the intrinsic antioxidant defenses during H₂O₂ stimulation. Similarly, to the NMDA stimulation, Npam43 was evaluated for its potential to protect cells from oxidative stress via 1 hour stimulation H₂O₂ at 600µM (Papadia et al., 2008)

7.2 Npam43 increased CREB phosphorylation that was prevented through GluN2A antagonism in cortical neurons

To determine whether Npam43 triggered the GluN2A-dependent, pro-survival signaling pathway, CREB phosphorylation was assayed in neurons treated with Npam43. Phosphorylation of CREB at the activator-site residue Serine 133 has been established as a reliable index of cell survival pathway activation. (Bonni et al., 1999; Hardingham et al., 2002; Terasaki et al., 2010; Yano et al., 1998; X. M. Zhang & Luo, 2013) To avoid off target effects associated with global NMDAR activation following treatment with NMDA, we, utilized either synaptically evoked bursts of action potentials (Hardingham et al., 2002) or the addition of Npam43 acutely on to neurons and measured the corresponding changes in basal and stimulated pCREB protein levels. Npam43 (10µM) was added to the medium of rat cortical cell cultures for 30 mins, after which the cells were harvested and proteins size-separated and immunoblotted. As a positive control, bicuculline (BIC; 10µM; 2min exposure) -induced action potentials were used to activate NMDARs which increased pCREB levels ~2 fold more than non-treated controls (10 μ M; n =10; 2.16 ± 0.65 ; P < 0.001; to control group) (Figure 7.1a). The addition of acute Npam43 alone to cortical neurons induced a significant elevation of pCREB levels. A ~2.5 fold increase (10µM; n = 10; 2.66 \pm 0.16; P < 0.001; to control group; 30 min exposure) of pCREB levels compared to vehicle-treated cells was observed in the presence of Npam43 (Figure 7.1a). This effect was abolished by applying the GluN1/GluN2A antagonist NVP-AAM077 (0.2µM) suggesting a requirement for GluN2A-containing NMDARs (0.2 μ M; n = 10; 0.719 \pm 0.10; P > 0.05; to control levels). The application of IF (3μ M) alone did not increase pCREB levels (3μ M; n = 10; 1.32 ± 0.17 ; P > 0.05; to control group) however co-application of Npam43 (10µM) with ifenprodril (IF) triggered an increase of pCREB levels (3µM; n = 10; 3.42 ± 0.48 ; P < 0.001; to control group) similar to Npam43 alone, indicating the GluN2B receptor inhibition fails to prevent enhanced pCREB induced by Npam43 (Figure 7.1a). A dose-dependent effect of the drug in relation to the increased pCREB levels was also observed (Figure 7.1b).



Figure 7.1 Npam43 enhancement of CREB phosphorylation in cortical neurons

(a) Immunocytochemical analysis of pCREB on Serine 133 at basal levels in cortical neurons exposed for 5 mins of bicuculline (BiC) (5µM) and 30 mins of Npam43 (10µM) in the presence and absence of NVP-AAM007 (0.2µM) or ifenprodril (3µM) compared to control levels. Quick stimulation of BiC (10µM; n = 10; 2.16 ± 0.65; P < 0.001; to control group) into neurons causes a dramatic increase in pCREB levels. Npam43 (10µM; n = 10; 2.72 ± 0.14; P < 0.001; to control group) independently, increased CREB phosphorylation (pCREB) in cortical neurons and this elevation is blocked by NVP-AAM007 (0.2µM; n = 10; 0.924 ± 0.13; P > 0.05; to control levels) and NVP-AAM007 (0.2µM; n = 10; 0.976 ± 0.18; P > 0.05; to control group) did not elevate

pCREB levels on its own. Whereas, Npam43 (10µM) maintained the enhancement of pCREB in the presence of ifenprodril (3μ M; n = 10; 3.42 ± 0.48 ; P < 0.001; to control group) and the if enprodril alone group ($3\mu M$; n = 10; 1.32 ± 0.17 ; P > 0.05; to control group) did not significantly increase pCREB levels. One-way ANOVA, P < 0.001, F(6, 63) = 13.225. *** P < 0.0010.001; Post- Hoc LSD; bars represent relative mean values \pm s.e.m. normalized to the total CREB protein and normalized to the control (white bar, arbitrarily set as 1). n represents individual experiments from at least 3 separate primary cultures. (b) Dose-dependent increase in pCREB by Npam43. Immunocytochemical analysis of CREB phosphorylation (pCREB) on Serine 133 at basal levels in cortical neurons. Npam43 significantly increased basal pCREB levels in a dose-dependent manner from 5-20 μ M. (5 μ M; n = 6; 1.64 \pm 0.08; P < 0.05; to control group), (10 μ M; n = 6; 2.19 \pm 0.10; P < 0.001; to control group), (15 μ M; n = 6; 2.52 \pm 0.18; P < 0.001; to control group), (20 μ M; n = 6; 2.84 \pm 0.18; P < 0.001; to control group) but not significantly for 0.1 & 1uM (0.1uM; n = 6; 1.02 ± 0.13; P > 0.05; to control group), (1 µM; n = 6; 1.35 \pm 0.07; P > 0.05; to control group). One-way ANOVA, P < 0.001, F(6, 35) = 11.304. *** P < 0.001, **P < 0.01 * P < 0.05; Post- Hoc LSD; bars represent relative mean values \pm s.e.m. normalized to the total CREB protein and normalized to the control (white bar, arbitrarily set as 1). *n* represents individual experiments from at least 3 separate primary cultures.

7.3 Npam43 increased CREB phosphorylation that was prevented through GluN2A antagonism in hippocampal slices

To ascertain whether pCREB levels could also be elevated in mouse hippocampal slices, Npam43 was applied for 30mins either alone or with NVP-AAM007 and the sample was homogenized and probed for pCREB. As seen in rat cortical neurons, Npam43 alone was able to increase pCREB levels (10μ M; n = 6; 2.35 ± 0.21 ; P < 0.001; to control group; 30min exposure) and this enhancement in CREB phosphorylation was again blocked by a GluN2A antagonist (NVP-AAM077) (0.2μ M; n = 6; 0.987 ± 0.28 ; P > 0.05; to control levels) (Figure 7.2). The positive control BIC also showed a dramatic increase in pCREB levels (10μ M; 3.85 ± 0.29 ; P < 0.001; to control group) and this was partially blocked by NVP-AAM007 (0.2μ M; n = 6; 2.33 ± 0.56 ; P > 0.001; to control group) (Figure 7.2). Finally, NVP-AAM077 alone did not change pCREB levels (0.2μ M; n = 6; 0.804 ± 0.18 ; P > 0.05; to control group) (Figure 33). Taken together these data suggest that Npam43 acting through GluN2A-containing NMDARs, is capable of initiating the activation of signaling cascades associated with pro-survival pathways in neurons and hippocampal slices.



Figure 7.2 Npam43 increased pCREB levels in hippocampal slices

(a) Immunocytochemical analysis of CREB phosphorylation (pCREB) on Serine 133 at basal levels in cortical neurons treated with bicuculline (BiC; 10 μ M; 30 min exposure) or Npam43 (10 μ M; 30 min exposure) in the presence and absence of NVP-AAM077 (0.2 μ M). Stimulation with BiC (10 μ M; n = 6; 3.85 \pm 0.29; P < 0.001; to control group) of neurons increased pCREB levels and this effect was reduced by b NVP-AAM007 (0.2 μ M; n = 6; 2.33 \pm 0.56; P < 0.001; to control level). Treatment with Npam43 (10 μ M; n = 6; 2.35 \pm 0.21; P < 0.001; to control group) significantly enhanced pCREB levels which was completely blocked in the presence of NVP-AAM077 (0.2 μ M; n = 6; 0.99 \pm 0.28; P > 0.05; to control levels. NVP-AAM077 (0.2 μ M; n = 6; 0.80 \pm 0.18; P > 0.05; compared to control) alone did not increase pCREB levels. One-way ANOVA, P < 0.001, F(5, 30) = 15.220. *P < 0.001; *Post- Hoc* LSD; bars represent relative mean values \pm SEM. normalized to total CREB protein and normalized to control (white bar, arbitrarily set as 1). *n* represents individual experiments from at least 3 separate primary cultures.

7.4 Npam43 is neuroprotective against NMDA-mediated excitotoxicity in primary neuron cultures

Given our observation of enhanced pCREB in response to Npam43, we hypothesized that NMDAR-dependent excitotoxicity might be reduced by treatment of Npam43. Cortical cultures were treated with Npam43, and assessed for NMDA-induced excitotoxicity after 20-24 h by measuring lactate dehydrogenase (LDH) release (Figure 7.4a). Cells were treated with NMDA $(75\mu M)$ for 1.5 h, after which, the media was exchanged with conditional medium. NMDA treated cells demonstrated a significant (~2 fold) increase in cell death as compared to vehicletreated controls (n = 15; 2.29 \pm 0.13; P < 0.001 to control) (Figure 7.4a); however, co-treatment with NMDA and Npam43 yielded a marked reduction in NMDA-induced excitotoxicity (Figure 7.4a). A dose-dependent neuroprotection was observed where excitotoxicity was significantly decreased at (5-50µM) compared to the NMDA treated condition (5µM; n = 15; 1.79 ± 0.03; P > 0.01; to NMDA group), (10 μ M; n = 15; 1.68 ± 0.10; P < 0.001; to NMDA group), (25 μ M; n =15; 1.54 ± 0.09 ; P < 0.001 to NMDA group), (50µM; n = 15; 1.19 ± 0.03 ; P < 0.001 relative to the NMDA alone group). To confirm that the effect was through GluN2A-mediated processes, NVP-AAM077 (0.2µM), NMDA and Npam43 were applied together which reversed the Npam43-dependent neuroprotection. (0.2µM; n = 15; 1.98 ± 0.08; P = 0.863 to NMDA group). Ifenprodil (3µM) was used as a positive control as inhibition of GluN2B which couples to procell death pathways completely blocked the excitotoxicity from the NMDA insult. Npam43 alone at 50µM was not toxic (Figure 7.4a). These results suggest that application of Npam43 can limit NMDA-induced neurotoxicity through a mechanism requiring GluN2A activation.

7.5 Npam43 protects neurons against oxidative stress in primary neuron cultures

We sought to determine whether the neuroprotective properties of Npam43 could also be observed following NMDA-independent insults. As shown in Figure 7.4b, exposure of cultured cortical neurons to H_2O_2 (600µM, 1h), a potent generator of oxidative stress-inducing reactive oxygen species, significantly increased neuronal death (approximately 4 fold relative to nontreated controls) (Figure 7.4b). Treatment with a selective GluN1/GluN2B antagonist ifenprodril $(3\mu M)$ did not protect against H₂O₂-induced neurotoxicity (Figure 7.4b). Similarly, GluN1/GluN2A antagonists (NVP-AAM077; 0.2µM) and TCN-201 (10µM) failed to protect against H₂O₂ exposure (Figure 7.4b). Given that Npam43 enhances pCREB; a biomarker for cell survival, we hypothesized that activating the cell survival pathway could reduce the H₂O₂induced neurotoxicity. Co-application of Npam43 (1,10 and 25µM) with H₂O₂, significantly reduced H₂O₂-induced neurotoxicity in a dose-dependent manner. Co-application of GluN1/GluN2A antagonists (TCN201 or NVP-AAM077) with Npam43 and H₂O₂ abolished the neuroprotective effects of Npam43, Along with our results demonstrating neuroprotection against NMDA treatment, these data indicate that Npam43 is neuroprotective against both NMDA insults and non-NMDAR-dependent oxidative stress suggesting that upregulation of cell survival pathway signaling by Npam43 is neuroprotective against diverse sources of cellular toxicity.



Figure 7.3 Npam43 protects against NMDA excitotoxicity and H₂O₂ cytotoxicity

(a) LDH assay revealed that NMDA treatment (75µM; 1.5 h) resulted in a significant increase in neuronal death 20 h after treatment (n = 15; 2.29 ± 0.13; P < 0.001 to control), which was rescued by application of ifenprodril (3µM; n = 15, 1.06 ± 0.03; P > 0.001 to control and P < 0.001 to NMDA group). NMDA-induced excitotoxicity was significantly reduced by Npam43 (5, 10, 25, 50µM; co-applied with NMDA and maintained throughout experiments; (5µM; n = 15; 1.79 ± 0.03; P < 0.01; to NMDA group), (10µM; n = 15; 1.68 ± 0.10; P < 0.001; to NMDA

group), (25µM; n = 15; 1.54 ± 0.09; P < 0.001 to NMDA group), (50µM; n = 15; 1.19 ± 0.03; P < 0.001 to NMDA group) and (1µM; n =15; 1.98 ± 0.06; P > 0.05). NVP-AAM0077 (0.2µM; n = 15; 1.98 ± 0.08 ; P = 0.863 to NMDA group) was able to rescue the NMDA-mediated cell death in the presence of Npam43 (10 μ M) (P = 0.901 to NMDA+Npam43 (10 μ M) group). NVP-AAM0077 (0.2µM; n = 15; 1.93 ± 0.10; P > 0.05; to NMDA group) was not reduce the NMDAmediated excitotoxicity and Npam43 at (50 μ M; n = 15; 1.06 \pm 0.03; P > 0.05 compared to control) did not induce toxicity on its own. One-way ANOVA, P < 0.001, F(10, 154) =31.892; Post-hoc LSD; *** P < 0.001 ** P < 0.01; bars represent relative mean values ± SEM. normalized to the control (white bar, arbitrarily set as 1). n represents individual experiments from at least 3 separate primary cultures. (b) LDH assay revealed that H_2O_2 treatment (600 μ M; 1h) resulted in a significant increase in neuronal death 20 h after treatment (n = 10; 3.59 ± 0.16 ; P < 0.001 to control). H₂O₂-induced neurotoxicity was significantly reduced by Npam43 (1, 10, 25μ M; co-applied with H₂O₂ and maintained throughout experiments (1 μ M; n = 10; 2.53 ± 0.16; P < 0.05 to H₂O₂); (10 μ M; n = 10; 1.98 ± 0.11; P < 0.001; to H₂O₂ group); (25 μ M; n = 8; 1.50 ± 0.06 ; P < 0.001; to H₂O₂ group) but not by NVP-AAM007 (0.2µM; n = 8; 3.26 ± 0.19 ; P > 0.05; to H₂O₂ group) nor ifenprodril (3μ M; n = 8; 3.48 ± 0.25 ; P > 0.05; to H₂O₂ group) and nor TCN201 (10 μ M; n = 8; 3.64 ± 0.25; P > 0.05 to H₂O₂ group). NVP-AAM007 (0.2 μ M; n = 8; 3.24 ± 0.30 ; P > 0.05; compared to H₂O₂) or TCN201 (10µM; n = 8; 3.24 ± 0.24 ; P > 0.05; to H₂O₂ group) abolished the neuroprotective effect of Npam43 (10µM), however ifenprodril $(3\mu M; n = 8; 1.92 \pm 0.10; P < 0.001;$ compared to H_2O_2) did not block the neuroprotection from Npam43 (10 μ M). TCN201 (10 μ M; n = 8; 1.01 \pm 0.07; P < 0.001; to control group), ifenprodril $(3\mu M; n = 8; 0.95 \pm 0.02; P < 0.001;$ to control group) and NVP-AAM077 $(0.2\mu M; n = 8; 0.93 \pm$ 0.02; P < 0.001; to control group) did not exhibit any toxic effects on their own. One-way ANOVA, P < 0.001, F(13, 104) = 40.189. *** P < 0.001, * P < 0.05; *Post- Hoc* LSD; bars represent relative mean values \pm SEM. normalized to the control (white bar, arbitrarily set as 1). *n* represents individual experiments from at least 3 separate primary cultures.

Chapter 8: Pharmacology- pharmacokinetics of Npam43 in-vivo

8.1 Introduction

Given the fact that the brain is shielded by the brain-blood barrier (BBB), it is first necessary to confirm that the candidate drug will reach the brain at the therapeutic concentrations tested *in-vitro*. The BBB is a network of capillaries lined by endothelial cells which are made of very tight junctions between the cells (Bauer, Krizbai, Bauer, & Traweger, 2014). This greatly prohibits the entry of molecules into the brain but there are a number of active transport mechanisms for transporting drugs into and out of the brain as well (Banks, 2009; Misra, Ganesh, Shahiwala, & Shah, 2003). Either way, the brain penetration is extremely important for drug efficacy *in-vivo* and majority of drugs do not pass the BBB due to their low hydrophobicity and high blood-plasma binding which prohibits the therapeutic unbound or therapeutic form of the drug to reach the target protein or may arrive at the site at too low concentrations (Banks, 2009; Pajouhesh & Lenz, 2005). Accordingly, we evaluated the ability for Npam43 to cross the BBB by collecting the cerebrospinal fluid from the cisterna magna in a rat and measuring the concentration of the drug from the CSF and serum. The concentration of the drug was quantified by using a high-performance-liquid-chromatography electrochemical detection (HPLC-ECD) to interpret the dose-concentration response and the half-life of the drug. This method was suitable to test Npam43 due to its propensity to oxidize and expel electrons that are electrochemically detected by the sensor. This method is extremely selective and sensitive detection technique in particular to compounds that are sensitive to oxidation or reduction changes.

For *in-vitro* studies it is usually possible to evaluate the compound as long as the compound is soluble in DMSO. However when moving to in-vivo studies for the further development of a new compound it is important to consider the potential clinical route(s) of administration the dosing regimen. Low aqueous solubility is the major problem encountered with formulation development of new chemical entities but these issues can be solved by several techniques; proper formulation mixtures (solvents/excipients), drug modification to improve solubility properties or convert drug into salt/prodrug form. In vitro testing of drug candidates usually leads to an increase of lipophilic compounds with poor solubility especially drugs that are designed to target the brain. Increasing the hydrophobicity of the drug generally increases the chances for it to cross the brain-blood-barrier (BBB) but at the same time decreasing the aqueous solubility profile. Poor solubility itself may lead to poor bioavailability of a potential drug. The growing demand for solubility data in lead phase of drug discovery is answered by a variety of simple solubility assays. One of the easiest ways to detect saturation in a solvent is the turbidity of the solution if precipitation occurs (J. S. Morrison, Nophsker, & Haskell, 2014). The turbidly caused by precipitation of poorly soluble compound can be detected by UV-Vis spectrophotometry. Precipitated particles lead to an increase in UV-absorbance due to light scattering and the precipitation defines the maximum solubility level. In this scheme, poorly soluble compounds are characterized by having solubility less than 10ug/ml, whereas good solubility is defined as solubility higher than 65ug/ml.

8.2 Characterization of the soluble state of Npam43 in water

To evaluate the solubility of Npam43 in water, a turbidity test was performed at different concentrations using UV-Vis spectrophotometery. The concentration range used in this experiment was 0-12500µM. It was determined that the solubility of Npam43 was found to be around 781µM in water, which translates to 267ug/mL. It has a fairly good solubility in water (higher than 65ug/ml) in these concentrations but it was calculated that at these concentrations Npam43 would not be suitable in an in-vivo setting (Figure 8.1). In light of this it was prudent to push Npam43 into a formulation development study to enhance the aqueous solubility of Npam43 at higher concentrations.



Figure 8.1 Turbidity assay of Npam43 in water

Transmittance versus the concentration of Npam43 in water Npam43 solubility in water. Npam43 was soluble at ~781 µM concentration in aqueous solution.

8.3 Formulation of Npam43

Because of its low aqueous solubility, Npam43 needed to be formulated in a vehicle that would be non-toxic, pharmacologically inert, non-reactive to the compound and especially does not affect the function of NMDARs. Pre-formulation of the drug involves the characterization of the drug's physical and chemical properties in order to choose what other ingredients (excipients) should be used in the preparation. Formulation studies take into account different factors such as particle size, polymorphism, pH and solubility. Initially, we believed that Npam43 would need a combination of a solvent, which will dissolve the drug at high concentrations and an excipient to help to keep the drug dissolved in aqueous layer (saline). Initially, we investigated different solvents other than water that could hold very high concentrations of the drug. The solvents used initially were ethanol, methanol, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF). All solvents worked extremely well but two solvents were not suitable to use in the context of targeting NMDARs. Many studies have shown that ethanol and methanol can inhibit the function NMDARs, which may interfere or counteract the positive allosteric modulation effects of Npam43. Moreover, DMF (aprotic) was also considered as poor choice due to its strong ability to cause reactions to occur or breaking bonds under certain conditions. On the other hand, DMSO was known also to have adverse effects invivo particularly at high concentrations. Numerous reports have shown that DMSO at low concentrations was suitable as vehicle, showing no noticeable adverse effects. In light if this, we used DMSO as the initial solvent to dissolve Npam43 at the highest possible concentration. The lowest possible volume of DMSO required to dissolve 1mg of Npam43 was 30µL, which translated to a 77mM stock solution. The next step was to select the appropriate excipient that

will help to prevent the drug from precipitating out of the aqueous solution. A series of commonly used excipients were used to choose the best formulation combination for Npam43. The excipients tested were 2-hydroxypropyl-cyclodextrin, Captisol, propylene glycol (PG), TWEEN 80, and polyoxyl-35 castor oil (cremophor EL). Among these excipients that were tested, polyoxyl-35 castor oil (cremophor EL) was found to the best at preventing Npam43 from precipitating out. Overall, we came to a formulation combination where Npam43 was first dissolved in DMSO (1.2%) initially and then mixed with the combination of saline (0.9%) and cremophor EL (1.6%). This formulation was then used for all subsequent *in-vivo* experiments.

8.4 Toxicity of vehicle

The chosen vehicle (DMSO, cremophor EL, saline) combination was then analyzed for its toxicity profile to eliminate the possibility that such a mixture would not counteract the neuroprotective effect of Npam43. Cortical cultures were treated with the vehicle itself (without Npam43), and assessed for the toxicity levels after 20-24 hours by measuring the LDH levels. The neurons were treated with vehicle at the same volumes used to formulate the drug. Figure 8.2 demonstrates that there was no significant difference between the control (no treatment) cells and the vehicle-treated cells, suggesting that this formulation matrix could be used in-vivo without affecting the neuroprotective properties of Npam43.



Figure 8.2 The vehicle used to dissolve Npam43 for *in-vivo* testing did not exhibit toxicity in primary neurons

LDH assay revealed that the vehicle formulation composed of (v:v 1.2% DMSO, 1.6% cremophor EL, 0.9% saline) did not increase in LDH levels 20 h after treatment (n = 8; 0.23 ± 0.02; P = 0.20) compared to control levels (n = 8; 0.21 ± 0.02). This formulation was used for the *in-vivo* testing based on the volumes described above. Two-tailed Student's t-test; bars represent relative mean values ± SEM.; *n* represents individual experiments from at least 3 separate primary cultures.

8.5 Detection of Npam43 using HPLC-ECD

In order to evaluate the clinical applicability of Npam43, a PK/PD and efficacy study of the compound was conducted *in-vivo*. Initially, a formulation development was implemented to ensure that the drug would be homogenously distributed throughout the body. The solubility of the drug was evaluated using different combinations of solvents and excipients and this yielded a suitable matrix (Formulation section of methods) to use in-vivo. This vehicle control was used in a LDH assay to ascertain whether the vehicle itself was toxic and the results showed that there was no noticeable cytotoxicity using the same formulation described in the methods (Figure 8.2). Subsequently, an *in-vivo* pharmacological profile of Npam43 was evaluated using Sprague Dawley mature (~300g) rats. Npam43 was injected intravenously (i.v) at different doses ranging from 0.5-5mg/kg and cerebrospinal fluid (CSF) and serum samples were extracted and analyzed using a high performance liquid chromatography electrochemical detection assay (HPLC-ECD). Npam43 was suitable to be tested using this analytical technique due to its ability to be oxidized in position 18 (Figure 6.3a) (Figure 8.3a) and this resulted in a limit of detection (LOD) of > 0.8μ M. The calibration of Npam43, (Figure 8.3b) shows a linear regression with the respect to electrical current and the concentration of the compound at retention time (T_R) of 3.33 min.



Figure 8.3 Npam43 was detectable using an HPLC-ECD and the response was concentration-dependent

(a) Chromatograph of Npam43 demonstrating its calibration standards from 0.76-50 μ M. Calibration of Npam43 in water showing a retention time 3.33 min and a limit of detection (LOD) > 0.76 μ M. (b) The linear regression with respect to area under curve (AUC) versus the concentration of Npam43.

8.6 Npam43 crosses the BBB and has a ~3 hours half-life via an IV-injection in mature rats

Initially, a dose dependent profile of Npam43 was undertaken to establish a dosing regimen for subsequent in-vivo experiments. The retention time of Npam43 in CSF was determined by adding the drug into blank CSF and comparing this to a rat CSF sample extracted 1 h post i.v. injection. Retention times were found to be identical (Figure 8.4a). The pharmacokinetic analysis of Npam43 following intravenous administration demonstrated that it was effective at penetrating the blood-brain-barrier (BBB) and had a moderate metabolic stability in the CSF and serum. At a 5mg/kg dose of Npam43 the maximum CSF drug concentration was established at approximately $108 \pm 7.8 \ \mu\text{M}$, 2.5 mg/kg was $47.7 \pm 6.8 \ \mu\text{M}$, 1mg/kg was 22.9 \pm 4.0 μ M and 0.5mg/kg was at 12.0 \pm 1.7 μ M (Figure 8.4b & Figure 8.5a). A linear relationship between the dose injected and the final concentration in the CSF was observed 1 h after injection (Figure 8.4c), suggesting its penetration through the BBB was efficient. To exclude the possibility of blood contamination in the CSF, Npam43 (1mg/kg) was co-injected with penicillin G (BBB penetration negative) and 1 hour after injection the CSF and serum were extracted. It was observed that amoxicillin was not detectable in the CSF (Figure 8.6a) suggesting that the presence of Npam43 in the CSF (10mg/L; 1mg/kg) was due to the penetration through the BBB and the quantity was comparable to what was seen previously in the doseresponse *in-vivo* (Figure 8.5a). More importantly, the half-life of Npam43 was estimated as 2.95 \pm 0.6 h in the CSF which was similar to the decay in serum (Figure 8.5b). Based on the *in-vitro* data obtained from primary neurons the therapeutic dose of Npam43 should be between 1-25µM and therefore the maximum CSF concentration should fall within this range. Based on the

maximum CSF concentration, the 1mg/kg dose was predicted to be the optimum dose, reaching approximately $22.9 \pm 4.0 \mu$ M levels. It is expected that the CSF concentration of the drug should remain within the predicted therapeutic window for at least 8 hours when delivered intravenously. Initial toxicity observations demonstrated no systemic toxicity for doses up to 10mg/kg with no observable negative behavioral affects in rats.



Figure 8.4 Npam43 detection in rat CSF (rCSF) using HPLC-ECD. Dose responsive effect of Npam43 post-1 hour extraction of rCSF

(a) Chromatograph demonstrating the detection of blank (water) (light green), rCSF blank (blue), Npam43 spiked in rCSF (purple), and post-1 hour extraction of rCSF of the i.v. injected Npam43 (dark green). Chromatograph showing Npam43 spiked in rCSF had the same retention time as the post-1 hour extraction of rCSF of the i.v. injected Npam43 at $T_R = 3.38$. The rCSF blank and 184 the blank water had no noticeable peak at this T_R . (b) Representative chromatograph of the doseresponse effect of Npam43 in rat CSF 1 h after i.v. injection. (c) Correlation between the dose injected and the concentration found in the CSF 1 h after injection. The correlation factor was R^2 = 0.98 reflecting the linear relationship between the dose-concentration curve.



Figure 8.5 Npam43 effectively crosses the BBB triggering the pro-survival pathway *in-vivo* (a) Dose-dependent presence of Npam43 versus its concentration in the CSF; cerebrospinal fluid taken 1 h after intravenous (i.v.) injection. Npam43 (0.5mg/kg, n = 6, $5.17 \pm 0.66 mg/L$), (1mg/kg; n = 6; $8.84 \pm 1.5 mg/L$), (2.5mg/kg; n = 6; $20.6 \pm 2.8 mg/L$), (5mg/kg; n = 6; $46.9 \pm 3.1 mg/L$) was concentration-dependently present in the CSF analyzed the HPLC-ECD. (b) Npam43 (1mg/kg; i.v.) was sub-administered and cortical and hippocampal tissue was collected (post-injection 2 h after) and pCREB/CREB was probed using immunocytochemical analysis. Npam43 caused a significant elevation in pCREB levels in the hippocampus (1mg/kg; n = 6; 2.05 ± 0.12 ; P < 0.001; normalized to the hippocampal control group) and in the cortical tissues (1mg/kg; n = 6; 2.50 ± 0.36 ; P < 0.001; normalized to cortical control). Two-tailed Student's t-test, *** P < 185

0.001; bars represent relative mean values \pm s.e.m. normalized to the total CREB protein (tCREB) and normalized to the control (white bar). *n* represents individual experiments from 6 separate rats.



Figure 8.6 Npam43 passes the BBB and exhibits a ~3 hours half-life

(a) The co-injection of Npam43 (1mg/kg; 1 h) and a BBB penetration negative antibiotic (amoxicillin) were used to determine if the presence of Npam43 in CSF was due to its ability to penetrate the BBB or through blood contamination. Npam43 reached levels of 10mg/L in the CSF and 12mg/L in the serum but there were no detectable levels of amoxicillin in the CSF suggesting contamination was most likely not a factor. (b) After Npam43 was injected i.v. at a dose of 1mg/kg, rat CSF and rat serum were extracted at different time points (0.5, 1, 2, 4, 8 and 12 h) and the samples were analyzed by HPLC-ECD. The half-life of the Npam43 in both serum and CSF was approximately 177 min and the reached a peak concentration of 18 and 12 mg/L respectively.

Chapter 9: Npam43 protects neurons against ischemic injuries in a mouse model of focal stroke *in-vivo*

9.1 Introduction

As a direct test of the therapeutic potential of Npam43, we employed an *in-vivo* model of stroke to see whether it can protect neurons against ischemic damage. As the project described here pertains to a mechanism to triggering the pro-survival pathways during an ischemic event, we thought that a transient model of middle cerebral artery occlusion (MCAo) would be a suitable model. The scientific reason for using this particular model was to mimic the conditions observed in human ischemic strokes as much as possible (Shyu, Lin, Chiang, et al., 2008; Shyu, Liu, et al., 2008; Sicard & Fisher, 2009; S. Zhang et al., 2013).

The MCAo represents the most common preclinical model for the evaluation of human ischemic stroke because it involves occlusion of the same artery (Traystman, 2003). This type of model also takes into account reperfusion of the blood, which is known to be representative in most ischemic strokes (Engel, Kolodziej, Dirnagl, & Prinz, 2011). With respect to the occlusion, we were inclined to use the distal MCAo model rather than the proximal MCAo model because the proximal model causes a large ischemic infarcts which include the striatum, cortex and thalamus/hypothalamus (Shyu, Lin, Yen, et al., 2008; Shyu, Liu, et al., 2008). This disadvantage of this type of damage is the fact that ischemia in the hypothalamus produces a hypothermic response in rats, which does not occur in humans and thus does not mimic the conditions seen in humans (Carmichael, 2005). A proximal MCAo is accomplished by inserting a suture into the

carotid artery and then blocking the MCA via a cranial suture at the junction point (Shyu, Lin, Yen, et al., 2008; Shyu, Liu, et al., 2008). On the other hand, the distal approach produces much smaller ischemic infracts, which include an ischemic core in the frontal and parietal cortex and a small region of dorsolateral striatum (Carmichael, 2005). The distal MCAo can be described as a three-vessel occlusion model because it includes an occlusion of the MCA on the surface of the brain above the rhinal fissure and the bilateral common carotid arteries (Shyu, Lin, Yen, et al., 2008; Shyu, Liu, et al., 2008). The occlusion process of the distal MCA can be achieved via a suture ligation (Tajiri et al., 2013). This method encompasses the ligation of the MCA with a square knot using a nylon suture and shown to be quite reproducible (Shyu, Lin, Yen, et al., 2008; Shyu, Liu, et al., 2008).

Overall, given the advantages and the disadvantages of the methods described above, we were inclined to use the suture ligation paradigm pertaining to the three-vessel ligation distal MCAo in rats to test whether Npam43 could promote the pro-survival mechanisms necessary to protect cells from the ischemic insult. Based on the pharmacology of the drug, we chose to use the intravenous route of administration because the pharmacokinetics of the drug through an intravenous a single injection clearly demonstrated that the drug had a 3 h half-life and passed the BBB efficiently.

9.2 Systemic application of Npam43 reduces neuronal damage in a MCAo model of focal ischemia in rat

Having established BBB permeability of Npam43, we next tested whether the Npam43 could facilitate neuroprotection following stroke *in-vivo*. Initially, we investigated the possibility that Npam43 in the brain could promote the cell-survival signaling pathway by enhancing phosphorylation of nuclear CREB in-vivo. Hippocampal and cortical brain sections were harvested 2 h post-treatment via i.v. injection of Npam43 (1mg/kg) and samples were probed for pCREB and tCREB levels via immunoblotting. Consistent with *in-vitro* assays, pCREB levels were elevated (2-2.5 fold increase) (Figure 8.5b). We next attempted to extrapolate these results to a well characterized, in-vivo rat stroke model, which involves 3-vessel ligation (bilateral common carotid arteries and right middle cerebral artery (MCA) (S. T. Chen, Hsu, Hogan, Maricq, & Balentine, 1986; Shyu, Lin, Chiang, et al., 2008). We tested whether the Npam43 modulation of GluN2A-containing NMDARs conferred neuroprotection in ischemic stroke 24 h post-ictus. As shown in Figure 9.1a, animals were subjected to a 90 min transient ischemic insult, 3.5 h after the stroke onset (i.e. 2 h following ischemia reperfusion), Npam43 at various concentrations (0.1, 0.5, 1 and 2.5mg/kg) or vehicle/saline, was administered intravenously in a rat model of middle cerebral artery occlusion (MCAo). In the contralateral side or infract area (shown in white) (Figure 9.1b), measurements on 2,3,5-Triphenyltetrazolium chloride (TTC) stained brain sections indicated a large infraction in the cortex that extended into the hippocampus region (Figure 9.1b). The ipsilateral side was used as control (shown in red). TTC staining in the Npam43-treated slices at 0.5, 1, and 2.5mg/kg doses, revealed a significant decrease in the infarct volume at 24 h post ischemia compared with the vehicle/saline group

[Kruskal-Wallis one-way analysis on ranks, [Npam43 (0.1mg/kg) $159 \pm 14 \text{ mm}^3 \text{ H}_5 = 2.156 \text{ P} > 0.05 n = 8$; (0.5mg/kg) $123 \pm 10 \text{ mm}^3 \text{ H}_5 = 4.773 \text{ P} = < 0.05, n = 8$; (1mg/kg) $88 \pm 19 \text{ mm}^3 \text{ H}_5 = 12.645 \text{ P} = < 0.001 n = 8$; (2.5mg/kg) $103 \pm 19 \text{ mm}^3 \text{ H}_5 = 12.343 \text{ P} = < 0.001 n = 8 \text{ versus}$ vehicle/saline control, $169 \pm 12 \text{ mm}^3$, n = 10] (Figure 9.1c). A reduction of the infarct area was observed at 1mg/kg, showing reduced lesion area in the hippocampus and cortex regions (Figure 9.1c). These results indicate that histological damage as measured by TTC is dose-dependently diminished in animals injected with 0.5, 1 and 2.5mg/kg of Npam43 but not the 0.1mg/kg dose in this reperfusion model of MCAo (Figure 9.1d). Consistent with the *in-vitro* findings in neurons, the *in-vivo* data indicated that Npam43 treatment protects against ischemia induced neuronal loss.



Figure 9.1 Npam43 reduces the infract volume of ischemic brain in-vivo

(a) Timeline of coronal brain sections collection for the analysis of infract areas in the rat MCAo model *in-vivo* using TTC; 2, 3, 5-triphenyltetrazolium chloride staining. (b) Sample images of TTC stained-brain sections collected at 48 h after ischemia onset with no treatment (control/vehicle) (c) Sample images of TTC stained-brain sections treated with 0, 0.1, 0.5, 1, 2.5mg/kg doses of Npam43 (3.5 hours after stroke induction) and collected 48 h after ischemia onset. (d) Bar graph quantification summarizing the results of the different doses of Npam43 (0.1mg/kg; n = 8; 159 ± 14 mm³; P > 0.05; to control group), (0.5mg/kg; n = 8; 123 ± 10 mm³; P < 0.05; to control group), (1mg/kg; n = 8; 88 ± 19 mm³; P < 0.001; to control group), (2.5mg/kg; n = 8; 103 ± 19 mm³; P < 0.001; compared to control group) given 3.5 hours after stroke-onset 191

significantly reduced infarct areas in comparison with the saline/vehicle control (n = 10; 169 ± 12 mm³). Mann-Whitney U test on ranks, U = 0.000 *P < 0.05 , *** P < 0.001; bars represent relative mean values ± SEM. n represents individual experiments from at least 8 separate rats.

9.3 Npam43 reduces infarct volume post-ictus as evaluated by 7 day magnetic nuclear resonance (MRI) imaging *in-vivo*

Based on the pharmacological profile of the drug and the efficacy observed 24 h after ischemia onset, the 1mg/kg dose of Npam43 was consequently used for a 7 day long-term morphological evaluation *in-vivo*. Due to the pharmacological profile of the drug and in an attempt to enhance its efficacy, we gave two additional doses of each drug at days 2 and 3 following stroke (Figure 9.2a). The neuroprotective effects of Npam43 versus vehicle/saline groups were then assessed using MRI 7 days after stroke onset (Figure 9.2a). Similarly, to the histological TTC staining, we observed the development of an infraction area (shown in white) (Figure 9.2b), localized to the hippocampus and adjacent cortical regions. As shown in Figure 43c, MRI scanning revealed that rats treated with Npam43, had dramatically reduced brain infarct volumes compared with vehicle/saline-treated rats [Mann-Whitney U test on ranks, U = 0.000 P = < 0.001; vehicle/saline control, $170 \pm 20 \text{ mm}^3$, n = 8; Npam43 81 \pm 19 mm³, n = 8] (Figure 9.2d). These results demonstrate that Npam43 is an effective neuroprotective agent *in-vivo*, and reduces infarct volume 7 days post-ictus.



Figure 9.2 Post-stoke treatment of Npam43 promotes a long-lasting reduction of neuronal damage and improves behavioral performances after a focal ischemic brain insult

(a) Timeline of coronal brain tissue collection for the analysis of infract volumes in the intact rat MCAo model *in-vivo* using MRI, magnetic resonance imaging. (b) Representative coronal scanning image of MRI taken at day 7 from vehicle-treated control stroke rat showed a well-defined infarct (white) area. (c) Representative MRI scanning images from control/vehicle or Npam43 (1mg/kg) from rostral (left) to caudal (right) levels were taken at day 7 from rats subjected to a 90 min transient focal ischemic insults. (d) Bar graph quantification summarizing the significant reduction in infract volume of Npam43 treatment (1mg/kg; $81 \pm 19 \text{ mm}^3$; P < 193
0.001) versus the control/vehicle group (n = 8; 175 ± 20 mm³) given 3.5 hours after stroke onset. Two-tailed Student's t-test, *P < 0.001; bars represent relative mean values ± SD.; *n* represents 8 rats from each the groups.

9.4 Post-stroke treatment with Npam43 improves motor behavior performance after focal ischemic brain insult *in-vivo*

To determine if Npam43 can preserve neuronal function at a behavioral level following transient MCAo, we evaluated post ictus functional recovery in both the presence and absence of Npam43 treatment. Post-stroke administration of Npam43 (1mg/kg) treatment followed by monitoring of neurobehavioral assays were used to determine the efficacy of Npam43 in functional rescue. Three locomotor activity (sensorimotor) modalities, vertical activity (the total number of beam interruptions that occurred in the vertical sensor), number of vertical movements (number of animal rears) and vertical movement time (the amount of time, in seconds, the animal rears) were measured. Over the 28 day recovery period following stroke, the vertical activity test revealed that rats treated with Npam43 exhibited a faster recovery in the locomotion behavior than those treated with saline/vehicle control (Figure 9.3a). By the fourth week, rats treated with Npam43 had largely recovered from their locomotion deficit [Mann-Whitney U test on ranks, $U = 0.000 P = \langle 0.001; vehicle/saline control, 1000 \pm 144 times, n = 8;$ Npam43 1900 \pm 188 times, n = 8]. Similarly, the vertical movement time as monitored in a computerized locomotor chamber showed that Npam43 treated rats displayed a faster and more pronounced improvement in locomotor activity over the 28 day recovery period in comparison with the saline/vehicle-treated rats (Figure 9.3b). By the end of the fourth week, a one-way

ANOVA analysis indicated a significant difference among groups which revealed that vertical movement time of Npam43-treated rats was significantly longer than that of saline/vehicle-treated rats [Mann-Whitney U test on ranks, U=0.000, P = < 0.001 vehicle/saline control, 222 ± 11 s, n = 8; Npam43, 400 ± 22 s, n = 8, p < 0.001]. Furthermore, results of the number of vertical movement test also revealed a more rapid recovery in rats treated with Npam43 at 28 d after stroke onset (Figure 9.3c) [Mann-Whitney U test on ranks, U= 0.000, P = < 0.001, vehicle/saline, 138 ± 22 times ; n = 8; Npam43 250 ± 22 times, n = 8]. Npam43 treatment, posticus, dramatically enhanced the number of vertical movements during recovery compared with the saline/vehicle treatment. Together, these results suggest that along with preventing cell loss following stroke, Npam43 facilitates recovery of functional neurological circuits damaged by ischemic insults.



Figure 9.3 Post-stoke treatment of Npam43 improves behavioral performances after a focal ischemic brain insult

(a) The 28 day, recovery period following stroke insult, showing the vertical activity test performance. Npam43-treated rats showed a faster recovery in the locomotion behavior (1mg/kg; 28^{th} day; n = 8; 1900 ± 188 times; P < 0.001) versus the control/vehicle group (28^{th} day; n = 8; 1000 ± 144 times). (b) At the 4th week recovery period, Npam43-treated rats were showing a dramatic enhancement in locomotor activity (1mg/kg; 28^{th} day; n = 8; 400 ± 22 s; P < 0.001) compared to vehicle/control rats (28^{th} day; n = 8; 222 ± 11 s). (c) The 28 day, recovery period following stroke insult, showing the vertical movement test performance. Npam43-treated rats

showed a faster recovery in the locomotor behavior $(1\text{mg/kg}; 28^{\text{th}} \text{ day}; n = 8; 250 \pm 22 \text{ times}; P < 0.001)$ versus the control/vehicle group $(28^{\text{th}} \text{ day}; n = 8; 138 \pm 22 \text{ times})$. Post-hoc analysis compares treatment groups with control/vehicle group after significant one-way ANOVA or Mann-Whitney U test on ranks, and significance is defined as *P < 0.001; line curves represent relative mean values \pm SD.; *n* represents 8 rats from each the groups.

Chapter 10: Discussion

10.1 Background

Recent evidence suggests that NMDARs exert differential functions in mediating synaptic plasticity and cell survival depending on the presence of GluN2 subunits (T. Bliss & Schoepfer, 2004; Seeburg, 1993b; Seeburg et al., 1995). In general, GluN2A-containing NMDARs promote the induction of long-term potentiation (LTP) important for learning and memory (L. Liu et al., 2004). Additionally, GluN2A-containing receptors promote neuronal survival, protecting neurons against excitotoxic injuries, whereas GluN2B-containing NMDARs contribute to cell death signaling cascades, thereby mediating excitotoxic neuronal injuries (Hardingham et al., 2002; Lai, Shyu, & Wang, 2011; Y. Liu et al., 2007; Yano et al., 1998). In general, GluN2A-containing NMDARs promotes the induction of long-term potentiation (LTP) important for learning and memory, and neuronal survival, protecting neurons against excitotoxic injuries; whereas GluN2B-containing NMDARs activates cell death signaling, thereby mediating excitotoxic neuronal injuries (Hardingham & Bading, 2002, 2010; Hardingham et al., 2002; Y. Liu et al., 2007; Wyllie et al., 2013). The dual, opposing outcomes of NMDARs in neuronal survival and death may contribute to failure of non-specific NMDAR antagonists to succeed in clinical trials (Albensi et al., 2004; Corbett & Nurse, 1998; Gladstone et al., 2002; Ikonomidou & Turski, 2002). Conventional NMDAR antagonists target the surface receptor, essentially blocking both neuronal survival-signaling and death-signaling pathways, limiting receptor function which contributes to off-target effects. The suspected detrimental effect of NMDAR (GluN2B-mediated) over-activation in mediating stroke brain damage has been confirmed along with the previously unappreciated beneficial action of NMDAR GluN2A-

mediated cell survival (Y. Liu et al, 2007). In support of the later finding, the data presented here provide multiple lines of evidence confirming that enhancement of GluN2A-containing NMDARs protects neurons against ischemic insults by specifically promoting neuronal survival mechanisms, with the added advantage of having fewer side effects and a wider therapeutic window. Data implicating NMDAR (GluN2B-mediated) over-activation as a common pathology contributing to brain damage associated with stroke may have overshadowed the potential utility of more selective NMDAR modulators as viable alternatives for stroke treatment (Hardingham et al., 2002; Shu et al., 2014). Progress in this strategy has been hampered by the lack of tools to specifically activate, GluN2A-NMDAR-mediated, pro-survival signaling. In light of this, we have screened thousands of compounds that could specifically modulate GluN2A-containing NMDARs and promote pro-survival action for the reduction of ischemic brain damage. Through this study, I have discovered and developed a series of low μ M (high nM) allosteric positive modulators (PAMs) that are specific for the GluN2A-containing NMDARs.

This series of PAMs have been discovered by a series of steps that included virtual screening using chemical libraries, target identification, hit identification, stability studies, biochemical evaluation *in-vitro*, analog testing, rational drug design, target validation, chemical synthesis, structure-activity relationships, potency and selectivity studies, formulation studies, safety pharmacology, pharmacokinetic profiling; half-life, BBB penetration, and *in-vivo* efficacy (Figure 10.1).



Figure 10.1 Schematic representation of the drug discovery pipeline used for the screening process, hit selection, and compound validation *in-vitro* and *in-vivo*

Stage I was focused on creating a homology model of GluN1/GluN2A and eventual virtual screen of ~200K from two chemical libraries; Stage II entailed the selection process of the insilico hits (~200 entities) based on molecular weight, chemical structure, ease of synthesis, brain-blood-barrier (BBB) penetration and toxicity characteristics, docking pose and score; *Stage III* involved the characterization of these top hits in whole-cell electrophysiology recordings cells expressing GluN1/GluN2A or using *HEK293* GluN1/GluN2B subunits and hippocampal/cortical primary neurons; Stage IV was dedicated on finding the binding site via site-directed mutagenesis for the top hit modulators found in the electrophysiological recordings in stage III; In Stage V entailed medicinal chemistry guided by rational drug design using the binding site information obtained from stage IV plus an analog search of the top hits generated

from the electrophysiology recording from *stage III*; *Stage VI* involved the testing of these analogs and medicinal chemistry prototypes in *HEK293* cells and primary neurons using electrophysiology and the evaluation of drug potency. Stage *VII* evaluated drug selectivity towards GluN1/GluN2A, aqueous solubility, toxicity, stability, CREB phosphorylation, and protection profile from NMDA excitotoxicity; *Stage VIII* entailed to selection of Npam43 as a lead candidate and was further tested in hippocampal slices for pCREB phosphorylation and electrophysiological recordings; *Stage IX's* aim was to evaluate the pharmacological profile of Npam43 which includes BBB in vivo penetration and half-life; *Stage X* involved the efficacy of Npam43 in vivo in a rat model of focal ischemia MCAo.

The aim of the project and my own personal goal was to proceed through a step-by-step approach to drug discovery that avoided skipping valuable steps in the pipeline (Figure 10.1). This philosophy helped to ensure robustness in the results while limiting future problems related to efficacy, selectivity and stability *in-vivo*. The resulting drugs were developed to be pharmacologically viable *in-vivo* and shown to represent a novel effective stroke treatment. Not only did they dramatically reduces brain infraction but also significantly improved the functional recovery of sensorimotor circuits in rats subjected to ischemia. The intervention remained effective 3.5 hours after the stroke insult and it is interestingly a longer time point than the time window established for intravenous injection of tPA (~3 hours) treatment. Moreover, this novel intervention causes no obvious side effects, as it only enhances the function of GluN2A-containing NMDARs and does not interfere or inhibit normal functioning of the receptors or other proteins.

10.2 Targeting GluN2A-containing NMDARs

A lot of effort (work and time) was spent designing and developing a lead compound that was highly potent and selective towards GluN2A-containing NMDARs. This research included a multitude of techniques including molecular modeling, *in-silico* screening, electrophysiology recordings (*in-vitro*, *ex-vivo*), chemistry, NMR, high-performance liquid chromatography (HPLC), mass spectrometry, biochemical analysis, pharmacological profiling, and *in-vivo* biological techniques, which were all necessary procedures to attain the best possible candidate for a new stroke treatment.

In the initial stage (*stage I*) of the project a 3D crystal structure model of the GluN1/GluN2A NMDAR was generated from the resolved crystal structure of the GluN1/GluN2B NMDAR. We probed the modelled structure of the GluN1/GluN2A NMDAR for possible binding sites that could endow a possible modulative effect on the receptor. The whole protein complex was initially probed to identify a suitable druggable pocket that could evoke an allosteric movement during channel activation. Past experiences on targeting multimeric complexes has revealed that targeting interfaces in between two subunits have shown to be beneficial at causing allosteric modulation effects (Axerio-Cilies et al., 2012; Cukuroglu, Engin, Gursoy, & Keskin, 2014; Zoraghi et al., 2011). Applying the same concept to the GluN1/GluN2A protein complex, we thought that targeting the interface in between the GluN1 and the GluN2A subunits was more likely to cause a conformational changes required for the receptor to be allosterically modulated. The selection criterion for the binding pocket that was imposed was as follows: 1) *the site is located far from the glutamate and glycine binding sites*;

therefore reducing potential dependency issues. This manifestation was observed for TCN201, a newly discovered selective GluN2A antagonist that targets the LBD near the glutamate and glycine binding sites. It was shown that the NAM loses significant efficacy as the concentration of glycine increases (Hansen, et al., 2012). This negative influence of glycine to the NAM was observed as low as 3µM and when glycine concentrations reach 300µM; the NAM activity is completely lost (Hansen et al., 2012). This phenomenon deterred the idea of targeting the LBD since glutamate concentrations have been reported as high as ~200µM and glycine concentrations can peak to ~20µM during ischemic stroke conditions and thus might render the drug less effective (Castillo, Davalos, & Noya, 1997). 2) druggable pocket; sites need to have good combination of hydrophobic and hydrophilic residues which would allow a ligand to bind with low µM to high nM affinity (Patschull, Gooptu, Ashford, Daviter, & Nobeli, 2012). Induced-fit docking using the model of GluN1/GluN2A NMDAR suggested that compounds within the pre-filtered database were capable of favorably binding to the pocket with high affinity and thus advocated this site to be druggable using our compound library. Reaching such high affinities is thought to be achieved by targeting sites that are not surface exposed but rather embedded within the protein ie. Interfaces or agonist binding sites which in turn can significantly decrease entropy costs of the ligand when bound to the protein and reflect positively to binding efficiency 3) Located between the interface of GluN1 and GluN2A; interfaces between two interlocking subunits are more likely to cause conformational changes, leading to functional alteration and modulation effects of the receptor (Axerio-Cilies et al., 2012; Cukuroglu et al., 2014; Zoraghi et al., 2011). 4) Extracellular NTD can mediate modulation effects that can be transmitted to the glutamate and glycine sites at the LBD. Past crystallization studies of the GluN1/GluN2B receptor suggest that the NTD during activation undergoes large conformational

changes from the R1 & R2 domain lobes of the NTD to the domains of the LBD, which could then give a possible negative or positive modulative effect on the receptor. 5) *There are more structural differences in the NTD than the LBD*. The extracellular NTDs of GluN2A and GluN2B share a 62% identity and 80% similarity whereas the LBD is highly conserved with an 86% identity and 93% similarity. Achieving drug selectivity will more likely be achieved through either differences in their primary sequences or subtle structural differences arising from the structural assembly of the different subunits of GluN1 and GluN2. The latter would be more evident in the interfaces as these are the main location of where the subunits interlock and directly bind. This identified site was used as a model for the virtual screening of 200K from our pre-filtered chemical library.

In *stage II* a selection process was employed to capture the best suitable candidates that had several characteristics including: 1) high-docking score, 2) binding position (pose) in relation to the nearby residues 3) ease of synthesis 4) brain-blood-barrier (BBB) penetration and 5) toxic and unstable moieties within their structure. Based on this analysis, a collection of ~200 entities were chosen to test via whole-cell patch clamp electrophysiological recordings to identify a potent and selective GluN1/GluN2A NMDAR PAMs.

In *stage III* all ~200 compounds were initially tested using *HEK293* cells expressing either GluN1/GluN2A or GluN1/GluN2B NMDARs. "Positive" hit compounds were then assayed with hippocampal/cortical neurons in the absence and presence of subunit selective antagonists for the NMDAR. One particular "hit" compound Npam02 was identified very early on as low potency (100 μ M; ~40% potentiation compared to basal levels) selective PAM for the GluN1/GluN2A NMDAR in 1) *HEK293* cells expressing GluN1/GluN2A 2) *HEK293* cells expressing GluN1/GluN2B NMDARs, 3) wild-type cortical neurons and 4) GluN2B-lacking mice cortical neurons. Its selectivity in wild-type and GluN2B-lacking cortical mice neurons were evaluated using GluN2A antagonist (NVP-AAM007; NVP) and GluN2B antagonist (ifenprodril; IF) to isolate the subtype specific NMDAR currents. Npam02 had shown a robust potentiation effect on NMDAR currents and selectivity towards the GluN2A subtype over the GluN2B form in all the *in-vitro* models. Moreover, since Npam02 showed no significant potentiation effects in the GluN2B-lacking cortical neurons, it strongly suggested that Npam02 was an early lead compound for which it could be exploited as a probe and could be potentially developed into a potent analog against GluN1/GluN2A NMDARs.

In *stage IV*, before a full blown medicinal chemistry effort was done, a site-directed mutagenesis (point mutations) against the predicted site was implemented to confirm if Npam02 was binding to the predicted binding pocket. Two residues were selected initially based on their propensity to interact directly to the ligand; Gln₁₁₁ and Phe₁₇₇. Gln₁₁₁ interacted directly with Npam02 through hydrogen bonding (H-bond) mechanisms and Phe₁₇₇ strongly interacted via hydrophobic contacts primarily through the aryl rings of Npam02 and Phe₁₇₇. The Gln₁₁₁Ala and Phe₁₇₇Ser conversions were expected to cause a loss of those H-bonding interactions (Gln \rightarrow Ala; polar to hydrophobic) and hydrophobic van-der-Waals contacts (Phe \rightarrow Ser; hydrophobic to polar). Both mutations independently (Gln₁₁₁Ala and Phe₁₇₇Ser) had significantly reduced the binding of Npam02 on the GluN1/GluN2A NMDAR complex suggesting that the interface of the GluN1 and GluN2A subunits in NTD may be sufficient to cause a functional alteration and in turn cause a positive modulative effect. Based on Npam02 selectivity towards GluN2A-

containing NMDARs, we believed that Npam02 could be used as a proof-of-concept (POC) lead compound to further develop it into a potent GluN1/GluN2A NMDAR PAM.

In stage V, a full medicinal chemistry effort and analog search was done based on Npam02 to generate structure-activity relationships (SARs) against the proposed binding pocket on the GluN1/GluN2A NMDAR. This will allow a clear understanding of the chemical features that are deemed responsible for potency and selectivity. Through electrophysiological testing, a subclass of methylated aryl derivatives of Npam02 were shown to be selective towards GluN1/GluN2A NMDAR, suggesting that the methyl group located in (position 21 in Figure 4.4 and Figure 10.2) was the main contributing factor for the preferential binding to GluN1/GluN2A subtype. It has been shown that the de-methylated versions for this sub-class of compounds were deemed non-specific to the two subtypes of NMDARs. In light of this, analogs were chemically synthesized with the intention to maintain the methyl functional group or a similar hydrophobic substituent to differentiate the potentiation effects from GluN2A and the GluN2B subunit forms. The SARs were analyzed based on nearly ~100 compounds to evaluate the functional groups that were responsible for the drug's potency. Several medicinal chemistry efforts afforded three analogs of Npam02 (Npam49, Npam50, and Npam43) which were classified as being 1000 times more potent than Npam02.



Figure 10.2 Lead optimization from a high μ M hit compound (Npam02) to a high nanomolar lead compounds

Red functional groups are chemically attached to the Npam02 scaffold. ** denotes functional group major contributor to its selectivity towards GluN1/GluN2A. Red functional groups were chemically attached to the Npam02 scaffold and generated the three most potent NMDA modulators.

In *stage VI and VII*, amongst the three top lead compounds, Npam43 was selected as our official lead compound because of its superior water solubility and therefore was scrutinized extensively through several biological assays to ascertain its effectiveness in the neuroprotective assays *in-vitro* assays and its *in-vivo* potential as a neuroprotective therapy against a model of ischemic stroke. The present research revealed that the chemically synthesized Npam43 specifically enhanced GluN2A-containing NMDARs function. The specific enhancement was evaluated by using the combination of Npam43 and GluN2A or GluN2B specific antagonists

with electrophysiological recordings in cortical neurons and in hippocampal slices. Indeed, Npam43 modulation effect was not affected with GluN2B antagonist (ifenprodril) but was blocked by using a GluN2A specific antagonist (NVP-AAM0077 or TCN201), demonstrating its effect was specific on GluN2A-containing NMDARs.

In *stage VIII*, the drug showed it could enhance the induction of LTP in hippocampal slices, suggesting that it may promote learning and memory processes. This also strengthens the evidence that Npam43 specifically modulates GluN2A-containing NMDARs since numerous reports have already demonstrated that GluN2A-containing NMDARs mediates the induction of LTP (L. Liu et al., 2004). Neural plasticity is the capacity of the brain to grow new neuronal connections, attain new functions, and adapt to impairments (Takeuchi & Izumi, 2015). These processes are extremely important for motor recovery after stroke (Takeuchi & Izumi, 2015). Therefore, this kind of treatment may represent a novel beneficial action during the stroke rehabilitation period, where the synaptic strength could be compromised(Takeuchi & Izumi, 2015). Such a treatment may help to regenerate those connections and help to improve learning and memory, encode new information and improve motor recovery (Takeuchi & Izumi, 2015).

The change in the degree of plasticity after stroke might play an important role in the outcome of the rehabilitation and Npam43 could have a synergistic effect on both the immediate cell death by promoting cell survival and by the strengthening the plasticity effects in later time points post-stroke (Pekna, Pekny, & Nilsson, 2012). The protection effect of Npam43 was evident by the stimulation of a high concentration of NMDA, which mimics the conditions seen

during stroke, where there is large amounts of glutamate released synaptically and there is spillover into extrasynaptic locations, which activate GluN2B-mediated cell death (Papouin & Oliet, 2014). The effects again were shown to be GluN2A-dependent and not by GluN2B receptors.

Interestingly, the modulation of GluN2A-containing NMDARs is not only effective against NMDAR-mediated cell death (primary neuronal injuries), but can also guard against non-NMDAR-mediated cell death (H₂O₂ cytotoxicity) (secondary neuronal injuries) (Papadia et al., 2008). This is of particular interest because there is growing evidence which supports the fact that some of the non-NMDAR-mediated mechanisms, while secondary to NMDAR activation, may contribute significantly to brain damage, particularly following severe stroke insults (M. Aarts et al., 2003; Xiong et al., 2004). Numerous reports demonstrate that mitochondria play a destructive role by producing harmful free radicals, which upon release can activate the degradative enzymes which lead to an acute cell death via necrosis (Fulda et al., 2010; Kristian & Siesjo, 1998; Zong & Thompson, 2006). Moreover, the present results from immunoblots show increased pCREB levels suggesting that potentiation of GluN2A-conatining NMDAR, do indeed promote the pro-survival pathway *in-vitro* and *in-vivo*.

Taken together these data suggest that Npam43 acting through GluN2A-containing NMDARs, is capable of initiating the activation of signaling cascades associated with prosurvival pathways in neurons. The protection observed from the H_2O_2 insult, suggests that the activation of the pro-survival signaling cascade afflicts a global effect against possibly different harmful stimuli, not just an excitotoxic mechanism. This suggests that the signaling cascades downstream, triggered by glutamate/NMDA stimulation, are possibly similar to or converge with the downstream signaling observed in other harmful stimuli. This highlights the versatility in this kind treatment for promoting cell survival signaling, during cell stress or a detrimental stimulus condition.

10.3 Novel modulation binding site on the GluN1/GluN2A NMDARs

We have further classified the drug as an allosteric modulator that targets the interface between the GluN1 and GluN2A subunits located in the N-terminal domain (NTD). Site-directed mutagenesis has revealed that GluN1 (Leu₁₃₅) and GluN2A (Phe₁₇₇, Pro₇₈, Phe₁₁₄, Qln₁₁₁, and Pro₁₇₈) form the Npam43 binding pocket between the GluN1 and GluN2A interface of NMDAR receptors in the NTD. This previously unexplored region is highly homologous when compared to the primary sequence of GluN2B (Figure 10.46a & b). Nonetheless, we were able to exploit the subtle differences which occur either from the relative positions of the residues attributed by the differences in the mode of binding of GluN1/GluN2A versus the GluN1/GluN2B subunits. Numerous reports have suggested that the distinction may be attributed to fundamental differences in the mode of subunit association between NR1/NR2A and NR1/NR2B in the NTD (Malherbe et al., 2003). This may confer spatial differences within the pocket that allows enough space for Npam43 to bind to the GluN1/GluN2A interface.

We cannot exclude the possibility that Npam43 does bind to GluN1/GluN2B-containing NMDARs but its binding may not cause the same conformational changes observed with GluN1/GluN2A-containing NMDARs. In the past, all of the structural studies on NMDARs have

been focused on the allosteric inhibition of the receptor. Previous crystal structures have accurately shown the structural features and the conformational state of the closed-form complex in the presence of a NAM in the NTD (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2016). Conversely, herein we report a modulator that can bind to the NTD and supposedly cause conformational changes which induces a positive modulation effect (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2009; C. H.

As there is no crystal structure of the GluN1/GluN2A complex structure, we can hypothesize that the changes induced by the compound would enhance the conformational changes seen in its active form, not its closed-form state (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2016). All structures of the NMDAR have been crystallized in its closed-form state; however a more recent study has shown a crystallized structure of GluN1/GluN2B NMDAR in its active form (Tajima et al., 2016). This demonstrates that in the absence of a NAM, the receptor can undergo large conformational changes both in the NTD and LBD (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2016).

The present research showed that the conformational changes that occur happen in the different domains during the activation process of GluN1/GluN2B NMDARs. This provides further indication that the activation requires opening of the bi-lobed architecture of the GluN2B NTD and re-positioning of the heterodimeric arrangement in the GluN1/GluN2B NTD. These changes lead to rotated movements of the GluN1/GluN2B heteromeric pairs in both the NTD and LBD causing an opening of the gating ring (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2016). This process may confirm the idea that the NTD during activation undergoes

significant conformational changes from the R1 & R2 domain lobes of the NTD to the S1 & S2 domains of the LBD, which could provide molecular movements for transmission of an allosteric effect to the D-serine/glycine and glutamate-binding LBD layer (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2016)(Figure 10.3).

These findings further strengthen the idea that the NTD can play an important role in modulation effect of the receptor and may suggest that a PAM located in the R1 domain of the GluN1/GluN2A NTD can facilitate or "choke" the receptor into transmitting these signals to the LBD layer through the same or similar movements observed by the heterodimeric arrangement of the GluN1/GluN2B NTD and rotated GluN1/GluN2B heteromeric pairs in both NTD and LTD as reported by Tajima et al. but the only way to confirm this would be to co-crystallize Npam43 with the GluN1/GluN2A complex and visualize the structural differences between the activated form of the two co-agonists (L-glutamate/glycine bound) versus the positive modulation allosteric state of three ligands; the two co-agonists plus Npam43 (L-glutamate/glycine/Npam43 bound) (Karakas et al., 2009; C. H. Lee et al., 2014; Tajima et al., 2016) (Figure 10.3a & b).



Figure 10.3 Structural architecture of the predicted GluN1/GluN2A NMDA receptor complex in the presence and absence of Npam43 at the NTD

(a) Three-dimensional structure of the GluN1/GluN2A receptor complex homology model created from the crystal structure (GluN1/GluN2B crystal structure) with the GluN1 subunits in cyan and the GluN2A subunits in purple. GluN1 (glycine) and GluN2B (glutamate) agonists are in space filling representation in the LTD. (b) The docked pose of Npam43 (yellow space-fill) occupying the N-terminal domain (NTD) relative to the whole GluN1/GluN2A NMDA structure which was confirmed by site-directed mutagenesis. GluN1 (glycine) and GluN2B (glutamate) agonists are in space filling representation in the LBD.(Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).(Pettersen et al., 2004)

10.4 Drug development of Npam43

In 2001, all clinical trials of NMDAR antagonists in human beings with stroke or traumatic brain injury were considered unsuccessful because of lack of efficacy (Gladstone et al., 2002; Ikonomidou & Turski, 2002). In particular, the overall quality of the molecules was poor in terms of pharmacokinetics and their inability to reach effective concentrations in the penumbra. In light of this, we were propelled to pharmacologically characterize our drug to see whether it could get to the brain at therapeutic concentrations. We characterized the drug based on its therapeutic potential *in-vivo* by evaluating its pharmacological profile and showed that Npam43 when administered intravenously achieved CSF concentration levels that were within range of its therapeutic dose *in-vitro* and had a half-life of ~3 hours, demonstrating that its ability to penetrate the brain-blood barrier (BBB) and its stability *in-vivo* was suitable to test in *in-vivo* using a stroke model. In fact, the dose administered was directly proportional to the concentration seen in the CSF, demonstrating the effectiveness of the drug to penetrate the BBB.

The intravenously injected Npam43 showed a CSF profile that suggested it could be administered intravenously with the maximum CSF drug concentrations was established at approximately 0.5mg/kg is ~10 μ M, 1mg/kg is ~20 μ M, 2.5mg/kg is ~50 μ M. Because of the high penetrating efficacy of Npam43, the therapeutic doses *in-vivo* could be reached at lower doses and avoids the necessity to use higher doses that maybe too toxic. The pharmacokinetic analysis of Npam43 demonstrated a moderate metabolic stability with a half-life of 3 hours. Based on the *in-vitro* data (EC₅₀ = 0.3 μ M), it was expected that the CSF concentration of the drug should remain within the predicted therapeutic window for at approximately 12 hours when delivered intravenously. Initial toxicity experiments demonstrated no visible toxicity, and doses up to and including 10mg/kg could be tolerated by the rats.

10.5 The efficacy in-vivo for Npam43

Arguably the biggest and most challenging step in drug discovery is the whether the treatment can translate from an *in-vitro* to an *in-vivo* model. There are many reasons and confounding factors that may be attributed to the loss of efficacy in *in-vivo*. Such reasons may include that the drug cannot get to the disease-impacting pathway, drug elimination issues, enzymatic drug inactivation or feedback pathways counteracting the drug's effect. Proper and rational drug design procedures enhance the chances of achieving a positive outcome in *in-vivo* such as pharmacological profiling of drug a priori.

In *stage VII and IX*, with this in mind, we have spent a considerable amount of time to study its pharmacokinetic and stability characteristics and have already observed a favorable result from Npam43, where it showed a good overall BBB-penetration, half-life and stability. Based on this data, we felt that Npam43 was suitable to test in an *in-vivo* model of stroke (MCAo).

In *stage X*, as expected, the administration of Npam43 (0.5, 1, 2.5mg/kg) 3.5 hours after the onset of stroke resulted in a remarkable reduction in total infarct volume after 24 hours depicted by histological evaluation (TTC staining). Previous studies indicate that some neuroprotective effects observed in animal studies may be temporary, essentially slowing down the neuronal damage rather than preventing or rescuing it. These factors, in addition to the narrow window of efficacy of NMDAR antagonists, may also partially contribute to the discrepancy between the effectiveness in basic research and failure in clinical trials of these therapies. These factors, in addition to the narrow window of efficacy of NMDAR antagonists, may also partially contribute the discrepancy between the effectiveness in basic research and failure in clinical trials of these therapies. To ascertain if the therapy used in this study was long-lasting and not just delaying the cell death, the rats were consequently monitored 7 days post ischemic stroke insult using MRI. As observed in the MRI, 7 days after stoke onset, the reduced infract area following selective modulation of GluN2A receptors by Npam43 was permanent and lead to a long-lasting protection. It has been clearly demonstrated that Npam43 had a significant neuroprotective outcome over morphological recovery of the ischemic stroke.

In the past it has been shown that reducing the infract volume does not always translate to a functional recovery in the brain and many drugs have shown this one-sided bias. It is well known that many treatments show their effects by slowing down the progression of the ischemic damage rather than introducing a long-lasting remedial outcome. It is known that the sensorimotor circuit is the major afflicted area during an ischemic event and thus it was prudent to test whether Npam43's ability to reduce the infract volume could be translatable to an improvement in sensorimotor performance in rats post-stroke (Schaar, Brenneman, & Savitz, 2010; Shyu, Lin, Yen, et al., 2008; Shyu, Liu, et al., 2008).

In light of this, the administration of Npam43 on rats subjected to MCA occlusion were evaluated weekly for up to 4 weeks to ascertain if the neuroprotective effect corresponds to a positive long-lasting behavioral outcome. Three locomotor activity (sensorimotor) modalities were used to evaluate the improvements or deficits in motor behavior performances. More specifically, the three modalities were the vertical activity (the total number of beam interruptions that occurred in the vertical sensor), the number of vertical movements (number of animal rears) and vertical movement time (the amount of time the animal rears) (Shen & Wang, 2010). These tests are often used to evaluate functional recovery of cortical neural circuits damaged by focal ischemic insult (Shen & Wang, 2010).

It was clear from the results, that Npam43 had a significant and more importantly a positive long-lasting behavioral change in locomotor activity in all three tasks. This demonstrates that the protective effect of Npam43 via the modulation of GluN2A-containing NMDARs, translates to a functional recovery lasting at least 1 month after the stroke onset. The *in-vivo* results obtained by the TTC staining, the MRI and the behavioral study were also very promising and demonstrated how the step-by-step approach (drug discovery pipeline Figure 10.1) implemented in this project increased the likelihood to have a biological and therapeutic effect *in-vivo*. I strongly believe that skipping steps during this process will decrease the chances of observing an effect *in-vivo* or lead to compounds that would never be conceivably a treatment but rather used simply as a tool or lost in translation.

10.6 Clinical relevance

Stroke occurs when the cerebral artery becomes blocked, due to poor blood flow or a blockage which results in considerable brain cell death (X. Liu et al., 2014; Shah & Abbruscato, 2014). This ultimately triggers cascades of events leading to a combination of progressive neuronal cell death (primary neuronal injury) and also mechanisms that release harmful chemicals that further worsen the mechanisms leading to death (secondary neuronal injury) (J. M. Lee et al., 2000).

Current treatments for stroke are very limited and the tissue plasminogen activator (tPA) is the only vascular-aimed treatment option for patients (X. Liu et al., 2014; Wardlaw, Warlow, & Counsell, 1997; Warlow & Wardlaw, 1997). tPA is a serine protease protein that accelerates the transformation of plasminogen to plasmin, the enzyme that facilitates the disintegration of blood clots (Adibhatla & Hatcher, 2008). The other type of therapy has been a neuroprotective-aimed approach, which helps to slow or stop the pro-death signaling cascades that damage or kill the cell after the stroke onset. Even though tPA has shown to be very effective at dislodging the block, the motivation to shift the paradigm from vascular to neuroprotective-aimed therapy is based on the fact that tPA has a small therapeutic window and therefore only useful and more importantly safe when given within 3-4 hours of the stroke onset (Hacke et al., 1998; "Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group," 1995)(Green & Shuaib, 2006). Another caveat for not using tPA is that patients need to screened to make sure that the stroke is not hemorrhagic because this therapy can facilitate conversion of an ischemic stroke into a hemorrhagic stroke

which can be severe or fatal and directly implicated to its safety profile (Brinker, Pillekamp, & Hossmann, 1999; Wardlaw et al., 1997). This restriction also leads to the difficultly to diagnose the stroke and this causes delays the treatment administration which lets the cell-death to progress and worsen (Brinker et al., 1999; Wardlaw, Lewis, Dennis, Counsell, & McDowall, 1998; Wardlaw et al., 1997) Consequently, stroke patients need to be tested at the hospital and scanned by MRI, (DWI, T1/T2-weighted) or CT to ascertain whether the stroke is hemorrhagic because of the possible exacerbation of the injury or even death and scanning needs to be done to evaluate the progression of the stroke such as if a HT has occurred in the acute stages (Allen et al., 2012; Green & Shuaib, 2006; van Everdingen et al., 1998; Wardlaw et al., 1998; J. Zhang et al., 2014). All of these negative attributes of tPA have dramatically diminished the ability to treat patients quickly and effectively and have been restrictive for patients in terms of therapeutic window, safety, side effects and stroke-type (Green & Shuaib, 2006).

Many molecular cascades have been documented to be responsible for the cell-death that is occurring after stroke onset and more than 1,026 neuroprotective agents that block these pathways have shown positive outcomes in experimental conditions (O'Collins et al., 2006) but have not translated to the clinic (Kidwell, Liebeskind, Starkman, & Saver, 2001; Saver, Kidwell, Liebeskind, & Starkman, 2001). These pharmacological neuroprotection therapies which have been used to stop and interfere with downstream pro-death signaling pathways have miserably failed clinically but on the other hand, the results accumulated in this work lead to new paradigm shift that aimed to naturally enhance the cell survival signaling mechanisms and allowing the cell to fight off these detrimental processes posed by the ischemic insult. This type of approach can potentially be used for both ischemic and hemorrhagic types of strokes but further studies need to be done to validate whether Npam43 would efficacious against hemorrhagic-type or permanent MCAo strokes (Green & Shuaib, 2006). Of particular note, Npam43 administration would not require pre-scanning at the hospital and theoretically can be administered without examination and may quickly to given in the ambulance van (Green & Shuaib, 2006). Another benefit of this type of treatment is that Npam43 neuroprotective therapy has a wider therapeutic window than tPA with already showing neuroprotective effects 3.5 hours after stroke onset and since it acts to naturally enhance the pro-survival signal it theoretically may not have a therapeutic window but more experiments are needed to confirm this.

Since, a large number of NMDAR antagonists were tested against ischemic strokes but unfortunately have failed due to:

- 1) poor pharmacokinetics; inability to reach effective concentrations in the penumbra
- inability to use the treatments at protective doses due to potential blocking of normal brain function and neuronal survival,
- 3) short neuroprotective times windows,
- 4) inappropriate receptor subunit selectivity,
- 5) mismatch of doses used in rodents and human beings resulting in high drug toxicity,
- 6) poor design of clinical trials,
- 7) heterogeneity in the patient population (Y. D. Cheng, Al-Khoury, & Zivin, 2004; Muir, 2006) (Albensi et al., 2004; Corbett & Nurse, 1998; Gladstone et al., 2002; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002).

While the last factor (6 & 7) requires better designed clinical trials, we focused the research presented in this dissertation on finding ways to lessen (1-5). The most two most important

reasons for the failures attributed to NMDAR antagonism is that they have short therapeutic windows and that they block synaptic transmission mediated by NMDARs, which hinders neuronal survival by blocking for instance pro-survival GluN2A NMDARs via CREB signaling (Hardingham & Bading, 2002, 2010; Y. Liu et al., 2007). Secondly, NMDARs are extremely important for a wide variety of physiological functions which includes synaptic plasticity (LTP, LTD) that play an important role in learning and memory (T. V. Bliss & Collingridge, 1993; Collingridge et al., 2004). Moreover, as this study suggests GluN2A-containing NMDARs and GluN2B-conatining NMDARs trigger different signaling pathways that mediate pro-survival and pro-death signaling respectively (Y. Liu et al., 2007). This is the reason why blocking both subtypes would not be beneficial since the block of GluN2A-containing NMDARs would limit the ability for the cell to trigger the cell-survival signaling cascade needed to keeping the cells alive (Y. Liu et al., 2007). In the current study, the limitations of NMDAR antagonists were bypassed by triggering the pro-survival signaling cascade. Unlike GluN2B antagonism, this novel intervention causes no obvious side effect, as it only enhances the function of GluN2Acontaining NMDARs and does not interfere or stop normal functioning of the receptors or other proteins. Therefore, triggering the cell survival signaling pathway post-ischemia, through the enhancement of GluN2A-containing NMDARs would theoretically have no therapeutic window limitation as it protects neurons against brain damage by promoting neuronal survival, rather than blocking the activation of a death signal initiated by the stroke insult (Y. Liu et al., 2007).

The enhancement of GluN2A-containing NMDARs promotes the induction of LTP processes, which potentially increase synaptic strength and could be used to treat pathological synaptic states by enhancing synaptic plasticity to improve conditions arising from a weakened

synaptic drive, such as in stroke conditions (T. V. Bliss & Cooke, 2011; T. H. Murphy & Corbett, 2009). We expect that this treatment may offer improved recovery as it have shown to increase LTP and thus may induce new synaptic growth and potentiate synaptic strength which are key events to help the functional re-organization for rehabilitation and recovery following stroke (Hosp & Luft, 2011; T. H. Murphy & Corbett, 2009; Yaka et al., 2007). Given that LTD of synaptic strength is associated with stroke and neural degeneration, Npam43 may shift intracellular signaling cascades in favor of pro-LTP and pro-survival pathways. Finally, the modulation of GluN2A-containing NMDARs was not only effective against NMDAR-mediated cell death (primary neuronal injuries), but can also guard against non-NMDAR-mediated cell death (H₂O₂ cytotoxicity), the cause of secondary neuronal injuries. This is of particular interest because there is growing evidence that some of the non-NMDAR-mediated mechanisms, while secondary to NMDAR activation, may contribute significantly to brain damage, particularly following severe stroke insults (X. Fan, Jin, Lu, Wang, & Wang, 2014; Papadia et al., 2008; Shirley et al., 2014).

Npam43 has been developed as a small molecule, which has several advantages over other peptide or protein therapies. Firstly, peptides that have not been properly chemically optimized or are naturally occurring are usually metabolically unstable, expensive, membrane impermeable, have a low oral bioavailability, and if not designed correctly they are not highly efficacious, and thus may target unspecific proteins which cause off-target effects (Di, 2015; Otvos & Wade, 2014; Sachdeva, Lobo, & Goswami, 2016). Unlike peptides, Npam43 is a small molecule, offering superior stability, low cost, higher membrane permeability, ease of synthesis, and potentially higher oral bioavailability (not tested yet), which could potentially allow this kind of treatment to be delivered orally.

At some point in the future, since the therapeutic time windows are a major concern for stroke especially if the patient inclusion times are within the range of 4-6 hours, it would be advantageous to have a therapy that is orally available or could be taken at home especially in the first week of the onset of stroke but preferably taken as soon as possible and then possibly continued for the first week. This will reduce cost at the hospitals; make the treatments more mainstream by offering patients more options, especially for people not residing close to a hospital such as in rural communities. Npam43 presents as new line of therapy for stroke treatment due to its versatility of use and ease of administration without the prerequisite of a CT test, availability of hospitals or medical administration procedures. This drawback significantly slows down the administration of tPA treatment given to the patient (Green & Shuaib, 2006). Another advantage of Npam43 over peptide therapies would be the straightforwardness to optimize its pharmacokinetic profile of the drug. Npam43 has already shown to be BBBpermeable and has a half-life of ~3 hours, therefore structural modification to improve the pharmacological profile of the drug and to maintain the drug's efficacy would be fairly easy. By comparison, a peptide at this stage with a similar half-life would be very difficult to modify and very likely to lose its efficacy once altered; therefore improvement strategies at this stage would most likely not be possible.

Since Npam43 is an allosteric modulator, the effect of the drug is only seen when GluN2A-containing NMDARs are activated by glutamate and glycine, which doesn't affect basal

transmission and avoids potential side effects compared to an intervention that inhibits a protein in a particular pathway. Because tPA cannot be used for hemorrhagic stroke, I recommend testing these PAMs for this type of stroke, which currently has no effective treatment option. Unlike, vascular-aimed therapeutics (e.g. tPA), Npam43 and its analogs, fall into the neuroprotection-aimed therapeutics, which are not thought to have any adverse effects on this type of stroke and should turn on the cell-signaling cascade that promotes cell survival rather than blocking the cell death mechanisms downstream and interfering with important beneficial physiological interactions occurring inside the cell. Moreover, unlike many early studies, the current experiments have been done in a step-by-step manner, progressing through each of the stages highlighted in a typical drug discovery pipeline. Proper progression through the stages is a necessary step to discover an effective therapy for stroke but also identify the faults and limitations of the drug. Also, unlike many stroke treatments which delay the ischemia injury process, Npam43 has been shown to provide a long-term neuroprotection that lasts up to 1 month post-stroke onset. Taken together, GluN2A PAM is not subject to the restrictions of other stroke treatments like TPA and other peptide therapies that are currently in clinical trials, which lack of long-term effects, no protection from secondary injuries (H₂O₂ cytotoxicity), narrow therapeutic windows, poor pharmacology, difficult administration procedures, and no long-term benefits from treatment during recovery. Npam43 appears to offer a viable treatment option without the negative issues found in current peptide therapies.

Finally, it would be interesting and prudent to try a co-administration of Npam43 with tPA or independently in combination as it might offer a synergistic effect for ischemic strokes. Given that excitotoxicity is thought to be a common neuropathology associated with a large

number of neurological disorders ranging from acute brain insults such as stroke to chronic neurodegenerative disorders such as Huntington's disease (Estrada Sanchez, Mejia-Toiber, & Massieu, 2008) and Parkinson's disease (Ambrosi, Cerri, & Blandini, 2014; Beal, 1998), our findings may have broad implications beyond stroke therapies and raises the potential and motivation for designing new therapeutic solutions for the clinical treatment of multiple neurological disorders.

Bibliography

- . (2015). In R. Graham, M. A. McCoy & A. M. Schultz (Eds.), *Strategies to Improve Cardiac Arrest Survival: A Time to Act*. Washington (DC).
- Aarts, M., Iihara, K., Wei, W. L., Xiong, Z. G., Arundine, M., Cerwinski, W., . . . Tymianski, M. (2003). A key role for TRPM7 channels in anoxic neuronal death. *Cell*, *115*(7), 863-877.
- Aarts, M., Liu, Y., Liu, L., Besshoh, S., Arundine, M., Gurd, J. W., . . . Tymianski, M. (2002). Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science*, 298(5594), 846-850. doi: 10.1126/science.1072873
- Aarts, M. M., & Tymianski, M. (2005). TRPMs and neuronal cell death. *Pflugers Arch, 451*(1), 243-249. doi: 10.1007/s00424-005-1439-x
- Abe, K., Aoki, M., Kawagoe, J., Yoshida, T., Hattori, A., Kogure, K., & Itoyama, Y. (1995).Ischemic delayed neuronal death. A mitochondrial hypothesis. *Stroke*, 26(8), 1478-1489.
- Adibhatla, R. M., & Hatcher, J. F. (2008). Tissue plasminogen activator (tPA) and matrix metalloproteinases in the pathogenesis of stroke: therapeutic strategies. CNS Neurol Disord Drug Targets, 7(3), 243-253.
- Albensi, B. C., Igoechi, C., Janigro, D., & Ilkanich, E. (2004). Why do many NMDA antagonists fail, while others are safe and effective at blocking excitotoxicity associated with dementia and acute injury? *Am J Alzheimers Dis Other Demen, 19*(5), 269-274.
- Allen, L. M., Hasso, A. N., Handwerker, J., & Farid, H. (2012). Sequence-specific MR imaging findings that are useful in dating ischemic stroke. *Radiographics*, 32(5), 1285-1297; discussion 1297-1289. doi: 10.1148/rg.325115760

- Ambrosi, G., Cerri, S., & Blandini, F. (2014). A further update on the role of excitotoxicity in the pathogenesis of Parkinson's disease. J Neural Transm (Vienna), 121(8), 849-859. doi: 10.1007/s00702-013-1149-z
- Amico-Ruvio, S. A., Murthy, S. E., Smith, T. P., & Popescu, G. K. (2011). Zinc effects on NMDA receptor gating kinetics. *Biophys J*, 100(8), 1910-1918. doi: 10.1016/j.bpj.2011.02.042
- Amico-Ruvio, S. A., Paganelli, M. A., Myers, J. M., & Popescu, G. K. (2012). Ifenprodil effects on GluN2B-containing glutamate receptors. *Mol Pharmacol*, 82(6), 1074-1081. doi: 10.1124/mol.112.078998
- Aniksztejn, L., Charton, G., & Ben-Ari, Y. (1987). Selective release of endogenous zinc from the hippocampal mossy fibers in situ. *Brain Res*, 404(1-2), 58-64.
- Araujo, I. M., Carreira, B. P., Pereira, T., Santos, P. F., Soulet, D., Inacio, A., . . . Carvalho, C. M. (2007). Changes in calcium dynamics following the reversal of the sodium-calcium exchanger have a key role in AMPA receptor-mediated neurodegeneration via calpain activation in hippocampal neurons. *Cell Death Differ, 14*(9), 1635-1646. doi: 10.1038/sj.cdd.4402171
- Arundine, M., & Tymianski, M. (2004). Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol Life Sci*, 61(6), 657-668. doi: 10.1007/s00018-003-3319-x
- Assaf, S. Y., & Chung, S. H. (1984). Release of endogenous Zn2+ from brain tissue during activity. *Nature*, 308(5961), 734-736.
- Auberson, Y. P., Allgeier, H., Bischoff, S., Lingenhoehl, K., Moretti, R., & Schmutz, M. (2002).5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a

preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett*, *12*(7), 1099-1102.

- Axerio-Cilies, P., Castaneda, I. P., Mirza, A., & Reynisson, J. (2009). Investigation of the incidence of "undesirable" molecular moieties for high-throughput screening compound libraries in marketed drug compounds. *Eur J Med Chem*, 44(3), 1128-1134. doi: 10.1016/j.ejmech.2008.06.013
- Axerio-Cilies, P., Lack, N. A., Nayana, M. R., Chan, K. H., Yeung, A., Leblanc, E., . . .
 Cherkasov, A. (2011). Inhibitors of androgen receptor activation function-2 (AF2) site identified through virtual screening. *J Med Chem*, 54(18), 6197-6205. doi: 10.1021/jm200532b
- Axerio-Cilies, P., See, R. H., Zoraghi, R., Worral, L., Lian, T., Stoynov, N., . . . Cherkasov, A. (2012). Cheminformatics-driven discovery of selective, nanomolar inhibitors for staphylococcal pyruvate kinase. ACS Chem Biol, 7(2), 350-359. doi: 10.1021/cb2003576
- Bae, C. Y., & Sun, H. S. (2011). TRPM7 in cerebral ischemia and potential target for drug development in stroke. Acta Pharmacol Sin, 32(6), 725-733. doi: 10.1038/aps.2011.60
- Bae, C. Y., & Sun, H. S. (2013). Current understanding of TRPM7 pharmacology and drug development for stroke. Acta Pharmacol Sin, 34(1), 10-16. doi: 10.1038/aps.2012.94
- Bajusz, D., Racz, A., & Heberger, K. (2015). Why is Tanimoto index an appropriate choice for fingerprint-based similarity calculations? *J Cheminform*, 7, 20. doi: 10.1186/s13321-015-0069-3
- Banks, W. A. (2009). Characteristics of compounds that cross the blood-brain barrier. *BMC Neurol*, 9 *Suppl 1*, S3. doi: 10.1186/1471-2377-9-S1-S3

- Bano, D., Young, K. W., Guerin, C. J., Lefeuvre, R., Rothwell, N. J., Naldini, L., . . . Nicotera, P. (2005). Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. *Cell*, *120*(2), 275-285. doi: 10.1016/j.cell.2004.11.049
- Bar-Shira, O., Maor, R., & Chechik, G. (2015). Gene Expression Switching of Receptor Subunits in Human Brain Development. *PLoS Comput Biol*, 11(12), e1004559. doi: 10.1371/journal.pcbi.1004559
- Baron, J. C., Yamauchi, H., Fujioka, M., & Endres, M. (2014). Selective neuronal loss in ischemic stroke and cerebrovascular disease. J Cereb Blood Flow Metab, 34(1), 2-18. doi: 10.1038/jcbfm.2013.188
- Bauer, H. C., Krizbai, I. A., Bauer, H., & Traweger, A. (2014). "You Shall Not Pass"-tight junctions of the blood brain barrier. *Front Neurosci*, 8, 392. doi: 10.3389/fnins.2014.00392
- Beal, M. F. (1998). Excitotoxicity and nitric oxide in Parkinson's disease pathogenesis. Ann Neurol, 44(3 Suppl 1), S110-114.
- Beitner-Johnson, D., Rust, R. T., Hsieh, T., & Millhorn, D. E. (2000). Regulation of CREB by moderate hypoxia in PC12 cells. *Adv Exp Med Biol*, 475, 143-152. doi: 10.1007/0-306-46825-5_14
- Bender, R. A., Lauterborn, J. C., Gall, C. M., Cariaga, W., & Baram, T. Z. (2001). Enhanced CREB phosphorylation in immature dentate gyrus granule cells precedes neurotrophin expression and indicates a specific role of CREB in granule cell differentiation. *Eur J Neurosci*, 13(4), 679-686.
- Bengtson, C. P., & Bading, H. (2012). Nuclear calcium signaling. Adv Exp Med Biol, 970, 377-405. doi: 10.1007/978-3-7091-0932-8_17
- Benveniste, H., Drejer, J., Schousboe, A., & Diemer, N. H. (1984). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem*, 43(5), 1369-1374.
- Benveniste, M., & Mayer, M. L. (1991). Kinetic analysis of antagonist action at N-methyl-Daspartic acid receptors. Two binding sites each for glutamate and glycine. *Biophys J*, 59(3), 560-573. doi: 10.1016/S0006-3495(91)82272-X
- Birenbaum, D., Bancroft, L. W., & Felsberg, G. J. (2011). Imaging in acute stroke. West J Emerg Med, 12(1), 67-76.
- Blanke, M. L., & VanDongen, A. M. J. (2009). Activation Mechanisms of the NMDA Receptor.In A. M. Van Dongen (Ed.), *Biology of the NMDA Receptor*. Boca Raton (FL).
- Bliss, T., & Schoepfer, R. (2004). Neuroscience. Controlling the ups and downs of synaptic strength. *Science*, *304*(5673), 973-974. doi: 10.1126/science.1098805
- Bliss, T. V., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, *361*(6407), 31-39. doi: 10.1038/361031a0
- Bliss, T. V., & Cooke, S. F. (2011). Long-term potentiation and long-term depression: a clinical perspective. *Clinics (Sao Paulo), 66 Suppl 1*, 3-17.
- Bodalia, A., Li, H., & Jackson, M. F. (2013). Loss of endoplasmic reticulum Ca2+ homeostasis:
 contribution to neuronal cell death during cerebral ischemia. *Acta Pharmacol Sin*, 34(1), 49-59. doi: 10.1038/aps.2012.139
- Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., & Greenberg, M. E. (1999). Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and independent mechanisms. *Science*, 286(5443), 1358-1362.

- Borza, I., & Domany, G. (2006). NR2B selective NMDA antagonists: the evolution of the ifenprodil-type pharmacophore. *Curr Top Med Chem*, 6(7), 687-695.
- Borza, I., Kolok, S., Gere, A., Agai-Csongor, E., Agai, B., Tarkanyi, G., . . . Domany, G. (2003).
 Indole-2-carboxamides as novel NR2B selective NMDA receptor antagonists. *Bioorg Med Chem Lett*, 13(21), 3859-3861.
- Bramlett, H. M., & Dietrich, W. D. (2004). Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. J Cereb Blood Flow Metab, 24(2), 133-150. doi: 10.1097/01.WCB.0000111614.19196.04
- Brigman, J. L., Feyder, M., Saksida, L. M., Bussey, T. J., Mishina, M., & Holmes, A. (2008). Impaired discrimination learning in mice lacking the NMDA receptor NR2A subunit. *Learn Mem*, 15(2), 50-54. doi: 10.1101/lm.777308
- Brigman, J. L., Wright, T., Talani, G., Prasad-Mulcare, S., Jinde, S., Seabold, G. K., . . . Holmes,
 A. (2010). Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. J Neurosci, 30(13), 4590-4600. doi: 10.1523/JNEUROSCI.0640-10.2010
- Brini, M., & Carafoli, E. (2011). The plasma membrane Ca(2)+ ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. *Cold Spring Harb Perspect Biol, 3*(2). doi: 10.1101/cshperspect.a004168
- Brinker, G., Pillekamp, F., & Hossmann, K. A. (1999). Brain hemorrhages after rt-PA treatment of embolic stroke in spontaneously hypertensive rats. *Neuroreport*, *10*(9), 1943-1946.
- Broughton, B. R., Reutens, D. C., & Sobey, C. G. (2009). Apoptotic mechanisms after cerebral ischemia. *Stroke*, 40(5), e331-339. doi: 10.1161/STROKEAHA.108.531632

- Brouns, R., & De Deyn, P. P. (2009). The complexity of neurobiological processes in acute ischemic stroke. *Clin Neurol Neurosurg*, 111(6), 483-495. doi: 10.1016/j.clineuro.2009.04.001
- Brouns, R., Heylen, E., Willemse, J. L., Sheorajpanday, R., De Surgeloose, D., Verkerk, R., . . . Hendriks, D. F. (2010). The decrease in procarboxypeptidase U (TAFI) concentration in acute ischemic stroke correlates with stroke severity, evolution and outcome. *J Thromb Haemost*, 8(1), 75-80. doi: 10.1111/j.1538-7836.2009.03663.x
- Brustovetsky, T., Bolshakov, A., & Brustovetsky, N. (2010). Calpain activation and Na+/Ca2+ exchanger degradation occur downstream of calcium deregulation in hippocampal neurons exposed to excitotoxic glutamate. J Neurosci Res, 88(6), 1317-1328. doi: 10.1002/jnr.22295
- Buller, A. L., & Monaghan, D. T. (1997). Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in Xenopus oocytes. *Eur J Pharmacol*, 320(1), 87-94.
- Cai, J., Yang, J., & Jones, D. P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim Biophys Acta*, 1366(1-2), 139-149.
- Cao, J., Semenova, M. M., Solovyan, V. T., Han, J., Coffey, E. T., & Courtney, M. J. (2004).
 Distinct requirements for p38alpha and c-Jun N-terminal kinase stress-activated protein kinases in different forms of apoptotic neuronal death. *J Biol Chem*, 279(34), 35903-35913. doi: 10.1074/jbc.M402353200
- Carmichael, S. T. (2005). Rodent models of focal stroke: size, mechanism, and purpose. *NeuroRx*, 2(3), 396-409. doi: 10.1602/neurorx.2.3.396

- Carvajal, F. J., Mattison, H. A., & Cerpa, W. (2016). Role of NMDA Receptor-Mediated Glutamatergic Signaling in Chronic and Acute Neuropathologies. *Neural Plast*, 2016, 2701526. doi: 10.1155/2016/2701526
- Casals, J. B., Pieri, N. C., Feitosa, M. L., Ercolin, A. C., Roballo, K. C., Barreto, R. S., . . . Ambrosio, C. E. (2011). The use of animal models for stroke research: a review. *Comp Med*, 61(4), 305-313.
- Castillo, J., Davalos, A., & Noya, M. (1997). Progression of ischaemic stroke and excitotoxic aminoacids. *Lancet*, *349*(9045), 79-83. doi: 10.1016/S0140-6736(96)04453-4
- Ceulemans, A. G., Zgavc, T., Kooijman, R., Hachimi-Idrissi, S., Sarre, S., & Michotte, Y. (2010). The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. *J Neuroinflammation*, 7, 74. doi: 10.1186/1742-2094-7-74
- Chang, H. R., & Kuo, C. C. (2008). The activation gate and gating mechanism of the NMDA receptor. *J Neurosci*, 28(7), 1546-1556. doi: 10.1523/JNEUROSCI.3485-07.2008
- Cheffings, C. M., & Colquhoun, D. (2000). Single channel analysis of a novel NMDA channel from Xenopus oocytes expressing recombinant NR1a, NR2A and NR2D subunits. J Physiol, 526 Pt 3, 481-491.
- Chen, C., & Tonegawa, S. (1997). Molecular genetic analysis of synaptic plasticity, activitydependent neural development, learning, and memory in the mammalian brain. *Annu Rev Neurosci, 20*, 157-184. doi: 10.1146/annurev.neuro.20.1.157
- Chen, M., Lu, T. J., Chen, X. J., Zhou, Y., Chen, Q., Feng, X. Y., . . . Xiong, Z. Q. (2008). Differential roles of NMDA receptor subtypes in ischemic neuronal cell death and ischemic tolerance. *Stroke*, 39(11), 3042-3048. doi: 10.1161/STROKEAHA.108.521898

- Chen, P. E., & Wyllie, D. J. (2006). Pharmacological insights obtained from structure-function studies of ionotropic glutamate receptors. *Br J Pharmacol*, 147(8), 839-853. doi: 10.1038/sj.bjp.0706689
- Chen, S. T., Hsu, C. Y., Hogan, E. L., Maricq, H., & Balentine, J. D. (1986). A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. *Stroke*, 17(4), 738-743.
- Cheng, G., Kong, R. H., Zhang, L. M., & Zhang, J. N. (2012). Mitochondria in traumatic brain injury and mitochondrial-targeted multipotential therapeutic strategies. *Br J Pharmacol*, 167(4), 699-719. doi: 10.1111/j.1476-5381.2012.02025.x
- Cheng, N. T., & Kim, A. S. (2015). Intravenous Thrombolysis for Acute Ischemic Stroke Within
 3 Hours Versus Between 3 and 4.5 Hours of Symptom Onset. *Neurohospitalist*, 5(3), 101-109. doi: 10.1177/1941874415583116
- Cheng, Y. D., Al-Khoury, L., & Zivin, J. A. (2004). Neuroprotection for ischemic stroke: two decades of success and failure. *NeuroRx*, *1*(1), 36-45. doi: 10.1602/neurorx.1.1.36
- Chesselet, M. F., & Carmichael, S. T. (2012). Animal models of neurological disorders. *Neurotherapeutics*, 9(2), 241-244. doi: 10.1007/s13311-012-0118-9
- Chevilley, A., Lesept, F., Lenoir, S., Ali, C., Parcq, J., & Vivien, D. (2015). Impacts of tissuetype plasminogen activator (tPA) on neuronal survival. *Front Cell Neurosci*, *9*, 415. doi: 10.3389/fncel.2015.00415
- Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, 1(8), 623-634.
- Christine, C. W., & Choi, D. W. (1990). Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. *J Neurosci, 10*(1), 108-116.

- Chung, J. W., Ryu, W. S., Kim, B. J., & Yoon, B. W. (2015). Elevated calcium after acute ischemic stroke: association with a poor short-term outcome and long-term mortality. J Stroke, 17(1), 54-59. doi: 10.5853/jos.2015.17.1.54
- Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med*, 48(6), 749-762. doi: 10.1016/j.freeradbiomed.2009.12.022
- Clements, J. D., Feltz, A., Sahara, Y., & Westbrook, G. L. (1998). Activation kinetics of AMPA receptor channels reveal the number of functional agonist binding sites. *J Neurosci, 18*(1), 119-127.
- Collingridge, G. L., Isaac, J. T., & Wang, Y. T. (2004). Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci*, 5(12), 952-962. doi: 10.1038/nrn1556
- Collingridge, G. L., Olsen, R. W., Peters, J., & Spedding, M. (2009). A nomenclature for ligandgated ion channels. *Neuropharmacology*, 56(1), 2-5. doi: 10.1016/j.neuropharm.2008.06.063
- Collingridge, G. L., Peineau, S., Howland, J. G., & Wang, Y. T. (2010). Long-term depression in the CNS. *Nat Rev Neurosci*, *11*(7), 459-473. doi: 10.1038/nrn2867
- Collingridge, G. L., Volianskis, A., Bannister, N., France, G., Hanna, L., Mercier, M., . . . Jane,
 D. E. (2013). The NMDA receptor as a target for cognitive enhancement. *Neuropharmacology*, 64, 13-26. doi: 10.1016/j.neuropharm.2012.06.051
- Collins, R. C. (1986). Selective vulnerability of brain: new insights from the excitatory synapse. *Metab Brain Dis*, *1*(4), 231-240.
- Collins, R. C., Dobkin, B. H., & Choi, D. W. (1989). Selective vulnerability of the brain: new insights into the pathophysiology of stroke. *Ann Intern Med*, *110*(12), 992-1000.

- Cook, D. J., Teves, L., & Tymianski, M. (2012). Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain. *Nature*, 483(7388), 213-217. doi: 10.1038/nature10841
- Corbett, D., & Nurse, S. (1998). The problem of assessing effective neuroprotection in experimental cerebral ischemia. *Prog Neurobiol*, *54*(5), 531-548.
- Cossins, B. P., & Lawson, A. D. (2015). Small Molecule Targeting of Protein-Protein Interactions through Allosteric Modulation of Dynamics. *Molecules*, 20(9), 16435-16445. doi: 10.3390/molecules200916435
- Courtine, G., Bunge, M. B., Fawcett, J. W., Grossman, R. G., Kaas, J. H., Lemon, R., . . . Edgerton, V. R. (2007). Can experiments in nonhuman primates expedite the translation of treatments for spinal cord injury in humans? *Nat Med, 13*(5), 561-566. doi: 10.1038/nm1595
- Cserep, C., Szabadits, E., Szonyi, A., Watanabe, M., Freund, T. F., & Nyiri, G. (2012). NMDA receptors in GABAergic synapses during postnatal development. *PLoS One*, 7(5), e37753. doi: 10.1371/journal.pone.0037753
- Cukuroglu, E., Engin, H. B., Gursoy, A., & Keskin, O. (2014). Hot spots in protein-protein interfaces: towards drug discovery. *Prog Biophys Mol Biol*, 116(2-3), 165-173. doi: 10.1016/j.pbiomolbio.2014.06.003
- Davalos, A., Shuaib, A., & Wahlgren, N. G. (2000). Neurotransmitters and pathophysiology of stroke: evidence for the release of glutamate and other transmitters/mediators in animals and humans. *J Stroke Cerebrovasc Dis*, 9(6 Pt 2), 2-8. doi: 10.1053/jscd.2000.18908
- Davis, A. M., Keeling, D. J., Steele, J., Tomkinson, N. P., & Tinker, A. C. (2005). Components of successful lead generation. *Curr Top Med Chem*, 5(4), 421-439.

- de Marchena, J., Roberts, A. C., Middlebrooks, P. G., Valakh, V., Yashiro, K., Wilfley, L. R., & Philpot, B. D. (2008). NMDA receptor antagonists reveal age-dependent differences in the properties of visual cortical plasticity. *J Neurophysiol*, 100(4), 1936-1948. doi: 10.1152/jn.90290.2008
- DeGracia, D. J., Neumar, R. W., White, B. C., & Krause, G. S. (1996). Global brain ischemia and reperfusion: modifications in eukaryotic initiation factors associated with inhibition of translation initiation. *J Neurochem*, 67(5), 2005-2012.
- Di, L. (2015). Strategic approaches to optimizing peptide ADME properties. *AAPS J*, *17*(1), 134-143. doi: 10.1208/s12248-014-9687-3
- Dick, O., & Bading, H. (2010). Synaptic activity and nuclear calcium signaling protect hippocampal neurons from death signal-associated nuclear translocation of FoxO3a induced by extrasynaptic N-methyl-D-aspartate receptors. *J Biol Chem*, 285(25), 19354-19361. doi: 10.1074/jbc.M110.127654
- Doll, D. N., Barr, T. L., & Simpkins, J. W. (2014). Cytokines: their role in stroke and potential use as biomarkers and therapeutic targets. *Aging Dis*, 5(5), 294-306. doi: 10.14336/AD.2014.0500294
- Dong, X. X., Wang, Y., & Qin, Z. H. (2009). Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin*, 30(4), 379-387. doi: 10.1038/aps.2009.24
- Dreier, J. P. (2011). The role of spreading depression, spreading depolarization and spreading ischemia in neurological disease. *Nat Med*, *17*(4), 439-447. doi: 10.1038/nm.2333

- Dupuis, J. P., Ladepeche, L., Seth, H., Bard, L., Varela, J., Mikasova, L., . . . Groc, L. (2014).
 Surface dynamics of GluN2B-NMDA receptors controls plasticity of maturing glutamate synapses. *EMBO J*, *33*(8), 842-861. doi: 10.1002/embj.201386356
- Dzamba, D., Honsa, P., Valny, M., Kriska, J., Valihrach, L., Novosadova, V., . . . Anderova, M. (2015). Quantitative Analysis of Glutamate Receptors in Glial Cells from the Cortex of GFAP/EGFP Mice Following Ischemic Injury: Focus on NMDA Receptors. *Cell Mol Neurobiol*, 35(8), 1187-1202. doi: 10.1007/s10571-015-0212-8
- Eliasson, M. J., Huang, Z., Ferrante, R. J., Sasamata, M., Molliver, M. E., Snyder, S. H., & Moskowitz, M. A. (1999). Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J Neurosci, 19*(14), 5910-5918.
- Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol*, *35*(4), 495-516. doi: 10.1080/01926230701320337
- Enard, D., Depaulis, F., & Roest Crollius, H. (2010). Human and non-human primate genomes share hotspots of positive selection. *PLoS Genet*, 6(2), e1000840. doi: 10.1371/journal.pgen.1000840
- Engel, O., Kolodziej, S., Dirnagl, U., & Prinz, V. (2011). Modeling stroke in mice middle cerebral artery occlusion with the filament model. *J Vis Exp*(47). doi: 10.3791/2423
- Erreger, K., Geballe, M. T., Kristensen, A., Chen, P. E., Hansen, K. B., Lee, C. J., . . . Traynelis,
 S. F. (2007). Subunit-specific agonist activity at NR2A-, NR2B-, NR2C-, and NR2Dcontaining N-methyl-D-aspartate glutamate receptors. *Mol Pharmacol*, *72*(4), 907-920. doi: 10.1124/mol.107.037333

- Estrada Sanchez, A. M., Mejia-Toiber, J., & Massieu, L. (2008). Excitotoxic neuronal death and the pathogenesis of Huntington's disease. *Arch Med Res*, 39(3), 265-276. doi: 10.1016/j.arcmed.2007.11.011
- Evans, R. C., Morera-Herreras, T., Cui, Y., Du, K., Sheehan, T., Kotaleski, J. H., . . . Blackwell,
 K. T. (2012). The effects of NMDA subunit composition on calcium influx and spike timing-dependent plasticity in striatal medium spiny neurons. *PLoS Comput Biol*, 8(4), e1002493. doi: 10.1371/journal.pcbi.1002493
- Ewald, R. C., & Cline, H. T. (2009). NMDA Receptors and Brain Development. In A. M. Van Dongen (Ed.), *Biology of the NMDA Receptor*. Boca Raton (FL).
- Fan, M. M., & Raymond, L. A. (2007). N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. *Prog Neurobiol*, 81(5-6), 272-293. doi: 10.1016/j.pneurobio.2006.11.003
- Fan, X., Jin, W. Y., Lu, J., Wang, J., & Wang, Y. T. (2014). Rapid and reversible knockdown of endogenous proteins by peptide-directed lysosomal degradation. *Nat Neurosci*, 17(3), 471-480. doi: 10.1038/nn.3637
- Fan, X., Jin, W. Y., & Wang, Y. T. (2014). The NMDA receptor complex: a multifunctional machine at the glutamatergic synapse. *Front Cell Neurosci*, 8, 160. doi: 10.3389/fncel.2014.00160
- Feng, B., Tse, H. W., Skifter, D. A., Morley, R., Jane, D. E., & Monaghan, D. T. (2004). Structure-activity analysis of a novel NR2C/NR2D-preferring NMDA receptor antagonist: 1-(phenanthrene-2-carbonyl) piperazine-2,3-dicarboxylic acid. *Br J Pharmacol*, 141(3), 508-516. doi: 10.1038/sj.bjp.0705644

- Fernandez-Lopez, D., Faustino, J., Daneman, R., Zhou, L., Lee, S. Y., Derugin, N., . . . Vexler, Z. S. (2012). Blood-brain barrier permeability is increased after acute adult stroke but not neonatal stroke in the rat. *J Neurosci, 32*(28), 9588-9600. doi: 10.1523/JNEUROSCI.5977-11.2012
- Festjens, N., Vanden Berghe, T., & Vandenabeele, P. (2006). Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta*, 1757(9-10), 1371-1387. doi: 10.1016/j.bbabio.2006.06.014
- Fink, S. L., & Cookson, B. T. (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun*, 73(4), 1907-1916. doi: 10.1128/IAI.73.4.1907-1916.2005
- Fischer, G., Mutel, V., Trube, G., Malherbe, P., Kew, J. N., Mohacsi, E., . . . Kemp, J. A. (1997).
 Ro 25-6981, a highly potent and selective blocker of N-methyl-D-aspartate receptors containing the NR2B subunit. Characterization in vitro. *J Pharmacol Exp Ther*, 283(3), 1285-1292.
- Fischer, G., Rossmann, M., & Hyvonen, M. (2015). Alternative modulation of protein-protein interactions by small molecules. *Curr Opin Biotechnol*, 35, 78-85. doi: 10.1016/j.copbio.2015.04.006
- Ford, G. A. (2008). Clinical pharmacological issues in the development of acute stroke therapies. *Br J Pharmacol*, 153 Suppl 1, S112-119. doi: 10.1038/sj.bjp.0707654
- Forstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *Eur Heart J*, 33(7), 829-837, 837a-837d. doi: 10.1093/eurheartj/ehr304

- Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: cell survival and cell death. *Int J Cell Biol*, 2010, 214074. doi: 10.1155/2010/214074
- Furukawa, H., & Gouaux, E. (2003). Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core. *EMBO J*, 22(12), 2873-2885. doi: 10.1093/emboj/cdg303
- Furukawa, H., Singh, S. K., Mancusso, R., & Gouaux, E. (2005). Subunit arrangement and function in NMDA receptors. *Nature*, 438(7065), 185-192. doi: 10.1038/nature04089
- Gaberel, T., Macrez, R., Gauberti, M., Montagne, A., Hebert, M., Petersen, K. U., ... Vivien, D. (2013). Immunotherapy blocking the tissue plasminogen activator-dependent activation of N-methyl-D-aspartate glutamate receptors improves hemorrhagic stroke outcome. *Neuropharmacology*, 67, 267-271. doi: 10.1016/j.neuropharm.2012.11.023
- Gao, L., Laude, K., & Cai, H. (2008). Mitochondrial pathophysiology, reactive oxygen species, and cardiovascular diseases. *Vet Clin North Am Small Anim Pract*, 38(1), 137-155, vi. doi: 10.1016/j.cvsm.2007.10.004
- Garcia, J. H., Yoshida, Y., Chen, H., Li, Y., Zhang, Z. G., Lian, J., . . . Chopp, M. (1993).Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol*, *142*(2), 623-635.
- Gebhardt, C., & Cull-Candy, S. G. (2006). Influence of agonist concentration on AMPA and kainate channels in CA1 pyramidal cells in rat hippocampal slices. *J Physiol*, 573(Pt 2), 371-394. doi: 10.1113/jphysiol.2005.102723
- Gladstone, D. J., Black, S. E., Hakim, A. M., Heart, & Stroke Foundation of Ontario Centre of Excellence in Stroke, Recovery. (2002). Toward wisdom from failure: lessons from neuroprotective stroke trials and new therapeutic directions. *Stroke*, 33(8), 2123-2136.

- Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Blaha, M. J., . . . Stroke Statistics, Subcommittee. (2014). Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation*, 129(3), e28-e292. doi: 10.1161/01.cir.0000441139.02102.80
- Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Borden, W. B., ... Stroke Statistics, Subcommittee. (2013). Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*, 127(1), e6-e245. doi: 10.1161/CIR.0b013e31828124ad
- Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2006). Multiple pathways of cytochrome c release from mitochondria in apoptosis. *Biochim Biophys Acta*, 1757(5-6), 639-647. doi: 10.1016/j.bbabio.2006.03.016
- Goldlust, E. J., Paczynski, R. P., He, Y. Y., Hsu, C. Y., & Goldberg, M. P. (1996). Automated measurement of infarct size with scanned images of triphenyltetrazolium chloride-stained rat brains. *Stroke*, *27*(9), 1657-1662.
- Gravanis, I., & Tsirka, S. E. (2008). Tissue-type plasminogen activator as a therapeutic target in stroke. *Expert Opin Ther Targets*, *12*(2), 159-170. doi: 10.1517/14728222.12.2.159
- Green, A. R. (2008). Pharmacological approaches to acute ischaemic stroke: reperfusion certainly, neuroprotection possibly. *Br J Pharmacol*, 153 Suppl 1, S325-338. doi: 10.1038/sj.bjp.0707594
- Green, A. R., & Shuaib, A. (2006). Therapeutic strategies for the treatment of stroke. *Drug Discov Today*, *11*(15-16), 681-693. doi: 10.1016/j.drudis.2006.06.001

- Gregory, T. F., Wright, J. L., Wise, L. D., Meltzer, L. T., Serpa, K. A., Konkoy, C. S., . . . Woodward, R. M. (2000). Parallel synthesis of a series of subtype-selective NMDA receptor antagonists. *Bioorg Med Chem Lett*, 10(6), 527-529.
- Gurusamy, K. S., Farooqui, N., Loizidou, M., Dijk, S., Taanman, J. W., Whiting, S., . . . Davidson, B. R. (2011). Influence of zinc and zinc chelator on HT-29 colorectal cell line. *Biometals*, 24(1), 143-151. doi: 10.1007/s10534-010-9382-5
- Haast, R. A., Gustafson, D. R., & Kiliaan, A. J. (2012). Sex differences in stroke. *J Cereb Blood Flow Metab*, *32*(12), 2100-2107. doi: 10.1038/jcbfm.2012.141
- Hacke, W., Kaste, M., Fieschi, C., von Kummer, R., Davalos, A., Meier, D., . . . Trouillas, P. (1998). Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Second European-Australasian Acute Stroke Study Investigators. *Lancet*, 352(9136), 1245-1251.
- Hackos, D. H., & Hanson, J. E. (2016). Diverse modes of NMDA receptor positive allosteric modulation: Mechanisms and consequences. *Neuropharmacology*. doi: 10.1016/j.neuropharm.2016.07.037
- Hagenston, A. M., & Bading, H. (2011). Calcium signaling in synapse-to-nucleus communication. *Cold Spring Harb Perspect Biol*, 3(11), a004564. doi: 10.1101/cshperspect.a004564
- Hansen, K. B., Ogden, K. K., & Traynelis, S. F. (2012). Subunit-selective allosteric inhibition of glycine binding to NMDA receptors. J Neurosci, 32(18), 6197-6208. doi: 10.1523/JNEUROSCI.5757-11.2012

- Hansen, K. B., Ogden, K. K., Yuan, H., & Traynelis, S. F. (2014). Distinct functional and pharmacological properties of Triheteromeric GluN1/GluN2A/GluN2B NMDA receptors. *Neuron*, 81(5), 1084-1096. doi: 10.1016/j.neuron.2014.01.035
- Hara, M. R., & Snyder, S. H. (2007). Cell signaling and neuronal death. *Annu Rev Pharmacol Toxicol*, 47, 117-141. doi: 10.1146/annurev.pharmtox.47.120505.105311
- Hardingham, G. E. (2006). Pro-survival signalling from the NMDA receptor. *Biochem Soc Trans*, 34(Pt 5), 936-938. doi: 10.1042/BST0340936
- Hardingham, G. E. (2009). Coupling of the NMDA receptor to neuroprotective and neurodestructive events. *Biochem Soc Trans*, 37(Pt 6), 1147-1160. doi: 10.1042/BST0371147
- Hardingham, G. E., Arnold, F. J., & Bading, H. (2001). A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. *Nat Neurosci*, 4(6), 565-566. doi: 10.1038/88380
- Hardingham, G. E., & Bading, H. (2002). Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. *Biochim Biophys Acta*, 1600(1-2), 148-153.
- Hardingham, G. E., & Bading, H. (2010). Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci*, 11(10), 682-696. doi: 10.1038/nrn2911
- Hardingham, G. E., Fukunaga, Y., & Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci*, 5(5), 405-414. doi: 10.1038/nn835

- He, S., Shao, L. R., Wang, Y., & Bausch, S. B. (2013). Synaptic and extrasynaptic plasticity in glutamatergic circuits involving dentate granule cells following chronic N-methyl-D-aspartate receptor inhibition. J Neurophysiol, 109(6), 1535-1547. doi: 10.1152/jn.00667.2012
- Heiss, W. D. (2012). The ischemic penumbra: how does tissue injury evolve? *Ann N Y Acad Sci, 1268*, 26-34. doi: 10.1111/j.1749-6632.2012.06668.x
- Henson, M. A., Roberts, A. C., Perez-Otano, I., & Philpot, B. D. (2010). Influence of the NR3A subunit on NMDA receptor functions. *Prog Neurobiol*, 91(1), 23-37. doi: 10.1016/j.pneurobio.2010.01.004
- Hertzberg, R. P., & Pope, A. J. (2000). High-throughput screening: new technology for the 21st century. *Curr Opin Chem Biol*, *4*(4), 445-451.
- Hestrin, S., Sah, P., & Nicoll, R. A. (1990). Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron*, 5(3), 247-253.
- Hetman, M., & Kharebava, G. (2006). Survival signaling pathways activated by NMDA receptors. *Curr Top Med Chem*, 6(8), 787-799.
- Hewitt, K., & Corbett, D. (1992). Combined treatment with MK-801 and nicardipine reduces global ischemic damage in the gerbil. *Stroke*, *23*(1), 82-86.
- Honer, M., Benke, D., Laube, B., Kuhse, J., Heckendorn, R., Allgeier, H., . . . Mohler, H. (1998).
 Differentiation of glycine antagonist sites of N-methyl-D-aspartate receptor subtypes.
 Preferential interaction of CGP 61594 with NR1/2B receptors. *J Biol Chem*, 273(18), 11158-11163.

- Hosp, J. A., & Luft, A. R. (2011). Cortical plasticity during motor learning and recovery after ischemic stroke. *Neural Plast*, 2011, 871296. doi: 10.1155/2011/871296
- Hossmann, K. A. (2006). Pathophysiology and therapy of experimental stroke. *Cell Mol Neurobiol*, 26(7-8), 1057-1083. doi: 10.1007/s10571-006-9008-1
- Hossmann, K. A. (2009). Pathophysiological basis of translational stroke research. *Folia Neuropathol*, 47(3), 213-227.
- Howe, J. R., Cull-Candy, S. G., & Colquhoun, D. (1991). Currents through single glutamate receptor channels in outside-out patches from rat cerebellar granule cells. *J Physiol*, 432, 143-202.
- Hoyte, L., Barber, P. A., Buchan, A. M., & Hill, M. D. (2004). The rise and fall of NMDA antagonists for ischemic stroke. *Curr Mol Med*, 4(2), 131-136.
- Hu, N. W., Klyubin, I., Anwyl, R., & Rowan, M. J. (2009). GluN2B subunit-containing NMDA receptor antagonists prevent Abeta-mediated synaptic plasticity disruption in vivo. *Proc Natl Acad Sci U S A*, 106(48), 20504-20509. doi: 10.1073/pnas.0908083106
- Hu, S. C., Chrivia, J., & Ghosh, A. (1999). Regulation of CBP-mediated transcription by neuronal calcium signaling. *Neuron*, 22(4), 799-808.
- Iadecola, C., & Anrather, J. (2011a). The immunology of stroke: from mechanisms to translation. *Nat Med*, *17*(7), 796-808. doi: 10.1038/nm.2399
- Iadecola, C., & Anrather, J. (2011b). Stroke research at a crossroad: asking the brain for directions. *Nat Neurosci, 14*(11), 1363-1368. doi: 10.1038/nn.2953
- Ikonomidou, C., & Turski, L. (2002). Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol*, *1*(6), 383-386.

- Impey, S., Obrietan, K., Wong, S. T., Poser, S., Yano, S., Wayman, G., . . . Storm, D. R. (1998). Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron*, 21(4), 869-883.
- Impey, S., Smith, D. M., Obrietan, K., Donahue, R., Wade, C., & Storm, D. R. (1998). Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nat Neurosci*, 1(7), 595-601. doi: 10.1038/2830
- Irvine, M. W., Costa, B. M., Volianskis, A., Fang, G., Ceolin, L., Collingridge, G. L., . . . Jane, D. E. (2012). Coumarin-3-carboxylic acid derivatives as potentiators and inhibitors of recombinant and native N-methyl-D-aspartate receptors. *Neurochem Int*, *61*(4), 593-600. doi: 10.1016/j.neuint.2011.12.020
- Irving, E. A., & Bamford, M. (2002). Role of mitogen- and stress-activated kinases in ischemic injury. J Cereb Blood Flow Metab, 22(6), 631-647. doi: 10.1097/00004647-200206000-00001
- Irwin, J. J., & Shoichet, B. K. (2005). ZINC--a free database of commercially available compounds for virtual screening. J Chem Inf Model, 45(1), 177-182. doi: 10.1021/ci049714+
- Jaffer, H., Morris, V. B., Stewart, D., & Labhasetwar, V. (2011). Advances in stroke therapy. Drug Deliv Transl Res, 1(6), 409-419. doi: 10.1007/s13346-011-0046-y
- Jahr, C. E., & Stevens, C. F. (1993). Calcium permeability of the N-methyl-D-aspartate receptor channel in hippocampal neurons in culture. *Proc Natl Acad Sci U S A*, 90(24), 11573-11577.

- Jia, M., Njapo, S. A., Rastogi, V., & Hedna, V. S. (2015). Taming glutamate excitotoxicity: strategic pathway modulation for neuroprotection. *CNS Drugs*, 29(2), 153-162. doi: 10.1007/s40263-015-0225-3
- Jimenez-Blasco, D., Santofimia-Castano, P., Gonzalez, A., Almeida, A., & Bolanos, J. P. (2015). Astrocyte NMDA receptors' activity sustains neuronal survival through a Cdk5-Nrf2 pathway. *Cell Death Differ*, 22(11), 1877-1889. doi: 10.1038/cdd.2015.49
- Jin, R., Yang, G., & Li, G. (2010). Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *J Leukoc Biol*, 87(5), 779-789. doi: 10.1189/jlb.1109766
- Jordan, L. C., & Hillis, A. E. (2007). Hemorrhagic stroke in children. *Pediatr Neurol*, *36*(2), 73-80. doi: 10.1016/j.pediatrneurol.2006.09.017
- Kaindl, A. M., Degos, V., Peineau, S., Gouadon, E., Chhor, V., Loron, G., . . . Gressens, P. (2012). Activation of microglial N-methyl-D-aspartate receptors triggers inflammation and neuronal cell death in the developing and mature brain. *Ann Neurol*, 72(4), 536-549. doi: 10.1002/ana.23626
- Kalogeris, T., Baines, C. P., Krenz, M., & Korthuis, R. J. (2012). Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol*, 298, 229-317. doi: 10.1016/B978-0-12-394309-5.00006-7
- Kalogeris, T., Bao, Y., & Korthuis, R. J. (2014). Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biol*, 2, 702-714. doi: 10.1016/j.redox.2014.05.006
- Kampfl, A., Posmantur, R. M., Zhao, X., Schmutzhard, E., Clifton, G. L., & Hayes, R. L. (1997).Mechanisms of calpain proteolysis following traumatic brain injury: implications for

pathology and therapy: implications for pathology and therapy: a review and update. J Neurotrauma, 14(3), 121-134. doi: 10.1089/neu.1997.14.121

- Kanthan, R., Shuaib, A., Griebel, R., & Miyashita, H. (1995). Intracerebral human microdialysis.
 In vivo study of an acute focal ischemic model of the human brain. *Stroke*, 26(5), 870-873.
- Kapetanovic, I. M. (2008). Computer-aided drug discovery and development (CADDD): in silico-chemico-biological approach. *Chem Biol Interact*, 171(2), 165-176. doi: 10.1016/j.cbi.2006.12.006
- Karakas, E., Simorowski, N., & Furukawa, H. (2009). Structure of the zinc-bound aminoterminal domain of the NMDA receptor NR2B subunit. *EMBO J*, 28(24), 3910-3920. doi: 10.1038/emboj.2009.338
- Karakas, E., Simorowski, N., & Furukawa, H. (2011). Subunit arrangement and phenylethanolamine binding in GluN1/GluN2B NMDA receptors. *Nature*, 475(7355), 249-253. doi: 10.1038/nature10180
- Kasawar, G. B., & Farooqui, M. N. (2010). Development and validation of HPLC method for the determination of pregabalin in capsules. *Indian J Pharm Sci*, 72(4), 517-519. doi: 10.4103/0250-474X.73935
- Kaufman, A. M., Milnerwood, A. J., Sepers, M. D., Coquinco, A., She, K., Wang, L., . . . Raymond, L. A. (2012). Opposing roles of synaptic and extrasynaptic NMDA receptor signaling in cocultured striatal and cortical neurons. *J Neurosci, 32*(12), 3992-4003. doi: 10.1523/JNEUROSCI.4129-11.2012
- Kemp, J. A., & McKernan, R. M. (2002). NMDA receptor pathways as drug targets. *Nat Neurosci, 5 Suppl*, 1039-1042. doi: 10.1038/nn936

- Kidwell, C. S., Liebeskind, D. S., Starkman, S., & Saver, J. L. (2001). Trends in acute ischemic stroke trials through the 20th century. *Stroke*, *32*(6), 1349-1359.
- Kiewert, C., Mdzinarishvili, A., Hartmann, J., Bickel, U., & Klein, J. (2010). Metabolic and transmitter changes in core and penumbra after middle cerebral artery occlusion in mice. *Brain Res, 1312*, 101-107. doi: 10.1016/j.brainres.2009.11.068
- Kimelberg, H. K. (2008). Tamoxifen as a powerful neuroprotectant in experimental stroke and implications for human stroke therapy. *Recent Pat CNS Drug Discov*, *3*(2), 104-108.
- Kiritoshi, S., Nishikawa, T., Sonoda, K., Kukidome, D., Senokuchi, T., Matsuo, T., . . . Araki, E. (2003). Reactive oxygen species from mitochondria induce cyclooxygenase-2 gene expression in human mesangial cells: potential role in diabetic nephropathy. *Diabetes*, 52(10), 2570-2577.
- Kitagawa, K. (2007). CREB and cAMP response element-mediated gene expression in the ischemic brain. *FEBS J*, 274(13), 3210-3217. doi: 10.1111/j.1742-4658.2007.05890.x
- Kleppe, I. C., & Robinson, H. P. (1999). Determining the activation time course of synaptic AMPA receptors from openings of colocalized NMDA receptors. *Biophys J*, 77(3), 1418-1427. doi: 10.1016/S0006-3495(99)76990-0
- Krieger, J., Bahar, I., & Greger, I. H. (2015). Structure, Dynamics, and Allosteric Potential of Ionotropic Glutamate Receptor N-Terminal Domains. *Biophys J*, 109(6), 1136-1148. doi: 10.1016/j.bpj.2015.06.061

Kristian, T., & Siesjo, B. K. (1998). Calcium in ischemic cell death. Stroke, 29(3), 705-718.

Kunz, A., & Iadecola, C. (2009). Cerebral vascular dysregulation in the ischemic brain. *Handb Clin Neurol*, 92, 283-305. doi: 10.1016/S0072-9752(08)01914-3

- Kutsuwada, T., Sakimura, K., Manabe, T., Takayama, C., Katakura, N., Kushiya, E., . . . Mishina, M. (1996). Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor epsilon 2 subunit mutant mice. *Neuron*, 16(2), 333-344.
- Lack, N. A., Axerio-Cilies, P., Tavassoli, P., Han, F. Q., Chan, K. H., Feau, C., . . . Cherkasov,
 A. (2011). Targeting the binding function 3 (BF3) site of the human androgen receptor through virtual screening. *J Med Chem*, 54(24), 8563-8573. doi: 10.1021/jm201098n
- Lai, T. W., Shyu, W. C., & Wang, Y. T. (2011). Stroke intervention pathways: NMDA receptors and beyond. *Trends Mol Med*, *17*(5), 266-275. doi: 10.1016/j.molmed.2010.12.008
- Lai, T. W., Zhang, S., & Wang, Y. T. (2014). Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Prog Neurobiol*, *115*, 157-188. doi: 10.1016/j.pneurobio.2013.11.006
- Lakhan, S. E., Kirchgessner, A., & Hofer, M. (2009). Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *J Transl Med*, *7*, 97. doi: 10.1186/1479-5876-7-97
- Lau, C. G., & Zukin, R. S. (2007). NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nat Rev Neurosci*, 8(6), 413-426. doi: 10.1038/nrn2153
- Lau, D., Bengtson, C. P., Buchthal, B., & Bading, H. (2015). BDNF Reduces Toxic Extrasynaptic NMDA Receptor Signaling via Synaptic NMDA Receptors and Nuclear-Calcium-Induced Transcription of inhba/Activin A. Cell Rep, 12(8), 1353-1366. doi: 10.1016/j.celrep.2015.07.038
- Lauritzen, M., Dreier, J. P., Fabricius, M., Hartings, J. A., Graf, R., & Strong, A. J. (2011). Clinical relevance of cortical spreading depression in neurological disorders: migraine,

malignant stroke, subarachnoid and intracranial hemorrhage, and traumatic brain injury. *J Cereb Blood Flow Metab*, *31*(1), 17-35. doi: 10.1038/jcbfm.2010.191

- Layton, M. E., Kelly, M. J., 3rd, & Rodzinak, K. J. (2006). Recent advances in the development of NR2B subtype-selective NMDA receptor antagonists. *Curr Top Med Chem*, 6(7), 697-709.
- Lee, B., Butcher, G. Q., Hoyt, K. R., Impey, S., & Obrietan, K. (2005). Activity-dependent neuroprotection and cAMP response element-binding protein (CREB): kinase coupling, stimulus intensity, and temporal regulation of CREB phosphorylation at serine 133. J Neurosci, 25(5), 1137-1148. doi: 10.1523/JNEUROSCI.4288-04.2005
- Lee, B. D., Kim, S., Hur, E. M., Park, Y. S., Kim, Y. H., Lee, T. G., . . . Ryu, S. H. (2005). Leumorphin has an anti-apoptotic effect by activating epidermal growth factor receptor kinase in rat pheochromocytoma PC12 cells. *J Neurochem*, 95(1), 56-67. doi: 10.1111/j.1471-4159.2005.03339.x
- Lee, C. H., Huang, H. C., & Juan, H. F. (2011). Reviewing ligand-based rational drug design: the search for an ATP synthase inhibitor. *Int J Mol Sci, 12*(8), 5304-5318. doi: 10.3390/ijms12085304
- Lee, C. H., Lu, W., Michel, J. C., Goehring, A., Du, J., Song, X., & Gouaux, E. (2014). NMDA receptor structures reveal subunit arrangement and pore architecture. *Nature*, 511(7508), 191-197. doi: 10.1038/nature13548
- Lee, J. M., Grabb, M. C., Zipfel, G. J., & Choi, D. W. (2000). Brain tissue responses to ischemia. *J Clin Invest, 106*(6), 723-731. doi: 10.1172/JCI11003
- Lee, J. M., Zipfel, G. J., & Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature*, 399(6738 Suppl), A7-14.

- Li, L., Fan, M., Icton, C. D., Chen, N., Leavitt, B. R., Hayden, M. R., . . . Raymond, L. A. (2003). Role of NR2B-type NMDA receptors in selective neurodegeneration in Huntington disease. *Neurobiol Aging*, 24(8), 1113-1121.
- Li, R., Huang, F. S., Abbas, A. K., & Wigstrom, H. (2007). Role of NMDA receptor subtypes in different forms of NMDA-dependent synaptic plasticity. *BMC Neurosci*, 8, 55. doi: 10.1186/1471-2202-8-55
- Li, Y., Chopp, M., Jiang, N., Zhang, Z. G., & Zaloga, C. (1995). Induction of DNA fragmentation after 10 to 120 minutes of focal cerebral ischemia in rats. *Stroke*, 26(7), 1252-1257; discussion 1257-1258.
- Liang, D., Bhatta, S., Gerzanich, V., & Simard, J. M. (2007). Cytotoxic edema: mechanisms of pathological cell swelling. *Neurosurg Focus*, 22(5), E2.
- Lipinski, C. A. (2000). Drug-like properties and the causes of poor solubility and poor permeability. *J Pharmacol Toxicol Methods*, 44(1), 235-249.
- Lipinski, C. A., Lombardo, F., Dominy, B. W., & Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev, 46*(1-3), 3-26.
- Lipton, P. (1999). Ischemic cell death in brain neurons. *Physiol Rev*, 79(4), 1431-1568.
- Lipton, S. A., & Nicotera, P. (1998). Calcium, free radicals and excitotoxins in neuronal apoptosis. *Cell Calcium*, 23(2-3), 165-171.
- Lipton, S. A., & Rosenberg, P. A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med, 330(9), 613-622. doi: 10.1056/NEJM199403033300907

- Liu, H., Zhang, X., Du, Y., Ji, H., Li, S., Li, L., . . . Cao, X. (2012). Leonurine protects brain injury by increased activities of UCP4, SOD, CAT and Bcl-2, decreased levels of MDA and Bax, and ameliorated ultrastructure of mitochondria in experimental stroke. *Brain Res*, 1474, 73-81. doi: 10.1016/j.brainres.2012.07.028
- Liu, L., Wong, T. P., Pozza, M. F., Lingenhoehl, K., Wang, Y., Sheng, M., . . . Wang, Y. T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*, 304(5673), 1021-1024. doi: 10.1126/science.1096615
- Liu, P. K., Robertson, C. S., & Valadka, A. (2002). The association between neuronal nitric oxide synthase and neuronal sensitivity in the brain after brain injury. *Ann N Y Acad Sci*, 962, 226-241.
- Liu, R. R., & Murphy, T. H. (2009). Reversible cyclosporin A-sensitive mitochondrial depolarization occurs within minutes of stroke onset in mouse somatosensory cortex in vivo: a two-photon imaging study. J Biol Chem, 284(52), 36109-36117. doi: 10.1074/jbc.M109.055301
- Liu, S., Levine, S. R., & Winn, H. R. (2010). Targeting ischemic penumbra: part I from pathophysiology to therapeutic strategy. *J Exp Stroke Transl Med*, *3*(1), 47-55.
- Liu, X. B., Murray, K. D., & Jones, E. G. (2004). Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. J *Neurosci*, 24(40), 8885-8895. doi: 10.1523/JNEUROSCI.2476-04.2004
- Liu, X., Ye, R., Yan, T., Yu, S. P., Wei, L., Xu, G., . . . Chen, J. (2014). Cell based therapies for ischemic stroke: from basic science to bedside. *Prog Neurobiol*, 115, 92-115. doi: 10.1016/j.pneurobio.2013.11.007

- Liu, Y., Wong, T. P., Aarts, M., Rooyakkers, A., Liu, L., Lai, T. W., ... Wang, Y. T. (2007).
 NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J Neurosci, 27*(11), 2846-2857. doi: 10.1523/JNEUROSCI.0116-07.2007
- Lo, E. H., Dalkara, T., & Moskowitz, M. A. (2003). Mechanisms, challenges and opportunities in stroke. *Nat Rev Neurosci*, *4*(5), 399-415. doi: 10.1038/nrn1106
- Lohmann, C., & Kessels, H. W. (2014). The developmental stages of synaptic plasticity. J Physiol, 592(1), 13-31. doi: 10.1113/jphysiol.2012.235119
- Lonze, B. E., & Ginty, D. D. (2002). Function and regulation of CREB family transcription factors in the nervous system. *Neuron*, *35*(4), 605-623.
- Lopez-Atalaya, J. P., Roussel, B. D., Levrat, D., Parcq, J., Nicole, O., Hommet, Y., . . . Vivien,
 D. (2008). Toward safer thrombolytic agents in stroke: molecular requirements for
 NMDA receptor-mediated neurotoxicity. *J Cereb Blood Flow Metab*, 28(6), 1212-1221.
 doi: 10.1038/jcbfm.2008.14
- Low, C. M., & Wee, K. S. (2010). New insights into the not-so-new NR3 subunits of N-methyl D-aspartate receptor: localization, structure, and function. *Mol Pharmacol*, 78(1), 1-11.
 doi: 10.1124/mol.110.064006
- Lucas, D. R., & Newhouse, J. P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. *AMA Arch Ophthalmol*, 58(2), 193-201.
- Luo, T., Wu, W. H., & Chen, B. S. (2011). NMDA receptor signaling: death or survival? *Front Biol (Beijing)*, 6(6), 468-476. doi: 10.1007/s11515-011-1187-6

- Luscher, C., & Malenka, R. C. (2012). NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). *Cold Spring Harb Perspect Biol*, 4(6). doi: 10.1101/cshperspect.a005710
- Ma, M. T., Yeo, J. F., Farooqui, A. A., & Ong, W. Y. (2011). Role of calcium independent phospholipase A2 in maintaining mitochondrial membrane potential and preventing excessive exocytosis in PC12 cells. *Neurochem Res*, 36(2), 347-354. doi: 10.1007/s11064-010-0340-y
- MacDermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J., & Barker, J. L. (1986). NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature*, 321(6069), 519-522. doi: 10.1038/321519a0
- Machado, V. M., Morte, M. I., Carreira, B. P., Azevedo, M. M., Takano, J., Iwata, N., . . . Araujo, I. M. (2015). Involvement of calpains in adult neurogenesis: implications for stroke. *Front Cell Neurosci*, 9, 22. doi: 10.3389/fncel.2015.00022
- Macrae, I. M. (2011). Preclinical stroke research--advantages and disadvantages of the most common rodent models of focal ischaemia. *Br J Pharmacol*, 164(4), 1062-1078. doi: 10.1111/j.1476-5381.2011.01398.x
- Magnusson, K. R. (2012). Aging of the NMDA receptor: from a mouse's point of view. *Future Neurol*, 7(5), 627-637. doi: 10.2217/fnl.12.54
- Majid, A. (2014). Neuroprotection in stroke: past, present, and future. *ISRN Neurol*, 2014, 515716. doi: 10.1155/2014/515716
- Malherbe, P., Mutel, V., Broger, C., Perin-Dureau, F., Kemp, J. A., Neyton, J., . . . Kew, J. N. (2003). Identification of critical residues in the amino terminal domain of the human

NR2B subunit involved in the RO 25-6981 binding pocket. *J Pharmacol Exp Ther*, 307(3), 897-905. doi: 10.1124/jpet.103.056291

Mander, T. (2000). Beyond uHTS: ridiculously HTS? Drug Discov Today, 5(6), 223-225.

- Marchand, P., Becerril-Ortega, J., Mony, L., Bouteiller, C., Paoletti, P., Nicole, O., ... Perrio, C. (2012). Confocal microscopy imaging of NR2B-containing NMDA receptors based on fluorescent ifenprodil-like conjugates. *Bioconjug Chem*, 23(1), 21-26. doi: 10.1021/bc100571g
- Mark, L. P., Prost, R. W., Ulmer, J. L., Smith, M. M., Daniels, D. L., Strottmann, J. M., . . . Hacein-Bey, L. (2001). Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging. *AJNR Am J Neuroradiol*, 22(10), 1813-1824.
- Maroto, R., & Perez-Polo, J. R. (1997). BCL-2-related protein expression in apoptosis: oxidative stress versus serum deprivation in PC12 cells. *J Neurochem*, 69(2), 514-523.
- Martel, M. A., Ryan, T. J., Bell, K. F., Fowler, J. H., McMahon, A., Al-Mubarak, B., . . . Hardingham, G. E. (2012). The subtype of GluN2 C-terminal domain determines the response to excitotoxic insults. *Neuron*, 74(3), 543-556. doi: 10.1016/j.neuron.2012.03.021
- Massey, P. V., Johnson, B. E., Moult, P. R., Auberson, Y. P., Brown, M. W., Molnar, E., . . .
 Bashir, Z. I. (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J Neurosci, 24*(36), 7821-7828. doi: 10.1523/JNEUROSCI.1697-04.2004
- Masuko, T., Kashiwagi, K., Kuno, T., Nguyen, N. D., Pahk, A. J., Fukuchi, J., . . . Williams, K. (1999). A regulatory domain (R1-R2) in the amino terminus of the N-methyl-D-aspartate

receptor: effects of spermine, protons, and ifenprodil, and structural similarity to bacterial leucine/isoleucine/valine binding protein. *Mol Pharmacol*, *55*(6), 957-969.

- Mattson, M. P. (1997). Neuroprotective signal transduction: relevance to stroke. *Neurosci Biobehav Rev*, 21(2), 193-206.
- Mattson, M. P. (2008). Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann N Y Acad Sci, 1144, 97-112. doi: 10.1196/annals.1418.005
- Mattson, M. P., Duan, W., Pedersen, W. A., & Culmsee, C. (2001). Neurodegenerative disorders and ischemic brain diseases. *Apoptosis*, 6(1-2), 69-81.
- Mayer, M. L., Westbrook, G. L., & Guthrie, P. B. (1984). Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature*, *309*(5965), 261-263.
- McCauley, J. A., Theberge, C. R., Romano, J. J., Billings, S. B., Anderson, K. D., Claremon, D.
 A., . . Liverton, N. J. (2004). NR2B-selective N-methyl-D-aspartate antagonists: synthesis and evaluation of 5-substituted benzimidazoles. *J Med Chem*, 47(8), 2089-2096. doi: 10.1021/jm030483s
- McCulloch, J. (1991). Ischaemic brain damage--prevention with competitive and noncompetitive antagonists of N-methyl-D-aspartate receptors. Arzneimittelforschung, 41(3A), 319-324.
- Mehan, S., Meena, H., Sharma, D., & Sankhla, R. (2011). JNK: a stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. *J Mol Neurosci*, 43(3), 376-390. doi: 10.1007/s12031-010-9454-6
- Micieli, G., Marcheselli, S., & Tosi, P. A. (2009). Safety and efficacy of alteplase in the treatment of acute ischemic stroke. *Vasc Health Risk Manag*, *5*(1), 397-409.

- Mielke, J. G., & Wang, Y. T. (2005). Insulin exerts neuroprotection by counteracting the decrease in cell-surface GABA receptors following oxygen-glucose deprivation in cultured cortical neurons. J Neurochem, 92(1), 103-113. doi: 10.1111/j.1471-4159.2004.02841.x
- Miller, D. J., Simpson, J. R., & Silver, B. (2011). Safety of thrombolysis in acute ischemic stroke: a review of complications, risk factors, and newer technologies. *Neurohospitalist*, 1(3), 138-147. doi: 10.1177/1941875211408731
- Minnerup, J., Sutherland, B. A., Buchan, A. M., & Kleinschnitz, C. (2012). Neuroprotection for stroke: current status and future perspectives. *Int J Mol Sci, 13*(9), 11753-11772. doi: 10.3390/ijms130911753
- Misra, A., Ganesh, S., Shahiwala, A., & Shah, S. P. (2003). Drug delivery to the central nervous system: a review. *J Pharm Pharm Sci*, 6(2), 252-273.
- Moha Ou Maati, H., Widmann, C., Sedjelmaci, D., Gallois, B., Heurteaux, C., Borsotto, M., & Hugues, M. (2013). Mapacalcine protects mouse neurons against hypoxia by blocking cell calcium overload. *PLoS One*, 8(7), e66194. doi: 10.1371/journal.pone.0066194
- Monaghan, D. T., Irvine, M. W., Costa, B. M., Fang, G., & Jane, D. E. (2012). Pharmacological modulation of NMDA receptor activity and the advent of negative and positive allosteric modulators. *Neurochem Int*, 61(4), 581-592. doi: 10.1016/j.neuint.2012.01.004
- Monaghan, D. T., & Jane, D. E. (2009). Pharmacology of NMDA Receptors. In A. M. Van Dongen (Ed.), *Biology of the NMDA Receptor*. Boca Raton (FL).
- Mongin, A. A. (2007). Disruption of ionic and cell volume homeostasis in cerebral ischemia:
 The perfect storm. *Pathophysiology*, 14(3-4), 183-193. doi: 10.1016/j.pathophys.2007.09.009

- Mony, L., Kew, J. N., Gunthorpe, M. J., & Paoletti, P. (2009). Allosteric modulators of NR2Bcontaining NMDA receptors: molecular mechanisms and therapeutic potential. *Br J Pharmacol*, 157(8), 1301-1317. doi: 10.1111/j.1476-5381.2009.00304.x
- Mony, L., Triballeau, N., Paoletti, P., Acher, F. C., & Bertrand, H. O. (2010). Identification of a novel NR2B-selective NMDA receptor antagonist using a virtual screening approach.
 Bioorg Med Chem Lett, 20(18), 5552-5558. doi: 10.1016/j.bmcl.2010.07.043
- Morley, R. M., Tse, H. W., Feng, B., Miller, J. C., Monaghan, D. T., & Jane, D. E. (2005). Synthesis and pharmacology of N1-substituted piperazine-2,3-dicarboxylic acid derivatives acting as NMDA receptor antagonists. *J Med Chem*, 48(7), 2627-2637. doi: 10.1021/jm0492498
- Morrison, J. S., Nophsker, M. J., & Haskell, R. J. (2014). A combination turbidity and supernatant microplate assay to rank-order the supersaturation limits of early drug candidates. *J Pharm Sci*, *103*(10), 3022-3032. doi: 10.1002/jps.24090
- Morrison, R. S., Kinoshita, Y., Johnson, M. D., Ghatan, S., Ho, J. T., & Garden, G. (2002). Neuronal survival and cell death signaling pathways. *Adv Exp Med Biol*, *513*, 41-86.
- Moskowitz, M. A., Lo, E. H., & Iadecola, C. (2010). The science of stroke: mechanisms in search of treatments. *Neuron*, 67(2), 181-198. doi: 10.1016/j.neuron.2010.07.002
- Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., . . . Stroke Statistics, Subcommittee. (2015). Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation*, 131(4), e29-322. doi: 10.1161/CIR.00000000000152
- Muir, K. W. (2006). Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists. *Curr Opin Pharmacol*, *6*(1), 53-60. doi: 10.1016/j.coph.2005.12.002

- Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem J*, 417(1), 1-13. doi: 10.1042/BJ20081386
- Murphy, T. H., & Corbett, D. (2009). Plasticity during stroke recovery: from synapse to behaviour. *Nat Rev Neurosci, 10*(12), 861-872. doi: 10.1038/nrn2735
- Mutel, V., Buchy, D., Klingelschmidt, A., Messer, J., Bleuel, Z., Kemp, J. A., & Richards, J. G. (1998). In vitro binding properties in rat brain of [3H]Ro 25-6981, a potent and selective antagonist of NMDA receptors containing NR2B subunits. *J Neurochem*, 70(5), 2147-2155.
- Myers, S. J., Dingledine, R., & Borges, K. (1999). Genetic regulation of glutamate receptor ion channels. *Annu Rev Pharmacol Toxicol*, 39, 221-241. doi: 10.1146/annurev.pharmtox.39.1.221
- Nedergaard, M., & Hansen, A. J. (1993). Characterization of cortical depolarizations evoked in focal cerebral ischemia. J Cereb Blood Flow Metab, 13(4), 568-574. doi: 10.1038/jcbfm.1993.74
- Nero, T. L., Morton, C. J., Holien, J. K., Wielens, J., & Parker, M. W. (2014). Oncogenic protein interfaces: small molecules, big challenges. *Nat Rev Cancer*, 14(4), 248-262. doi: 10.1038/nrc3690
- Neyton, J., & Paoletti, P. (2006). Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. *J Neurosci*, 26(5), 1331-1333. doi: 10.1523/JNEUROSCI.5242-05.2006
- Nicholls, D. G. (2009). Mitochondrial calcium function and dysfunction in the central nervous system. *Biochim Biophys Acta*, *1787*(11), 1416-1424. doi: 10.1016/j.bbabio.2009.03.010

- Nicotera, P. (2003). Molecular switches deciding the death of injured neurons. *Toxicol Sci*, 74(1), 4-9. doi: 10.1093/toxsci/kfg109
- Nikoletopoulou, V., & Tavernarakis, N. (2012). Calcium homeostasis in aging neurons. *Front Genet*, *3*, 200. doi: 10.3389/fgene.2012.00200
- Northington, F. J., Chavez-Valdez, R., & Martin, L. J. (2011). Neuronal cell death in neonatal hypoxia-ischemia. *Ann Neurol*, *69*(5), 743-758. doi: 10.1002/ana.22419
- Nowak, G., Li, Y., & Paul, I. A. (2000). Chronic glycine treatment desensitizes the behavioral response to 1-aminocyclopropanecarboxylic acid (ACPC), a partial agonist at the strychnine-insensitive glycine site of the NMDA receptor complex. *J Neural Transm* (*Vienna*), 107(2), 123-131. doi: 10.1007/s007020050011
- Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., & Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, *307*(5950), 462-465.
- O'Collins, V. E., Macleod, M. R., Donnan, G. A., Horky, L. L., van der Worp, B. H., & Howells,
 D. W. (2006). 1,026 experimental treatments in acute stroke. *Ann Neurol*, 59(3), 467-477.
 doi: 10.1002/ana.20741
- Ogden, K. K., & Traynelis, S. F. (2011). New advances in NMDA receptor pharmacology. *Trends Pharmacol Sci*, 32(12), 726-733. doi: 10.1016/j.tips.2011.08.003
- Ong, W. Y., Kim, J. H., He, X., Chen, P., Farooqui, A. A., & Jenner, A. M. (2010). Changes in brain cholesterol metabolome after excitotoxicity. *Mol Neurobiol*, 41(2-3), 299-313. doi: 10.1007/s12035-010-8099-3
- Oprea, T. I. (2000). Property distribution of drug-related chemical databases. *J Comput Aided Mol Des*, 14(3), 251-264.

- Oprea, T. I., Davis, A. M., Teague, S. J., & Leeson, P. D. (2001). Is there a difference between leads and drugs? A historical perspective. *J Chem Inf Comput Sci*, *41*(5), 1308-1315.
- Otvos, L., Jr., & Wade, J. D. (2014). Current challenges in peptide-based drug discovery. *Front Chem*, 2, 62. doi: 10.3389/fchem.2014.00062
- Ouyang, Y. B., & Giffard, R. G. (2012). ER-Mitochondria Crosstalk during Cerebral Ischemia: Molecular Chaperones and ER-Mitochondrial Calcium Transfer. *Int J Cell Biol*, 2012, 493934. doi: 10.1155/2012/493934
- Ovbiagele, B., Goldstein, L. B., Higashida, R. T., Howard, V. J., Johnston, S. C., Khavjou, O. A., . . . Stroke, Council. (2013). Forecasting the future of stroke in the United States: a policy statement from the American Heart Association and American Stroke Association. *Stroke*, *44*(8), 2361-2375. doi: 10.1161/STR.0b013e31829734f2
- Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*, 87(1), 315-424. doi: 10.1152/physrev.00029.2006
- Pajouhesh, H., & Lenz, G. R. (2005). Medicinal chemical properties of successful central nervous system drugs. *NeuroRx*, 2(4), 541-553. doi: 10.1602/neurorx.2.4.541
- Palmer, G. C., Miller, J. A., Cregan, E. F., Gendron, P., & Peeling, J. (1997). Low-affinity NMDA receptor antagonists. The neuroprotective potential of ARL 15896AR. Ann N Y Acad Sci, 825, 220-231.
- Paoletti, P., Ascher, P., & Neyton, J. (1997). High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J Neurosci, 17(15), 5711-5725.
- Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci*, 14(6), 383-400. doi: 10.1038/nrn3504

- Paoletti, P., & Neyton, J. (2007). NMDA receptor subunits: function and pharmacology. *Curr Opin Pharmacol*, 7(1), 39-47. doi: 10.1016/j.coph.2006.08.011
- Paoletti, P., Perin-Dureau, F., Fayyazuddin, A., Le Goff, A., Callebaut, I., & Neyton, J. (2000). Molecular organization of a zinc binding n-terminal modulatory domain in a NMDA receptor subunit. *Neuron*, 28(3), 911-925.
- Papadia, S., & Hardingham, G. E. (2007). The dichotomy of NMDA receptor signaling. *Neuroscientist*, *13*(6), 572-579. doi: 10.1177/10738584070130060401
- Papadia, S., Soriano, F. X., Leveille, F., Martel, M. A., Dakin, K. A., Hansen, H. H., . . . Hardingham, G. E. (2008). Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. *Nat Neurosci*, 11(4), 476-487. doi: 10.1038/nn2071
- Papouin, T., Ladepeche, L., Ruel, J., Sacchi, S., Labasque, M., Hanini, M., . . . Oliet, S. H.
 (2012). Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. *Cell*, *150*(3), 633-646. doi: 10.1016/j.cell.2012.06.029
- Papouin, T., & Oliet, S. H. (2014). Organization, control and function of extrasynaptic NMDA receptors. *Philos Trans R Soc Lond B Biol Sci*, 369(1654), 20130601. doi: 10.1098/rstb.2013.0601
- Parnis, J., Montana, V., Delgado-Martinez, I., Matyash, V., Parpura, V., Kettenmann, H., . . .
 Nolte, C. (2013). Mitochondrial exchanger NCLX plays a major role in the intracellular
 Ca2+ signaling, gliotransmission, and proliferation of astrocytes. *J Neurosci, 33*(17), 7206-7219. doi: 10.1523/JNEUROSCI.5721-12.2013
- Paschen, W., & Frandsen, A. (2001). Endoplasmic reticulum dysfunction--a common denominator for cell injury in acute and degenerative diseases of the brain? *J Neurochem*, 79(4), 719-725.

- Patschull, A. O., Gooptu, B., Ashford, P., Daviter, T., & Nobeli, I. (2012). In silico assessment of potential druggable pockets on the surface of alpha1-antitrypsin conformers. *PLoS One*, 7(5), e36612. doi: 10.1371/journal.pone.0036612
- Pei, J., You, X., & Fu, Q. (2015). Inflammation in the pathogenesis of ischemic stroke. Front Biosci (Landmark Ed), 20, 772-783.
- Pekna, M., Pekny, M., & Nilsson, M. (2012). Modulation of neural plasticity as a basis for stroke rehabilitation. *Stroke*, 43(10), 2819-2828. doi: 10.1161/STROKEAHA.112.654228
- Perin-Dureau, F., Rachline, J., Neyton, J., & Paoletti, P. (2002). Mapping the binding site of the neuroprotectant ifenprodil on NMDA receptors. *J Neurosci*, 22(14), 5955-5965. doi: 20026631
- Perng, D. W., Wu, Y. C., Chang, K. T., Wu, M. T., Chiou, Y. C., Su, K. C., . . . Lee, Y. C. (2006). Leukotriene C4 induces TGF-beta1 production in airway epithelium via p38 kinase pathway. Am J Respir Cell Mol Biol, 34(1), 101-107. doi: 10.1165/rcmb.2005-00680C
- Petito, C. K., Feldmann, E., Pulsinelli, W. A., & Plum, F. (1987). Delayed hippocampal damage in humans following cardiorespiratory arrest. *Neurology*, *37*(8), 1281-1286.
- Petralia, R. S. (2012). Distribution of extrasynaptic NMDA receptors on neurons. *ScientificWorldJournal*, 2012, 267120. doi: 10.1100/2012/267120
- Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*, 25(13), 1605-1612. doi: 10.1002/jcc.20084
- Pettigrew, L. C., Holtz, M. L., Craddock, S. D., Minger, S. L., Hall, N., & Geddes, J. W. (1996). Microtubular proteolysis in focal cerebral ischemia. J Cereb Blood Flow Metab, 16(6), 1189-1202. doi: 10.1097/00004647-199611000-00013
- Pilati, N., Linley, D. M., Selvaskandan, H., Uchitel, O., Hennig, M. H., Kopp-Scheinpflug, C., & Forsythe, I. D. (2016). Acoustic trauma slows AMPA receptor-mediated EPSCs in the auditory brainstem, reducing GluA4 subunit expression as a mechanism to rescue binaural function. *J Physiol*, 594(13), 3683-3703. doi: 10.1113/JP271929
- Popp, A., Jaenisch, N., Witte, O. W., & Frahm, C. (2009). Identification of ischemic regions in a rat model of stroke. *PLoS One*, *4*(3), e4764. doi: 10.1371/journal.pone.0004764
- Porras, A., Zuluaga, S., Black, E., Valladares, A., Alvarez, A. M., Ambrosino, C., . . . Nebreda,
 A. R. (2004). P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. *Mol Biol Cell*, 15(2), 922-933. doi: 10.1091/mbc.E03-08-0592
- Prass, K., & Dirnagl, U. (1998). Glutamate antagonists in therapy of stroke. *Restor Neurol Neurosci*, 13(1-2), 3-10.
- Punnakkal, P., Jendritza, P., & Kohr, G. (2012). Influence of the intracellular GluN2 C-terminal domain on NMDA receptor function. *Neuropharmacology*, 62(5-6), 1985-1992. doi: 10.1016/j.neuropharm.2011.12.018
- Qu, X. X., Cai, J., Li, M. J., Chi, Y. N., Liao, F. F., Liu, F. Y., . . . Xing, G. G. (2009). Role of the spinal cord NR2B-containing NMDA receptors in the development of neuropathic pain. *Exp Neurol*, 215(2), 298-307. doi: 10.1016/j.expneurol.2008.10.018

- Rainer, M., Wuschitz, A., Jagsch, C., Erb, C., Chirikdjian, J. J., & Mucke, H. A. (2011).
 Memantine in moderate to severe Alzheimer's disease: an observational post-marketing study. *J Neural Transm (Vienna)*, *118*(8), 1255-1259. doi: 10.1007/s00702-011-0623-8
- Rammes, G., Danysz, W., & Parsons, C. G. (2008). Pharmacodynamics of memantine: an update. *Curr Neuropharmacol*, *6*(1), 55-78. doi: 10.2174/157015908783769671
- Regan, M. C., Romero-Hernandez, A., & Furukawa, H. (2015). A structural biology perspective on NMDA receptor pharmacology and function. *Curr Opin Struct Biol*, 33, 68-75. doi: 10.1016/j.sbi.2015.07.012
- Ribary, U., & Lichtensteiger, W. (1989). Effects of acute and chronic prenatal nicotine treatment on central catecholamine systems of male and female rat fetuses and offspring. J Pharmacol Exp Ther, 248(2), 786-792.
- Rink, C., & Khanna, S. (2011). Significance of brain tissue oxygenation and the arachidonic acid cascade in stroke. *Antioxid Redox Signal*, *14*(10), 1889-1903. doi: 10.1089/ars.2010.3474
- Rock, K. L., & Kono, H. (2008). The inflammatory response to cell death. *Annu Rev Pathol, 3*, 99-126. doi: 10.1146/annurev.pathmechdis.3.121806.151456
- Rossi, D. J., Brady, J. D., & Mohr, C. (2007). Astrocyte metabolism and signaling during brain ischemia. *Nat Neurosci, 10*(11), 1377-1386. doi: 10.1038/nn2004
- Rothman, S. M. (1983). Synaptic activity mediates death of hypoxic neurons. *Science*, 220(4596), 536-537.
- Rothman, S. M., & Olney, J. W. (1995). Excitotoxicity and the NMDA receptor--still lethal after eight years. *Trends Neurosci*, *18*(2), 57-58.

- Rungta, R. L., Choi, H. B., Tyson, J. R., Malik, A., Dissing-Olesen, L., Lin, P. J., . . . MacVicar,
 B. A. (2015). The cellular mechanisms of neuronal swelling underlying cytotoxic edema. *Cell*, 161(3), 610-621. doi: 10.1016/j.cell.2015.03.029
- Ryan, T. J., Emes, R. D., Grant, S. G., & Komiyama, N. H. (2008). Evolution of NMDA receptor cytoplasmic interaction domains: implications for organisation of synaptic signalling complexes. *BMC Neurosci*, 9, 6. doi: 10.1186/1471-2202-9-6
- Saatman, K. E., Creed, J., & Raghupathi, R. (2010). Calpain as a therapeutic target in traumatic brain injury. *Neurotherapeutics*, 7(1), 31-42. doi: 10.1016/j.nurt.2009.11.002
- Sachdeva, S., Lobo, S., & Goswami, T. (2016). What is the future of noninvasive routes for protein- and peptide-based drugs? *Ther Deliv*, *7*(6), 355-357. doi: 10.4155/tde-2016-0031
- Sakimura, K., Kutsuwada, T., Ito, I., Manabe, T., Takayama, C., Kushiya, E., . . . et al. (1995).
 Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon
 1 subunit. *Nature*, *373*(6510), 151-155. doi: 10.1038/373151a0
- Sattler, R., & Tymianski, M. (2000). Molecular mechanisms of calcium-dependent excitotoxicity. *J Mol Med (Berl)*, 78(1), 3-13.
- Saver, J. L., Kidwell, C. S., Liebeskind, D. S., & Starkman, S. (2001). Acute ischemic stroke trials. *Stroke*, *32*(1), 275-278.
- Scatton, B. (1994). Excitatory amino acid receptor antagonists: a novel treatment for ischemic cerebrovascular diseases. *Life Sci*, *55*(25-26), 2115-2124.
- Schaar, K. L., Brenneman, M. M., & Savitz, S. I. (2010). Functional assessments in the rodent stroke model. *Exp Transl Stroke Med*, 2(1), 13. doi: 10.1186/2040-7378-2-13

- Schmidt, B. J., Hochman, S., & MacLean, J. N. (1998). NMDA receptor-mediated oscillatory properties: potential role in rhythm generation in the mammalian spinal cord. Ann N Y Acad Sci, 860, 189-202.
- Seeburg, P. H. (1993a). The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci*, *16*(9), 359-365.
- Seeburg, P. H. (1993b). The TiPS/TINS lecture: the molecular biology of mammalian glutamate receptor channels. *Trends Pharmacol Sci*, 14(8), 297-303. doi: 10.1016/0165-6147(93)90047-N
- Seeburg, P. H., Burnashev, N., Kohr, G., Kuner, T., Sprengel, R., & Monyer, H. (1995). The NMDA receptor channel: molecular design of a coincidence detector. *Recent Prog Horm Res*, 50, 19-34.
- Seidenstein, A. H., Barone, F. C., & Lytton, W. W. (2015). Computer modeling of ischemic stroke. *Scholarpedia J*, 10(3). doi: 10.4249/scholarpedia.32015
- Semenova, M. M., Maki-Hokkonen, A. M., Cao, J., Komarovski, V., Forsberg, K. M., Koistinaho, M., . . . Courtney, M. J. (2007). Rho mediates calcium-dependent activation of p38alpha and subsequent excitotoxic cell death. *Nat Neurosci, 10*(4), 436-443. doi: 10.1038/nn1869
- Shah, K., & Abbruscato, T. (2014). The role of blood-brain barrier transporters in pathophysiology and pharmacotherapy of stroke. *Curr Pharm Des*, 20(10), 1510-1522.
- Shen, H., & Wang, Y. (2010). Correlation of locomotor activity and brain infarction in rats with transient focal ischemia. J Neurosci Methods, 186(2), 150-154. doi: 10.1016/j.jneumeth.2009.11.008

- Shipton, O. A., & Paulsen, O. (2014). GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. *Philos Trans R Soc Lond B Biol Sci, 369*(1633), 20130163. doi: 10.1098/rstb.2013.0163
- Shirley, R., Ord, E. N., & Work, L. M. (2014). Oxidative Stress and the Use of Antioxidants in Stroke. *Antioxidants (Basel)*, *3*(3), 472-501. doi: 10.3390/antiox3030472
- Shleper, M., Kartvelishvily, E., & Wolosker, H. (2005). D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. J Neurosci, 25(41), 9413-9417. doi: 10.1523/JNEUROSCI.3190-05.2005
- Shohami, E., & Biegon, A. (2014). Novel approach to the role of NMDA receptors in traumatic brain injury. *CNS Neurol Disord Drug Targets, 13*(4), 567-573.
- Shu, S., Pei, L., & Lu, Y. (2014). Promising targets of cell death signaling of NR2B receptor subunit in stroke pathogenesis. *Regen Med Res*, 2(1), 8. doi: 10.1186/2050-490X-2-8
- Shyu, W. C., Lin, S. Z., Chiang, M. F., Chen, D. C., Su, C. Y., Wang, H. J., . . . Li, H. (2008). Secretoneurin promotes neuroprotection and neuronal plasticity via the Jak2/Stat3 pathway in murine models of stroke. *J Clin Invest*, *118*(1), 133-148. doi: 10.1172/JCI32723
- Shyu, W. C., Lin, S. Z., Yen, P. S., Su, C. Y., Chen, D. C., Wang, H. J., & Li, H. (2008). Stromal cell-derived factor-1 alpha promotes neuroprotection, angiogenesis, and mobilization/homing of bone marrow-derived cells in stroke rats. *J Pharmacol Exp Ther*, 324(2), 834-849. doi: 10.1124/jpet.107.127746
- Shyu, W. C., Liu, D. D., Lin, S. Z., Li, W. W., Su, C. Y., Chang, Y. C., . . . Li, H. (2008). Implantation of olfactory ensheathing cells promotes neuroplasticity in murine models of stroke. *J Clin Invest*, 118(7), 2482-2495. doi: 10.1172/JCI34363

- Sicard, K. M., & Fisher, M. (2009). Animal models of focal brain ischemia. *Exp Transl Stroke Med*, 1, 7. doi: 10.1186/2040-7378-1-7
- Simon, R. P., Swan, J. H., Griffiths, T., & Meldrum, B. S. (1984). Blockade of N-methyl-Daspartate receptors may protect against ischemic damage in the brain. *Science*, 226(4676), 850-852.
- Slemmer, J. E., Shacka, J. J., Sweeney, M. I., & Weber, J. T. (2008). Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. *Curr Med Chem*, 15(4), 404-414.
- Sliwoski, G., Kothiwale, S., Meiler, J., & Lowe, E. W., Jr. (2014). Computational methods in drug discovery. *Pharmacol Rev*, 66(1), 334-395. doi: 10.1124/pr.112.007336
- Sobolevsky, A. I., & Yelshansky, M. V. (2000). The trapping block of NMDA receptor channels in acutely isolated rat hippocampal neurones. *J Physiol*, *526 Pt 3*, 493-506.
- Soriano, F. X., & Hardingham, G. E. (2007). Compartmentalized NMDA receptor signalling to survival and death. *J Physiol*, 584(Pt 2), 381-387. doi: 10.1113/jphysiol.2007.138875
- Soriano, F. X., Papadia, S., Hofmann, F., Hardingham, N. R., Bading, H., & Hardingham, G. E. (2006). Preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability. *J Neurosci*, 26(17), 4509-4518. doi: 10.1523/JNEUROSCI.0455-06.2006
- Sorkin, A., & Waters, C. M. (1993). Endocytosis of growth factor receptors. *Bioessays*, 15(6), 375-382. doi: 10.1002/bies.950150603
- Sozmen, E. G., Hinman, J. D., & Carmichael, S. T. (2012). Models that matter: white matter stroke models. *Neurotherapeutics*, 9(2), 349-358. doi: 10.1007/s13311-012-0106-0

- Sprengel, R., Suchanek, B., Amico, C., Brusa, R., Burnashev, N., Rozov, A., . . . Seeburg, P. H. (1998). Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell*, 92(2), 279-289.
- Storey, G. P., Opitz-Araya, X., & Barria, A. (2011). Molecular determinants controlling NMDA receptor synaptic incorporation. J Neurosci, 31(17), 6311-6316. doi: 10.1523/JNEUROSCI.5553-10.2011
- Strominger, M. B., Slamovits, T. L., Herskovitz, S., & Lipton, R. B. (1994). Transient worsening of optic neuropathy as a sequela of the Jarisch-Herxheimer reaction in the treatment of Lyme disease. *J Neuroophthalmol*, 14(2), 77-80.
- Strong, K. L., Jing, Y., Prosser, A. R., Traynelis, S. F., & Liotta, D. C. (2014). NMDA receptor modulators: an updated patent review (2013-2014). *Expert Opin Ther Pat, 24*(12), 1349-1366. doi: 10.1517/13543776.2014.972938
- Sun, H. S., Jackson, M. F., Martin, L. J., Jansen, K., Teves, L., Cui, H., . . . Tymianski, M. (2009). Suppression of hippocampal TRPM7 protein prevents delayed neuronal death in brain ischemia. *Nat Neurosci, 12*(10), 1300-1307. doi: 10.1038/nn.2395
- Tait, S. W., & Green, D. R. (2013). Mitochondrial regulation of cell death. Cold Spring Harb Perspect Biol, 5(9). doi: 10.1101/cshperspect.a008706
- Tajima, N., Karakas, E., Grant, T., Simorowski, N., Diaz-Avalos, R., Grigorieff, N., & Furukawa, H. (2016). Activation of NMDA receptors and the mechanism of inhibition by ifenprodil. *Nature*, 534(7605), 63-68. doi: 10.1038/nature17679
- Tajiri, N., Dailey, T., Metcalf, C., Mosley, Y. I., Lau, T., Staples, M., . . . Borlongan, C. V. (2013). In vivo animal stroke models: a rationale for rodent and non-human primate models. *Transl Stroke Res*, 4(3), 308-321. doi: 10.1007/s12975-012-0241-2

- Takeuchi, N., & Izumi, S. (2015). Combinations of stroke neurorehabilitation to facilitate motor recovery: perspectives on Hebbian plasticity and homeostatic metaplasticity. *Front Hum Neurosci*, 9, 349. doi: 10.3389/fnhum.2015.00349
- Tamiz, A. P., Cai, S. X., Zhou, Z. L., Yuen, P. W., Schelkun, R. M., Whittemore, E. R., . . . Keana, J. F. (1999). Structure-activity relationship of N-(phenylalkyl)cinnamides as novel NR2B subtype-selective NMDA receptor antagonists. *J Med Chem*, 42(17), 3412-3420. doi: 10.1021/jm990199u
- Tang, Y. P., Shimizu, E., Dube, G. R., Rampon, C., Kerchner, G. A., Zhuo, M., . . . Tsien, J. Z.
 (1999). Genetic enhancement of learning and memory in mice. *Nature*, 401(6748), 63-69.
 doi: 10.1038/43432
- Tatlisumak, T. (2002). Is CT or MRI the method of choice for imaging patients with acute stroke? Why should men divide if fate has united? *Stroke*, *33*(9), 2144-2145.
- Teague, S. J., Davis, A. M., Leeson, P. D., & Oprea, T. (1999). The Design of Leadlike Combinatorial Libraries. *Angew Chem Int Ed Engl*, *38*(24), 3743-3748.
- Terasaki, Y., Sasaki, T., Yagita, Y., Okazaki, S., Sugiyama, Y., Oyama, N., . . . Kitagawa, K. (2010). Activation of NR2A receptors induces ischemic tolerance through CREB signaling. J Cereb Blood Flow Metab, 30(8), 1441-1449. doi: 10.1038/jcbfm.2010.18
- Thomas, C. G., Miller, A. J., & Westbrook, G. L. (2006). Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J Neurophysiol*, 95(3), 1727-1734. doi: 10.1152/jn.00771.2005
- Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. (1995). *N Engl J Med*, *333*(24), 1581-1587. doi: 10.1056/NEJM199512143332401

- Tong, C. K., & MacDermott, A. B. (2014). Synaptic GluN2A and GluN2B containing NMDA receptors within the superficial dorsal horn activated following primary afferent stimulation. *J Neurosci*, 34(33), 10808-10820. doi: 10.1523/JNEUROSCI.0145-14.2014
- Tovar, K. R., & Westbrook, G. L. (1999). The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci, 19*(10), 4180-4188.
- Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., . . . Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev*, 62(3), 405-496. doi: 10.1124/pr.109.002451
- Traystman, R. J. (2003). Animal models of focal and global cerebral ischemia. *ILAR J*, 44(2), 85-95.
- Trimble, B., & Morgenstern, L. B. (2008). Stroke in minorities. *Neurol Clin*, 26(4), 1177-1190,
 xi. doi: 10.1016/j.ncl.2008.05.010
- Tu, Y. C., & Kuo, C. C. (2015). The differential contribution of GluN1 and GluN2 to the gating operation of the NMDA receptor channel. *Pflugers Arch*, 467(9), 1899-1917. doi: 10.1007/s00424-014-1630-z
- Uchino, H., Ogihara, Y., Fukui, H., Chijiiwa, M., Sekine, S., Hara, N., & Elmer, E. (2016). Brain injury following cardiac arrest: pathophysiology for neurocritical care. *J Intensive Care*, *4*, 31. doi: 10.1186/s40560-016-0140-9
- Unterberg, A. W., Stover, J., Kress, B., & Kiening, K. L. (2004). Edema and brain trauma. *Neuroscience*, *129*(4), 1021-1029. doi: 10.1016/j.neuroscience.2004.06.046
- Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant

therapeutic options. *Curr Neuropharmacol*, 7(1), 65-74. doi: 10.2174/157015909787602823

- van Everdingen, K. J., van der Grond, J., Kappelle, L. J., Ramos, L. M., & Mali, W. P. (1998). Diffusion-weighted magnetic resonance imaging in acute stroke. *Stroke*, 29(9), 1783-1790.
- Vance, K. M., Simorowski, N., Traynelis, S. F., & Furukawa, H. (2011). Ligand-specific deactivation time course of GluN1/GluN2D NMDA receptors. *Nat Commun*, 2, 294. doi: 10.1038/ncomms1295
- Verhagen Metman, L., Morris, M. J., Farmer, C., Gillespie, M., Mosby, K., Wuu, J., & Chase, T. N. (2002). Huntington's disease: a randomized, controlled trial using the NMDA-antagonist amantadine. *Neurology*, 59(5), 694-699.
- Voglis, G., & Tavernarakis, N. (2006). The role of synaptic ion channels in synaptic plasticity. *EMBO Rep*, 7(11), 1104-1110. doi: 10.1038/sj.embor.7400830
- Volgraf, M., Sellers, B. D., Jiang, Y., Wu, G., Ly, C. Q., Villemure, E., . . . Schwarz, J. B. (2016). Discovery of GluN2A-Selective NMDA Receptor Positive Allosteric Modulators (PAMs): Tuning Deactivation Kinetics via Structure-Based Design. *J Med Chem*, 59(6), 2760-2779. doi: 10.1021/acs.jmedchem.5b02010
- Vosler, P. S., Brennan, C. S., & Chen, J. (2008). Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Mol Neurobiol*, 38(1), 78-100. doi: 10.1007/s12035-008-8036-x
- Walton, M., Woodgate, A. M., Muravlev, A., Xu, R., During, M. J., & Dragunow, M. (1999). CREB phosphorylation promotes nerve cell survival. *J Neurochem*, *73*(5), 1836-1842.

- Wang, C. X., & Shuaib, A. (2007). Neuroprotective effects of free radical scavengers in stroke. Drugs Aging, 24(7), 537-546.
- Wang, C., & Youle, R. J. (2009). The role of mitochondria in apoptosis*. *Annu Rev Genet, 43*, 95-118. doi: 10.1146/annurev-genet-102108-134850
- Wang, G. J., Jackson, J. G., & Thayer, S. A. (2003). Altered distribution of mitochondria impairs calcium homeostasis in rat hippocampal neurons in culture. *J Neurochem*, 87(1), 85-94.
- Wang, L., Du, F., & Wang, X. (2008). TNF-alpha induces two distinct caspase-8 activation pathways. *Cell*, *133*(4), 693-703. doi: 10.1016/j.cell.2008.03.036
- Wang, Q., Tang, X. N., & Yenari, M. A. (2007). The inflammatory response in stroke. J Neuroimmunol, 184(1-2), 53-68. doi: 10.1016/j.jneuroim.2006.11.014
- Wang, Y., Ju, W., Liu, L., Fam, S., D'Souza, S., Taghibiglou, C., . . . Wang, Y. T. (2004). alpha-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype glutamate receptor (AMPAR) endocytosis is essential for N-methyl-D-aspartate-induced neuronal apoptosis. *J Biol Chem*, 279(40), 41267-41270. doi: 10.1074/jbc.C400199200
- Wang, Y. T., & Linden, D. J. (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron*, 25(3), 635-647.
- Ward, M. W., Kushnareva, Y., Greenwood, S., & Connolly, C. N. (2005). Cellular and subcellular calcium accumulation during glutamate-induced injury in cerebellar granule neurons. *J Neurochem*, 92(5), 1081-1090. doi: 10.1111/j.1471-4159.2004.02928.x
- Ward, M. W., Rego, A. C., Frenguelli, B. G., & Nicholls, D. G. (2000). Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. J *Neurosci*, 20(19), 7208-7219.

- Wardlaw, J. M., Lewis, S. C., Dennis, M. S., Counsell, C., & McDowall, M. (1998). Is visible infarction on computed tomography associated with an adverse prognosis in acute ischemic stroke? *Stroke*, 29(7), 1315-1319.
- Wardlaw, J. M., Warlow, C. P., & Counsell, C. (1997). Systematic review of evidence on thrombolytic therapy for acute ischaemic stroke. *Lancet*, 350(9078), 607-614.
- Warlow, C., & Wardlaw, J. (1997). The drug trials that have influenced our clinical practice in acute ischaemic stroke. *Thromb Haemost*, 78(1), 558-561.
- Watkins, J. C. (1981). Pharmacology of excitatory amino acid transmitters. *Adv Biochem Psychopharmacol*, 29, 205-212.
- Wayman, G. A., Lee, Y. S., Tokumitsu, H., Silva, A. J., & Soderling, T. R. (2008). Calmodulinkinases: modulators of neuronal development and plasticity. *Neuron*, 59(6), 914-931. doi: 10.1016/j.neuron.2008.08.021
- Webb, B., & Sali, A. (2014a). Comparative Protein Structure Modeling Using MODELLER. *Curr Protoc Bioinformatics*, 47, 5 6 1-32. doi: 10.1002/0471250953.bi0506s47
- Webb, B., & Sali, A. (2014b). Protein structure modeling with MODELLER. *Methods Mol Biol*, *1137*, 1-15. doi: 10.1007/978-1-4939-0366-5_1
- Weber, J. T. (2012). Altered calcium signaling following traumatic brain injury. *Front Pharmacol*, *3*, 60. doi: 10.3389/fphar.2012.00060
- Webster, K. A. (2012). Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species. *Future Cardiol*, 8(6), 863-884. doi: 10.2217/fca.12.58

- Welsh, F. A., Marcy, V. R., & Sims, R. E. (1991). NADH fluorescence and regional energy metabolites during focal ischemia and reperfusion of rat brain. J Cereb Blood Flow Metab, 11(3), 459-465. doi: 10.1038/jcbfm.1991.89
- Wenzel, A., Fritschy, J. M., Mohler, H., & Benke, D. (1997). NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. *J Neurochem*, 68(2), 469-478.
- Wenzel, A., Villa, M., Mohler, H., & Benke, D. (1996). Developmental and regional expression of NMDA receptor subtypes containing the NR2D subunit in rat brain. J Neurochem, 66(3), 1240-1248.
- West, A. E., Griffith, E. C., & Greenberg, M. E. (2002). Regulation of transcription factors by neuronal activity. *Nat Rev Neurosci*, 3(12), 921-931. doi: 10.1038/nrn987
- Williams, C. (2006). Reverse fingerprinting, similarity searching by group fusion and fingerprint bit importance. *Mol Divers*, *10*(3), 311-332. doi: 10.1007/s11030-006-9039-z
- Williams, K. (2009). Extracellular Modulation of NMDA Receptors. In A. M. Van Dongen (Ed.), *Biology of the NMDA Receptor*. Boca Raton (FL).
- Williams, K., Russell, S. L., Shen, Y. M., & Molinoff, P. B. (1993). Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. *Neuron*, 10(2), 267-278.
- Woodgett, J. R., Avruch, J., & Kyriakis, J. (1996). The stress activated protein kinase pathway. *Cancer Surv*, 27, 127-138.
- Woodruff, T. M., Thundyil, J., Tang, S. C., Sobey, C. G., Taylor, S. M., & Arumugam, T. V. (2011). Pathophysiology, treatment, and animal and cellular models of human ischemic stroke. *Mol Neurodegener*, 6(1), 11. doi: 10.1186/1750-1326-6-11

- Wright, J. L., Gregory, T. F., Kesten, S. R., Boxer, P. A., Serpa, K. A., Meltzer, L. T., . . . Woodward, R. M. (2000). Subtype-selective N-methyl-D-aspartate receptor antagonists: synthesis and biological evaluation of 1-(heteroarylalkynyl)-4-benzylpiperidines. *J Med Chem*, 43(18), 3408-3419.
- Writing Group, Members, Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., . . . Stroke Statistics, Subcommittee. (2016). Heart Disease and Stroke Statistics-2016
 Update: A Report From the American Heart Association. *Circulation*, 133(4), e38-360. doi: 10.1161/CIR.00000000000350
- Wu, L. J., Xu, H., Ren, M., Cao, X., & Zhuo, M. (2007). Pharmacological isolation of postsynaptic currents mediated by NR2A- and NR2B-containing NMDA receptors in the anterior cingulate cortex. *Mol Pain*, 3, 11. doi: 10.1186/1744-8069-3-11
- Wyllie, D. J., Livesey, M. R., & Hardingham, G. E. (2013). Influence of GluN2 subunit identity on NMDA receptor function. *Neuropharmacology*, 74, 4-17. doi: 10.1016/j.neuropharm.2013.01.016
- Xing, C., Arai, K., Lo, E. H., & Hommel, M. (2012). Pathophysiologic cascades in ischemic stroke. *Int J Stroke*, *7*(5), 378-385. doi: 10.1111/j.1747-4949.2012.00839.x
- Xiong, Z. G., Zhu, X. M., Chu, X. P., Minami, M., Hey, J., Wei, W. L., . . . Simon, R. P. (2004). Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell*, 118(6), 687-698. doi: 10.1016/j.cell.2004.08.026
- Yaka, R., Biegon, A., Grigoriadis, N., Simeonidou, C., Grigoriadis, S., Alexandrovich, A. G., . . .
 Shohami, E. (2007). D-cycloserine improves functional recovery and reinstates long-term potentiation (LTP) in a mouse model of closed head injury. *FASEB J, 21*(9), 2033-2041. doi: 10.1096/fj.06-7856com

- Yano, S., Tokumitsu, H., & Soderling, T. R. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature*, 396(6711), 584-587. doi: 10.1038/25147
- Yu, G., Wu, F., & Wang, E. S. (2015). BQ-869, a novel NMDA receptor antagonist, protects against excitotoxicity and attenuates cerebral ischemic injury in stroke. *Int J Clin Exp Pathol*, 8(2), 1213-1225.
- Zaidi, A. (2010). Plasma membrane Ca-ATPases: Targets of oxidative stress in brain aging and neurodegeneration. World J Biol Chem, 1(9), 271-280. doi: 10.4331/wjbc.v1.i9.271
- Zanke, B. W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L. A., Zon, L., . . . Woodgett, J. R. (1996). The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr Biol*, 6(5), 606-613.
- Zhang, J., Yang, Y., Sun, H., & Xing, Y. (2014). Hemorrhagic transformation after cerebral infarction: current concepts and challenges. *Ann Transl Med*, 2(8), 81. doi: 10.3978/j.issn.2305-5839.2014.08.08
- Zhang, S. J., Zou, M., Lu, L., Lau, D., Ditzel, D. A., Delucinge-Vivier, C., . . . Bading, H. (2009). Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. *PLoS Genet*, 5(8), e1000604. doi: 10.1371/journal.pgen.1000604
- Zhang, S., Taghibiglou, C., Girling, K., Dong, Z., Lin, S. Z., Lee, W., . . . Wang, Y. T. (2013). Critical role of increased PTEN nuclear translocation in excitotoxic and ischemic neuronal injuries. *J Neurosci*, 33(18), 7997-8008. doi: 10.1523/JNEUROSCI.5661-12.2013

- Zhang, X. M., & Luo, J. H. (2013). GluN2A versus GluN2B: twins, but quite different. *Neurosci Bull*, 29(6), 761-772. doi: 10.1007/s12264-013-1336-9
- Zhang, Y., Galloway, J. M., Welty, T. K., Wiebers, D. O., Whisnant, J. P., Devereux, R. B., ...
 Lee, E. T. (2008). Incidence and risk factors for stroke in American Indians: the Strong
 Heart Study. *Circulation*, *118*(15), 1577-1584. doi: 10.1161/CIRCULATIONAHA.108.772285
- Zhou, Q., & Sheng, M. (2013). NMDA receptors in nervous system diseases. *Neuropharmacology*, 74, 69-75. doi: 10.1016/j.neuropharm.2013.03.030
- Zhu, H., Yoshimoto, T., Imajo-Ohmi, S., Dazortsava, M., Mathivanan, A., & Yamashima, T. (2012). Why are hippocampal CA1 neurons vulnerable but motor cortex neurons resistant to transient ischemia? *J Neurochem*, *120*(4), 574-585. doi: 10.1111/j.1471-4159.2011.07550.x
- Zhu, S., & Paoletti, P. (2015). Allosteric modulators of NMDA receptors: multiple sites and mechanisms. *Curr Opin Pharmacol*, 20, 14-23. doi: 10.1016/j.coph.2014.10.009
- Zhu, S., Stein, R. A., Yoshioka, C., Lee, C. H., Goehring, A., McHaourab, H. S., & Gouaux, E. (2016). Mechanism of NMDA Receptor Inhibition and Activation. *Cell*, 165(3), 704-714. doi: 10.1016/j.cell.2016.03.028
- Zong, W. X., & Thompson, C. B. (2006). Necrotic death as a cell fate. *Genes Dev, 20*(1), 1-15. doi: 10.1101/gad.1376506
- Zoraghi, R., See, R. H., Axerio-Cilies, P., Kumar, N. S., Gong, H., Moreau, A., . . . Reiner, N. E. (2011). Identification of pyruvate kinase in methicillin-resistant Staphylococcus aureus as a novel antimicrobial drug target. *Antimicrob Agents Chemother*, 55(5), 2042-2053. doi: 10.1128/AAC.01250-10

Appendices

Appendix A

PART I: 2-hydroxy-3-ethoxybenzaldehyde (1.0 g, 5.2 mmol) was dissolved in acetic acid (20 mL) and N-chlorosuccinimide (NCS) (1.4 g, 11 mmol) was added all at once. The reaction mixture was stirred overnight at 80°C, and then cooled to room temperature. Water and CH_2Cl_2 were then added, the phases were separated and the water phase was further extracted with CH_2Cl_2 , dried over MgSO₄ and evaporated under vacuo. The crude product was purified by flash chromatography (CH_2Cl_2 /hexane) to afford the pure product (1.2 g, 89 %) as a yellow solid.

PART II: Dissolve equimolar amounts of hydrazine (0.30mmol) and aldehyde (0.30mmol) in THF (0.5 M solution). Add 2 equivalents MgSO₄ and heat to reflux for 1 h. The product may precipitate. If not, the reaction was checked by TLC or NMR to ascertain consumption of the aldehyde. If the product precipitated, it was recovered by filtration and washed with water (2x2ml) to remove residual MgSO₄. If the product did not precipitate the reaction mixture was diluted with water until the product precipitated. The mixture was acidified to pH 3 with dilute HCl for the removal of residual hydrazine and consequently filtered and if necessary it was recrystallized. The compound is a high-melting solid (mp 210-212 °C). Extraction was avoided. If no precipitation was observed, the solvent was evaporated in vacuo and the compound was purified by flash column chromatography.

¹H-NMR (400 MHz, DMSO-d₆): $\delta = 11.55$ (s, 1H), 10.33 (s, 1H), 7.264 (s, 1H), 4.092 (d, J = 6.6 Hz, 2H), 2.500 (s, 1H), 1.344 (t, J = 4.5 Hz, 3H). MS (EI): calcd for C₉H₈Cl₂O₃ ⁻ M⁻ 232.986, found 233.1

Npam43-(white solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.59$ (s, 1H), 10.41 (s, 1H), 8.89 (s, 1H), 7.61-7.34 (m, 4H), 7.08 (s, 1H), 4.11 (q, J = 6.9 Hz, 2H), 1.41 (t, J = 7.0 Hz, 3H). MS (EI): calcd for C₁₆H₁₃BrCl₂N₂O₃⁺ M⁺ 433.1, found 433.0; calcd for C₁₆H₁₃BrCl₂N₂O₃⁺ Na 455.1, found 455.0.

Npam38-(grey powder solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.2$ (s, 1H), 10.35 (s, 1H), 8.59 (s, 1H), 8.15-7.35 (m, 5H), 7.05 (s, 1H), 4.11 (q, J = 6.9 Hz, 2H), 1.41 (t, J = 7.0Hz, 3H), MS (EI): calcd for C₁₆H₁₄Br₂N₂O₃⁻ M⁻ 441.11, found 441.0

Npam32-(white solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.75$ (s, 1H), 9.05 (s, 1H), 8.15-7.30 (m, 4H), 7.10 (s, 1H), 4.10 (q, J = 6.9 Hz, 2H), 1.40 (t, J = 7.0 Hz, 3H). MS (EI): calcd for C₁₆H₁₃BrCl₂N₂O₃⁺ M⁺ 433.1, found 433.0; calcd for C₁₆H₁₃BrCl₂N₂O₃⁺ Na 455.1, found 455.0.

Npam44-(white solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.65$ (s, 1H), 8.9 (s, 1H), 7.5-7.25 (m, 3H), 7.12 (s, 1H), 4.11 (q, J = 6.9 Hz, 2H), 2.50 (s, 3H), 1.40 (t, J = 7.0 Hz, 3H). MS (EI): calcd for C₁₇H₁₅Cl₃N₂O₃⁺ M⁺ 401.0, found 401.2, calcd for C₁₇H₁₅Cl₃N₂O₃⁺ Na 423.0, found 423.1.

Npam35-(white solid) ¹H NMR (400 MHz, DMSO-d₆), $\delta = 13.1$ (s, 1H), 12.35 (s, 1H), 9.05 (s, 1H), 8.9-7.55 (m, 4H), 7.25 (s, 1H), 3.85 (s, 3H), 1.40 (s, 9H), C19H20Br2N2O3, 484.19 Npam58-(grey powder solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.1$ (s, 1H), 10.7 (s, 1H), 8.45 (s, 1H), 7.75-7.65 (m, 1H) 7.55-7.35 (m, 4H), 7.05 (s, 1H), 4.05 (q, J = 6.9 Hz, 2H), 1.35 (dt, J = 7.0Hz, 3H). MS (EI): calcd for C₁₆H₁₄Br₂N₂O₃⁻ M⁻ 441.11, found 441.0 Npam46-(white solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.0$ (s, 1H), 8.42 (s, 1H), 7.5-6.9 (m, 5H), 4.20 (q, J = 6.9 Hz, 2H), 2.50 (s, 3H), 1.40 (dt, J = 7.0 Hz, 3H). MS (EI): calcd for C₁₇H₁₆BrClN₂O₃⁺ M⁺ 411.0, found 411.1, calcd for C₁₇H₁₆BrClN₂O₃⁺ Na 435.1, found 435.1.

Npam50-(white solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 8.88$ (s, 1H), 7.78-7.73 (m, 1H),

7.53-7.48 (m, 1H), 7.29-7.24 (m, 1H), 7.17 (s, 1H), 4.12 (q, J = 6.9 Hz, 2H), 2.53 (s, 3H), 1.47 (t,

$$J = 7.0 \text{ Hz}, 3\text{H}$$
). HRMS (EI): calcd for $C_{17}H_{16}BrCl_2N_2O_3^+M^+$ 447.0, found 446.9648

Npam49-(white solid) ¹H NMR (400 MHz, DMSO-d₆): δ = 8.75 (s, 1H), 7.62 (m, 1H), 7.54 (m, 1H), 7.41-7.35 (m, 1H), 7.07 (s, 1H), 4.02 (q, J = 6.9 Hz, 2H), 1.33 (t, J = 7.0 Hz, 3H). HRMS (EI): calcd for C₁₆H₁₃BrCl₃N₂O₃ ⁺ M⁺ 467, found 466.9085

Npam59-(white solid) ¹H NMR (400 MHz, DMSO-d₆) δ = 13.05 (s, 1H), 12.45 (s, 1H), 9.10 (s, 1H), 8.0-7.5 (m, 5H), 7.3 (s, 1H), 3.85 (s, 3H). MS (EI): calcd for C₁₅H₁₂Br₂N₂O₃⁺ M⁺ 429.0, found 428.9, calcd for C₁₅H₁₂Br₂N₂O₃⁺ Na 451.0, found 450.9.

Npam22-(white solid) ¹H NMR (400 MHz, DMSO-d₆), $\delta = 12.1$ (s, 1H), 11.1 (s, 1H), 8.59 (s, 1H), 8.0-7.45 (m, 5H), 7.3 (s, 1H), 7.05 (s, 1H), 4.1 (q, J = 6.9 Hz, 2H), 1.40 (t, J = 7.0 Hz, 3H), C16H15BrN2O3, 363.21

Npam75-(grey powder solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.1$ (s, 1H), 10.65 (s, 1H), 8.45 (s, 1H), 7.75-6.82 (m, 6H), 3.85 (s, 3H), MS (EI): calcd for C₁₅H₁₂BrClN₂O₃ ⁻ M⁻ 382.63, found 383.0

Npam17- (yellow solid) ¹H NMR (400 MHz, DMSO-d₆): $\delta = 12.84$ (s, 1H), 8.865 (s, 1H), 7.608-7.184 (m, 4H), 4.076 (q, J= 6.9 Hz, 2H), 2.496 (s, 3H), 1.361 (t, J = 7.0 Hz, 3H). (EI): calcd for C₁₇H₁₆Cl₂N₂O₃⁺ M⁺ 367.1, found 367.1, calcd for C₁₇H₁₆Cl₂N₂O₃⁺ Na 389.1, found 389.1 Npam48-(white solid) ¹H NMR (400 MHz, DMSO-d₆), $\delta = 12.8$ (s, 1H), 12.15 (s, 1H), 9.0 (s, 1H), 7.95-7.05 (m, 4H), 4.12 (q, J=6.9 Hz, 2H), 3.00 (s, 3H), 1.40 (t, J = 7.0 Hz, 3H),

Appendix B

B.1 Potentiation and inhibition effects of the "hit" compounds of initial screen validated in *HEK293* cells expressing GluN1/GluN2A or GluN1/GluN2B NMDARs via whole-cell voltage clamp recordings

	R2 H R1							
Name	R1	R2	Normalized Potentiation GluN1/GluN2A	Normalized Potentiation GluN1/GluN2B	n			
Npam01	н₀		26.18 ± 2.24 % @ 100μM	-28.00 ± 5.11 % @ 100µM	6			
Npam02		CH.	36.88 ± 3.76 % @ 100µM	-4.820 ± 2.83 % @ 100µM	6			
Npam04	но	CH ₃	33.71 ± 4.84 % @ 100µM	-54.59 ± 5.48 % @ 100µM	б			

Name	R1	R2	Normalized Potentiation GluN1/GluN2A	Normalized Potentiation GluN1/GluN2B	n
Npam30	но н		52.67 ± 4.87 % @ 100μM	$\begin{array}{c} -100 \pm 2.83 \ \% \\ @ \ 100 \mu M; \ - \\ 74.36 \pm 6.98 \ \% \\ @ \ 25 \mu M \end{array}$	4
Npam65	HO LO	CH ₂	51.83 ± 6.55 % @ 50µM	-46.87 ± 8.94 % @ 50μM	4
Npam66	HO CH3		45.69 ± 3.87 % @ 100μM	-20.89 ± 3.69 % @ 25µM	4
Npam68	но	но	35.14 ± 4.85 % @ 50µM	-56.56 ± 7.45 % @ 50μM	4
Npam69	HO CH3		11.11 ± 1.98 % @ 50µM	$-34.01 \pm 2.47 \%$ @ 50 μ M	4

Name	R1	R2	Normalized Potentiation GluN1/GluN2A	Normalized Potentiation GluN1/GluN2B	n
Npam16	ĕ ₽	Br	$\begin{array}{c} 75.41 \pm 7.69 \ \% \\ @ \ 40 \mu M; \ 116.17 \\ \pm \ 16.35 \ \% \ @ \\ 100 \mu M \end{array}$	$\begin{array}{c} -50.808 \pm 4.69 \\ \% @ 40 \mu M; - \\ 80.24 \pm 9.63 \ \% \\ @ 50 \mu M \end{array}$	4
Npam70	но	HO CH	-24.08 ± 2.47 % @ 50μM	-45.57 ± 3.67 % @ 50μM	4
Npam72	H ₃ c ⁻⁰ → → → → → → → → → → → → → → → → → → →	CH3	118.45 ± 18.46 % @ 100µM	-24.24 ± 4.55 % @ 100μM	4
Npam73	e ₽	Br	30.70 ± 4.76 % @ 50µM	-49.85 ± 6.87 % @ 50μM	4
Npam71	но	H ₃ C	$\begin{array}{c} 41.68 \pm 6.78 \ \% \\ 50 \mu M \end{array}$	-48.56 ± 4.77 % @ 50μM	4

Name	R1	R2	Normalized Potentiation GluN1/GluN2A	Normalized Potentiation GluN1/GluN2B	n
Npam75	HO CH3	Br C	57.18 ± 3.78 % @ 100µM	-17.00 ± 2.14 % @ 100μM	4
Npam64	HO LO	0- ^N	28.17 ± 2.47 % @ 100µM	-54.57 ± 5.87 % @ 100µM	4

B.2 Positive modulation effects (potentiation) of different chemically synthesized analogs for Npam02 on NMDAR-mediated currents in cortical neurons and their SARs analysis based on closely related analogs

Appendix Table 2:



Name	R1	R2	Normalized Potentiaton	n	Potency	SAR
Npam43	\mathcal{H}	Br	363.52 ± 27.3 %	6.0	+++	Not the best addition of a para hydrophobic group (Me, Cl) will increase potency (Npam46, 44)
Npam52		N CH3	175.53 ± 25.9 %	4.0	++	Maybe benefical addition as it increases the potency with respect to Npam55 and helps metabolism

Name	R1	R2	Normalized Potentiaton	n	Potency	SARs
Npam53		o ^{−cH} ³	110.23 ± 4.70 %	4.0	++	OMe detrimental (volume, steric clashes) , comparsion with Npam43 (R2)
Npam40	O ^{CH3} OH Br	Br	42.51 ± 3.79 %	4.0	+	OH detrimental in para-position (R1)
Npam51	CH3 CH3 CH3 CH3 CH3 CH3	H ₃ C	33.37 ± 6.39 %	4.0	+	Failure to match - Methylated addition of OH detrimental (R1)
Npam50		H ₃ C	441.11 ± 36.50 %	5.0	++++	Addition of Me increases potency (R2)
Npam49		H ₃ C F	530.06 ± 114.00 %	6.0	++++	Adddition of hydrophobic substients (R2) around the ring enhances potency

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam31	HO HO HO	Br	15.11 ± 8.78 %	4.0	+	No hydrophobic substuients (R1) losses significant potency
Npam42	HO CH3	H ²	43.04 ± 6.55 %	4.0	+	No hydrophobic substuients (R1) losses significant potency
Npam56		° → ^H Z →	21.48 ± 2.89 %	4.0	+	Elongation of (R2) and absence of substients on ring losses potency
Npam54		Br	43.41 ± 13.48 %	4.0	+	ortho hydrophbic substiuent (R1) is essential of potency if m- Cl is present (Npam43)
Npam20		Br	131.85 ± 23.20 %	5.0	++	ortho hydrophobic substiuent (R1) is essential of potency if m- Cl is present

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam08	Ho CH3 CH3		362.76 ± 38.44 %	4.0	+++	elongation of (R2) and presence of substients on ring generates a decent potency (compare Npam43)
Npam57			105.85 ± 12.62 %	4.0	++	Loss of the carbonyl losses stability of the compound
Npam21		NH ₂	37.27 ± 4.64 %	4.0	+	Failure of matching - NH2 in R2 losses potency (compare to Npam58)
Npam58		Br	163.60 ± 13.94 %	4.0	++	Not as good as Npam43 but meta-Br is better

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam48		o I CH ₃	441.05 ± 150.41 %	4.0	+++	Para- substieunet in R2 is more effective towards potency than the ortho- substiuent (Npam43)
Npam23			87.00 ± 6.48 %	4.0	+	Steric clashes
Npam32		Br	374.58 ± 19.07 %	4.0	+++	Comparable to Npam43

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam38	HO HO HO HO HO HO HO HO HO HO HO HO HO H	Br	426.44 ± 87.65 %	4.0	+++	Hydrophobic subsituent in (R2) is needed for potency (compared with Npam21), ortho-Br is better compared with Npam58
Npam46	HO HO HO HO HO HO HO HO HO HO HO	H ₃ c	466.57 ± 37.78 %	4.0	+++	Combination of para and ortho substiunet was the best combination in terms of potency and selectivity
Npam44		H ₃ c	443.25 ± 83.42 %	4.0	+++	Combination of para and ortho substiunet was the best combination in terms of potency and selectivity

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam07	HO HO HO	CI CH3	103.77 ± 11.00 %	4.0	++	
Npam06	HO CO	Br	201.19 ± 14.63 %	4.0	++	N inside the ring was detramental to the potency (Npam43)
Npam55	HO HO CI		32.10 ± 3.02 %	7.0	+	the ortho Br was crucial for selectivity and potency absence of substienut in R2 reduced potency (Npam12)
Npam37		F C	435.94 ± 156.01 %	4.0	+++	
Npam59			441.7 ± 65.24 %	6.0	+++	

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam12		о ^{сн} з	118.15 ± 35.54 %	4.0	++	
Npam45		u □	284.50 ± 85.20 %	4.0	++	Chloro subsutient in R2 was detrminetal to potency compared with Npam59 with 2 Br in R1
Npam47		Br	224.79 ± 24.78 %	4.0	++	
Npam28			30.25 ± 2.87 %	4.0	+	No hydrophobic substuients (R2) losses significant potency and nitrogen in ring does not help

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam22		F C C C C C C C C C C C C C C C C C C C	59.83 ± 7.55 %	4.0	+	No large hydrophobic substituent in R2 losses signifincat potency, with one Br in R1, potency increases with either a meta & para substuient or a meta Brortho-Br is not preferred
Npam29	HO CI	Br	120.68 ± 9.61 %	3.0	++	

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam27	сн _з о он вг	Br	-0.278 ± 5.62 %	4.0		Re- arrangement of from m-OH to the p-OH abolishes the potentiation
Npam39			51.95 ± 11.29 %	4.0	+	No hydrophobic substiient in R2 greatly reduces the potency
Npam01	HC OCH3		$2.61 \pm 9.69 \\ \%$	4.0		Blocking OH of R1 will not elicit binding
Npam02		OH OH	243.57 ± 24.81 %	4.0	++	Polar OH of R2 on ring causes match failure
Npam03	HO CH ₂	Br	97.62 ± 6.03 %	4.0	+	Elongated allyl group was not effective as much as a Cl or Br

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam04		CH3	292.08 ± 44.70 %	4.0	++	
Npam05		Br	196.54 ± 21.20 %	4.0	++	
Npam10		Br	206.28 ± 33.62 %	4.0	++	
Npam13		cl Cl	195.38 ± 40.11 %	6.0	++	
Npam15		H ₃ C	89.84 ± 27.86 %	4.0	+	

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam17		CH3	130.54 ± 13.63 %	4.0	++	
Npam18	HO HO CH3 CI	Br	213.58 ± 43.58 %	4.0	++	
Npam24	H ² H ² H ² H ² H ² H ² H ² H ²	₽	61.57 ± 8.15 %	4.0	+	
Npam25			-14.32 ± 2.20 %	3.0		
Npam26		Br	175.08 ± 14.42 %	4.0	++	
Name	R1	R2	Normalized Response	n	Potency	SARs
--------	-------------	------------------------------------	------------------------	-----	---------	------
Npam30	H H		3.99 ± 4.92 %	4.0		
Npam16	но сн	Br	36.90 ± 5.62 %	3.0		
Npam33	oH →	∧ NH	-11.76 ± 14.57 %	3.0		
Npam34	e H H	E-	-12.47 ± 2.06 %	4.0		
Npam35		H ₃ C - CH ₃	512.24 ± 28.67 %	4.0	++++	

Name	R1	R2	Normalized Response	n	Potency	SARs
Npam36	₽		4.02 ± 4.79 %	3.0		