Role of the NR2 subunit composition and intracellular C-terminal domain in N-methyl-D-aspartate receptor signalling.

by

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Abstract

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ionotropic receptors. When activated, NMDARs let extracellular sodium and calcium ions enter neurons. This calcium influx, depending on its duration, intensity and the presence of nearby signalling proteins can signal to synaptic plasticity. Additionally, physiological NMDAR activity promotes pro-survival cascades and gene transcription, whereas both lack of activation and overactivation of these receptors trigger pro-death signals. Several neurodegenerative pathologies such as stroke/ischemia and Alzheimer’s disease are thought to involve NMDAR overactivation, so-called “excitotoxicity”, but since NMDARs are important for normal neuronal physiology, potential therapeutical approaches needs to go beyond simple antagonism. Here, we studied the receptor subunit composition and the molecular cascades downstream of the receptor activation to try and isolate the pro-death pathways in NMDAR-mediated excitotoxicity. We found that the NR2 subunit composition did not dictate the type of NMDAR-mediated signals, as receptors comprised of NR2B subunits were able to signal to death, survival and plasticity. However, we also found that the intracellular tail of the NR2B subunit was more efficient at triggering neuronal death compared to the NR2A C-terminus, which suggests that different pro-death signalling complexes are associated to each subunit. Two pro-death signals, the p38 and c-Jun N-terminal kinase (JNK) cascades, are key mediators of neuronal excitotoxicity. In a non-neuronal cell line, NMDAR-mediated cell death could be reconstituted but was found to rely solely on JNK and not p38. This was due to the lack of pro-death signals from the NR2B-PDZ domain, a cytoplasmic interacting domain which forms a signalling cassette with the neuronal proteins PSD-95 and neuronal nitric oxide synthase. This PDZ-ligand recruits the p38 cascade in neurons, but was absent in non-neuronal cells. The pro-death p38 pathway could be inhibited in neurons by disrupting the PDZ domain interactions, which protects against excitotoxicity. This disruption was not affecting normal synaptic transmission, potentiation or survival signalling, suggesting that this could be a therapeutically viable avenue. Thus, this work has expanded the understanding of how NMDAR subunits and
their cytoplasmic domains mediate signalling leading to a variety of cellular outcomes; a crucial point for the development of a strategy specifically targeting NMDAR-mediated pro-death signalling.
<table>
<thead>
<tr>
<th>Table of contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xv</td>
</tr>
<tr>
<td><strong>Chapter 1: Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1. Glutamate and its receptors</td>
<td></td>
</tr>
<tr>
<td>1.1 The AMPA receptor</td>
<td>2</td>
</tr>
<tr>
<td>2. The NMDA receptor</td>
<td></td>
</tr>
<tr>
<td>2.1 The NR1 subunit</td>
<td>3</td>
</tr>
<tr>
<td>2.2 The NR2 subunit</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1 The developmental and regional pattern of NR2 subunit expression</td>
<td>5</td>
</tr>
<tr>
<td>2.2.2 The NR2 subunit structure</td>
<td>5</td>
</tr>
<tr>
<td>2.3 The NR3 subunit</td>
<td>8</td>
</tr>
<tr>
<td>2.4 Extracellular NMDAR modulation</td>
<td>8</td>
</tr>
<tr>
<td>2.5 The C-terminus of NMDAR interacts with intracellular proteins</td>
<td>9</td>
</tr>
<tr>
<td>3. Physiological and pathophysiological signalling downstream of NMDARs</td>
<td>10</td>
</tr>
<tr>
<td>3.1 NMDAR-dependent plasticity</td>
<td>10</td>
</tr>
<tr>
<td>3.1.1 Long-term potentiation and depression</td>
<td>10</td>
</tr>
<tr>
<td>3.2 The role of NR2 subunit composition in NMDAR-dependent plasticity</td>
<td>11</td>
</tr>
<tr>
<td>3.2.1 Studies from transgenic animal models</td>
<td>12</td>
</tr>
<tr>
<td>3.2.2 Studies using pharmacological agents</td>
<td>13</td>
</tr>
</tbody>
</table>
3.3 NMDAR-dependent pro-survival and pro-death signalling

3.3.1 Pro-survival NMDAR signalling
3.3.1a The CREB pathway is induced by synaptic NMDAR activity
3.3.1b The PI3K/Akt pathway

3.3.2 Pro-death NMDAR signalling
3.3.2a The CREB and ERK1/2 pathways
3.3.2b Calpain-mediated proteolysis
3.3.2c Mitochondrial damage
3.3.2d The JNK pathway
3.3.2e The p38 pathway
3.3.2f The PSD-95 MAGUK

3.4 The role of NR2 subunit composition of NMDARs in pro-death and pro-survival signals

3.5 A brief overview of NMDAR-dependent processes in diseases

3.6 Overview, research aims and chapter content

3.6.1 Chapter 3
3.6.2 Chapter 4
3.6.3 Chapter 5
3.6.4 Chapter 6

Chapter 2: Material and methods

1. Neuronal culture
2. AtT20 cell culture
3. Transfection procedure
4. Stimulation protocols
   4.1 Neuronal cell death
   4.2 Enhancement of synaptic NMDAR activity in neuronal cultures
5. Luciferase assay
6. Immunohistochemistry
7. Calcium assay (cell line and neurons) 41
8. Western blotting and antibodies 42
9. Electrophysiology 43
   9.1 Measurements of NMDAR-mediated currents 44
   9.2 Measurement of spontaneous EPSC frequency 45
   9.3 Analysis of extrasynaptic NMDAR currents 45
   9.4 Models of NMDAR-dependent synaptic potentiation 45
   9.5 Model of NMDAR-dependent synaptic depression 46
10. Plasmid preparation and chimera construction 46
    10.1 Digestion of pDNA and mutagenesis 47
    10.2 Chimera construction 48
11. Data and statistical analysis 49

Chapter 3: NR2B-containing NMDARs can mediate signalling to neuronal survival, death and synaptic plasticity 50
1. Chapter introduction 51
2. Results 54
   2.1 Ifenprodil sensitivity of whole-cell NMDAR currents 54
   2.2 Ifenprodil sensitivity of extrasynaptic NMDAR currents 59
   2.3 NR2B-NMDARs can mediate pro-death signalling 65
   2.4 NR2B-NMDARs can mediate pro-survival signalling 67
   2.5 NR2B-NMDAR activation can trigger synaptic potentiation at DIV 8-11 73
   2.6 NR2B-NMDARs are able to signal to synaptic depression at 75
3. Chapter discussion 79

Chapter 4: Pro-death p38 signalling requires a neuronal context and does not contribute to NMDAR-induced non-neuronal cell death 86
Chapter 5: The PDZ-ligand pro-death signalling is neuron-specific and can be disrupted without affecting other NMDAR-mediated signals

1. Chapter introduction
2. Results
   2.1 NR2B-NMDARs mediate death of cortical neurons
   2.2 TAT-NR2B9c is neuroprotective against agonist-induced neuronal death
   2.3 Disruption of the PDZ-signalling cassette does not affect excitability or potentiation signalling
   2.4 Specific signalling to p38 by the PDZ-signalling complex
   2.5 mEPSC potentiation signalling is independent of the NR2B PDZ-ligand but not of the p38 cascade
   2.6 NR2B-PDZ binding domain does not signal to death in non-neuronal cells
3. Chapter discussion
Chapter 6: The C-terminus tail of the NR2 subunit influences NMDAR-mediated neuronal death

1. Chapter introduction 138

2. Results 141

2.1 Overexpression of NR2 affects the pool of functional NMDARs in hippocampal neurons 141

2.2 Overexpression of NR2 subunits in hippocampal neurons influences excitotoxicity 145

2.3 Creation of NR2 chimeras with swapped C-terminus domains 147

2.4 Overexpression of NR2A(2B^{CTD}) and NR2B(2A^{CTD}) in neurons affects current in the same manner as NR2A and NR2B overexpression 151

2.5 The NR2-C-terminus influences the subunit’s potency for excitotoxicity 154

2.6 Excitotoxicity also depends on the NR2 subunit regardless of their C-terminal domain 156

3. Chapter discussion 160

Summary of experimental findings 164

Bibliography 168
List of Figures

Figure 1.1: The N-methyl-D-aspartate receptor (NMDAR). 7

Figure 1.2: Pro-survival and pro-death signalling from the NMDAR. 16

Figure 1.3: A schematic of the NMDAR-associated protein complex. 23

Figure 2.1: Luciferase chemiluminescence reaction. 40

Figure 3.1: Hippocampal neurons exhibit increasingly larger NMDAR-mediated currents with time in vitro. 55

Figure 3.2: The decrease of ifenprodil sensitivity of NMDAR currents starts from DIV 12. 57

Figure 3.3: DIV 12-18 hippocampal neurons contain a lower proportion of NR2B-containing NMDARs than DIV 7-11 neurons. 58

Figure 3.4: Developmental loss of ifenprodil sensitivity of NMDAR currents is not solely restricted to synaptic locations. 62

Figure 3.5: The proportion of synaptic and extrasynaptic NMDAR currents remains similar between DIV 7-11 and DIV 12-18 hippocampal neurons. 64

Figure 3.6: NR2B-containing NMDARs can mediate pro-death NMDAR signalling. 66
Figure 3.7: Endogenous network firing recruits synaptic NMDAR activity.

Figure 3.8: NR2B-containing NMDARs can mediate pro-survival signalling.

Figure 3.9: NR2B-containing NMDARs mediate activity-dependent potentiation of mEPSC frequency.

Figure 3.10: A short stimulation of agonist at low concentration does not induce cell death.

Figure 3.11: NR2B-containing NMDARs mediate agonist-induced depression of spontaneous EPSC (sEPSC) frequency.

Figure 4.1: In neurons, both p38 and JNK SAPKs participate in excitotoxic signalling downstream of NMDARs.

Figure 4.2: Transfection of plasmids encoding for the NR1 and NR2B subunit leads to expression of functional, surface-expressed NMDARs in AtT20 cell line.

Figure 4.3: NMDAR-dependent cell death is reconstituted in non-neuronal cell line AtT20 expressing NR1 and NR2B subunits.

Figure 4.4: Excitotoxicity in AtT20 cells expressing NR2B-containing NMDARs is dependent on JNK SAPK but not on p38 SAPK.

Figure 4.5: The p38 SAPK pathway is present and inducible in the AtT20 cell line.
Figure 4.6: Determination of the amount of Ca$^{2+}$ influx needed for half-maximal toxicity in cortical neurons and AtT20 cells.

Figure 4.7: Increased NMDAR-mediated Ca$^{2+}$ influx is required to kill NMDAR-expressing AtT20 cells than to kill neurons.

Figure 5.1: NR2B-containing NMDARs mediate excitotoxicity in cortical neurons (DIV 8-10).

Figure 5.2: Disruption of the PDZ domain interactions is neuroprotective against NMDAR-mediated toxicity.

Figure 5.3: Neurons protected from excitotoxicity by the TAT-NR2B9c peptide show normal excitability and network activity.

Figure 5.4: Incubation with the TAT-NR2B9c peptide does alter basal mEPSC frequency or sEPSC frequency.

Figure 5.5: Disruption of the NR2B/PSD-95 interaction by TAT-NR2B9c does not block synaptic plasticity signalling unlike conventional NMDAR antagonists.

Figure 5.6: mEPSC frequency potentiation is impaired when p38 is globally inhibited.

Figure 5.7: In NMDAR-expressing AtT20 cells, disruption of PDZ ligand does not protect cells against agonist-induced toxicity.

Figure 5.8: Truncation of the PDZ domain of the NR2B subunit does not
alter NMDAR-mediated Ca$^{2+}$ influx or toxicity in non-neuronal cells AtT20.

Figure 5.9: Significant protein levels of PSD-95 and neuronal nitric oxide synthase (nNOS) are absent from AtT20 cell homogenates.

Figure 6.1: Overexpression of NR2A or NR2B increases NMDA-induced currents in hippocampal neurons.

Figure 6.2: A large proportion of NMDARs incorporate exogenously provided subunits in hippocampal neurons overexpressing NR2 subunits.

Figure 6.3: Overexpression of NR2A and NR2B subunits influences agonist-induced neuronal death differentially.

Figure 6.4: Mutant NR2 subunits with swapped C-terminal domains.

Figure 6.5: NMDARs composed of NR2 subunits with their C-terminal domain swapped retain the ifenprodil sensitivity of their wild-type counterparts.

Figure 6.6: Chimeric subunit overexpression increases NMDAR-mediated current in transfected hippocampal neurons.

Figure 6.7: Ifenprodil sensitivity of NMDA-induced currents shows that an equally large proportion of NMDARs incorporates C-terminal mutant NR2 subunits compared to wild-type when overexpressed in neurons.
Figure 6.8: Changing the C-terminal domain of the NR2 subunit affects the amount of agonist-induced cell death in overexpressing hippocampal neurons. 155

Figure 6.9: The C-terminal domain does not alter the NMDAR-mediated current, although the rest of the subunit does. 157

Figure 6.10: The C-terminal domain of the NR2B subunit is more effective at promoting death than the C-terminal domain of NR2A. 159
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-isoxazole propionate</td>
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<td>AMPAR</td>
<td>AMPA receptor</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-associated death promoter</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CGC</td>
<td>cerebellar granule cell</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CREB</td>
<td>c-AMP response element binding protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIV</td>
<td>days <em>in vitro</em></td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated protein kinase 1/2</td>
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<td>FOXO</td>
<td>forkhead box subgroup O</td>
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<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<td>GluR</td>
<td>glutamate receptor</td>
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<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney cell 293</td>
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<tr>
<td>ifenprodil</td>
<td>4-[2-[4-(cyclohexylmethyl)-1-piperidinyl]-1-hydroxypropyl]phenol</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
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<tr>
<td>LTP</td>
<td>long term potentiation</td>
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<tr>
<td>MAGUK</td>
<td>membrane-associated guanylate kinase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>mEPSC</td>
<td>miniature excitatory postsynaptic current</td>
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<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>MK-801</td>
<td>(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine</td>
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<td>M KK7</td>
<td>MAPK kinase 7</td>
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<tr>
<td>NCX3</td>
<td>Na(^+)/Ca(^{2+}) exchanger 3</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>neuronal nitric oxide synthase</td>
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<td>NVP-AAM077</td>
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</tr>
<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<tr>
<td>PDZ</td>
<td>PSD-95/Disc large/Zona occludens 1</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase A</td>
</tr>
<tr>
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<td>protein phosphatase 1</td>
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<td>protein phosphatase 3</td>
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<tr>
<td>PSD</td>
<td>postsynaptic density</td>
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<td>PTX</td>
<td>picrotoxin</td>
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<td>reactive oxygen species</td>
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<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
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<tr>
<td>sEPSC</td>
<td>spontaneous excitatory postsynaptic current</td>
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<td>TARP</td>
<td>transmembrane AMPAR regulatory protein</td>
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<td>TTX</td>
<td>tetrodotoxin</td>
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<tr>
<td>VGCC</td>
<td>voltage-gated calcium channel</td>
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</table>
Chapter 1
Introduction
1. Glutamate and its receptors

Glutamate is the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS). Accordingly, neurons responsive to the glutamate neurotransmitter exhibit various glutamate receptors (GluRs). These receptors are either ionotropic, letting certain cations pass through their pore, or metabotropic (metabotropic glutamate receptors; mGluRs). The ionotropic GluRs are assembled in tetramers of subunits which form a cationic channel (Dingledine et al., 1999). They are classified in three categories, bearing the name of the agonists which selectively activate them: kainate receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs). The work presented in this thesis focuses on NMDARs, key mediators of intracellular signals that follow glutamatergic activity.

1.1 The AMPA receptor

During the fast glutamatergic synaptic transmission, the majority of the postsynaptic ion flux is mediated by AMPARs. AMPARs quickly respond to presynaptic glutamate release by allowing $\text{Na}^+$ to enter the postsynaptic neuron, effectively converting the chemical signal of glutamate to an electric signal -depolarization- that will propagate through the neuronal membrane. The neuronal excitability to glutamate can be regulated by the number of AMPARs at the postsynaptic density, which can be modulated through development or following certain patterns of activity. Indeed, the modulation of surface AMPARs is one of the mechanisms of synaptic plasticity: from a readily accessible pool of subsynaptic vesicles containing receptors, AMPARs can be rapidly inserted or retrieved from the postsynaptic synapse following a given cellular signal -often initiated by NMDARs. Additionally, AMPAR channel properties can be altered by the phosphorylation of their C-terminal intracellular tail, by association with transmembrane AMPAR regulatory proteins (TARPs) or by alternative editing. Such a wide range of modifications gives to glutamatergic neurons the molecular tools to control the strength of individual synapses, which is believed to be the cellular and molecular basis for
learning and memory formation (Bliss and Lomo, 1973; Dingledine et al., 1999; Gomes et al., 2003; Milstein and Nicoll, 2008).

2. The NMDA receptor

The main focus of the work presented here is on the NMDAR subclass of GluR. In addition to glutamate, the NMDAR also requires the binding of co-agonist glycine. Among the glutamate-gated receptors, NMDARs possess the highest affinity for glutamate (Dingledine et al., 1999). They are expressed in many types of neurons throughout the CNS, but also in other cell types like oligodendrocytes, osteoclasts and lymphocytes where their function is ill-defined (Papadia and Hardingham, 2007).

Whilst the other GluRs can be permeable to Ca\(^{2+}\) only in certain conditions, NMDARs exhibit a relatively high permeability to calcium ions. Since calcium ions act as second messengers in many cellular events, the rise in postsynaptic calcium levels following NMDAR activation can have several physiological and pathophysiological consequences.

At the resting, negative membrane potential of the neuron, the NMDAR pore is directly blocked by Mg\(^{2+}\) ions; the cation being driven from the extracellular medium into the channel by the electrochemical gradient. Thus, only when activity depolarizes the neuron does the magnesium ion escape the pore and let other cations (Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\)) through. Consequently NMDAR are both ligand- and voltage-dependent, for their opening necessitates both the binding of glutamate and co-agonist glycine, in addition to membrane depolarization. Effectively, this only occurs when presynaptic glutamate release is synchronized with postsynaptic depolarization (often mediated by AMPAR activation), earning the NMDARs the epithet of “coincidence detectors”.

The postsynaptic density has been estimated to contain only around 20 NMDARs, which amount for a relatively low proportion of the proteins present in the structure (Sheng and Hoogenraad, 2007). Whilst the quantity of glutamate released in the synaptic cleft is understandably difficult to measure, it is thought that under low frequency firing the neurotransmitter does not saturate synaptic NMDARs, causing only a small number of receptors to open (Nimchinsky et al., 2004). Thus, along with their
high Ca²⁺/Na⁺ permeability ratio, this gives NMDARs unique physiological—and pathophysiological—roles in neurons, as the magnitude of their activation depends on the intensity and frequency of the glutamate release.

2.1 The NR1 subunit
Each NMDAR is composed of four subunits, two of which are NR1 subunits. The NR1 subunit has the binding site for co-agonist glycine or D-serine. The exact identity, origin and role of the co-agonist are still unclear, as well as the level of the glycine-site occupancy of NMDARs (Berger et al., 1998; Wolosker, 2007). Alternative splicing of the mRNA leads to eight possible products, NR1-1a, NR1-1b to NR4-4b (Stephenson, 2006). The different NR1 splice variants can modulate NMDAR properties (Jin and Woodward, 2006; Stephenson, 2006). Moreover, it has been reported that NR1 alternative splicing is developmentally regulated in some areas of the brain, suggesting that the NR1 subunit composition may affect NMDAR functions in some cases (Prybylowski and Wolfe, 2000; Cull-Candy et al., 2001). However, the NR1-1a subtype is the most widely expressed in the CNS (Cull-Candy et al., 2001) and is usually referred to simply as NR1.

2.2 The NR2 subunit
The two remaining subunits can be from the NR2 or the NR3 subfamily. The bulk of the NMDAR population in the mammal forebrain is comprised of receptors composed of the two glycine-binding NR1 subunits and two NR2 glutamate-binding subunits. The four isoforms of NR2, NR2A to –D, are encoded by the Grin2a-d gene set and confer the channel with distinct biophysical properties. Any combination of NR2 subunits can assemble into a functional channel with NR1s, thus determining the receptor channel deactivation, desensitization and pharmacological properties (Dingledine et al., 1999).

NR2A- or NR2B-containing NMDARs are more sensitive to the Mg²⁺ pore blockade than those containing NR2C or NR2D (Monyer et al., 1992; Kuner and Schoepfer, 1996; Wrighton et al., 2008). Compared to NR2B, the NR2A subunit confers to the NMDAR faster opening and peak currents, as well as increased desensitization
and deactivation kinetics (Chen et al., 1999; Erreger et al., 2005; Erreger et al., 2007). The potency of various agonists and analogs also changes between each NR2 subunits, generally increasing from 2A to 2D; albeit all four NR2 subunits exhibit a relatively high potency (low EC\textsubscript{50}) for glutamate ranging between 0.51 and 3.30 µM (Erreger et al., 2007). Since glutamate may not saturate NMDARs during low frequency synaptic transmission, the subunit composition may affect the receptor response and Ca\textsuperscript{2+} influx in such cases (Nimchinsky et al., 2004). On the other hand, it appears that there is no direct correlation between the agonist EC\textsubscript{50} and its efficacy on channel gating; for example, the agonist NMDA has a higher potency for NR2B compared to NR2A (42 µM vs 64 µM) but exhibits a lower efficacy of channel opening (78% vs 93%; Frizelle et al., 2006; Erreger et al., 2007).

### 2.2.1 The developmental and regional pattern of NR2 subunit expression

The NR2 subunit expression follows a specific temporal and regional distribution in the rodent brain. Whereas NR1 is ubiquitously expressed in all brain structures at all stages in development, only NR2B and NR2D are present at the embryonic and early postnatal stages (Monyer et al., 1994), with NR2B being the predominant subunit in the forebrain structures, which includes the cortex and hippocampus. NR2C mRNA expression increases shortly after birth (after P7) in the cerebellum. At the same period, the NR2A subunit also emerges in the forebrain areas (Monyer et al., 1994). Thus mature cortical and hippocampal neurons are considered to contain predominantly NMDARs composed of either NR2A or NR2B subunits –along with NR1-1a (Waxman and Lynch, 2005a). In these regions, the proportion of NR2A has been estimated to reach 50-75% of the total subunit population at tissue maturation (Tovar and Westbrook, 1999; Nakayama et al., 2005; Thomas et al., 2006; Harris and Pettit, 2007).

### 2.2.2 The NR2 subunit structure

The NR2 subunit has a similar transmembrane structure to the other canonical glutamate receptor subunits, as it is comprised of an extracellular N-terminal domain, three transmembrane spanning domains, one re-entrant membrane loop and an intracellular C-
terminal domain (figure 1.1; reviewed in Chen and Wyllie, 2006). NR2A and NR2B, the two predominant subunits in forebrain neurons, are composed of 1464 and 1482-1484 amino acids, respectively and depending on the species. Note that this numbering includes a signal peptide of 19-20 amino acids cleaved in the final product. The NR2 protein sequence is highly conserved between species: the human, mouse and rat NR2A proteins share approximately 96% of their amino acids –and more than 98% for NR2B. The glutamate-binding site of the NR2 subunits is formed by the S1 region, preceding the first transmembrane domain (M1), and S2 region consisting of the extracellular re-entrant loop between M3 and M4; the same can be said for the glycine-binding site of NR1 (Chen and Wyllie, 2006).

The transmembrane domains of NR2A and NR2B are highly conserved between the two subunits, and the extracellular domains share more than 60% of their amino acid sequences. On the other hand, the C-terminal domain sequences of NR2A and NR2B exhibit only around 30% of amino acid homology.
Figure 1.1: The N-methyl-D-aspartate receptor (NMDAR). A. In forebrain neurons, the NMDAR is a tetrameric assembly of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits. When activated by agonists and membrane depolarization, the NMDAR channel lets extracellular Ca\(^{2+}\) enter the cytoplasm. B. Schematic of the NR2 subunit: the glutamate-binding site formed by domains S1 and S2; three transmembrane spanning domains and a re-entrant loop (M1-M4); a large intracellular C-terminal portion of which the last 4 amino acids (aa) consist in a PSD-95/Disc large/Zona occludens 1 (PDZ) motif.
2.3 The NR3 subunit
Although this particular subfamily of NMDAR subunit has not been considered in the present study, the role of NR3 subunits in modulating NMDAR function is gathering growing research interest. NR3A is expressed throughout the brain early in development whereas NR3B is enriched in motor neuron populations in the adult (Wong et al., 2002; Yao et al., 2008). Presence of the NR3 subunit in the heterotetramer confers to the NMDAR a reduced blockade by magnesium ions, as well as decreased calcium permeability. Like the NR1 subunit, the NR3 subunit subclass has glycine as an agonist. Thus, NR1/NR3 di-heteromeric receptors are predicted to form glycine-gated NMDARs, however such receptors have not yet been observed in neurons (Ulbrich and Isacoff, 2008). Since NR3A coimmunoprecipitates with NR1 and NR2 subunits in cortex homogenates, it is thought that NR3 subunits may assemble in tri-heteromeric NR1/NR2/NR3 complexes (Dingledine et al., 1999; Ulbrich and Isacoff, 2008). Moreover, genetic ablation of NR3A expression has been shown to increase NMDAR-mediated currents, suggesting that this subunit regulates currents amplitude or perhaps trafficking of NMDARs (Das et al., 1998). It is suspected that the NR3A subunit plays a role in synaptogenesis early in the development of the neuronal network (Wong et al., 2002). Certain types of glial cells have been found to exhibit NMDA-evoked currents that are relatively insensitive to Mg\(^{2+}\) blockade, which may indicate a predominant expression of the NR3 subunit in glial-NMDARs (Verkhratsky and Kirchhoff, 2007).

2.4 Extracellular NMDAR modulation
The extracellular portion of the NMDAR can be modulated allosterically at certain sites. The NR1 subunit N-terminal sequence is sensitive to inhibition by protons (H\(^+\)). In fact, the NMDAR channels are 50% inhibited by protons at the physiological pH of 7.3 (Traynelis et al., 1995). Thus, the proton regulation might be an intrinsic mechanism by which NMDAR-mediated calcium influx is limited in acidic conditions like hypoxia or ischemia (Yamakura and Shimoji, 1999). Polyamines also interact on the extracellular side of NMDAR to produce either a stimulatory or inhibitory effect, as does some reducing/oxidizing agents (Traynelis et al., 1995; Yamakura and Shimoji, 1999).
2.5 The C-terminus of NMDAR interacts with intracellular proteins

Of all the NR subunits the NR2A and NR2B subunits have the largest cytoplasmic tails (around 650 amino acids), which can interact with diverse signalling and scaffolding proteins (Sheng and Pak, 1999; Hardingham and Bading, 2003; Kim and Sheng, 2004).

The PSD-95-like family of membrane-associated guanylate kinases (MAGUKs) is comprised of PSD-95, synapse-associated protein 90 (SAP90), PSD-93 and SAP102. The PSD-95-like MAGUKs are submembranous proteins able to multimerize and bind to a vast array of proteins possessing a PSD-95/Disc large/Zona occludens 1 (PDZ) motif (Gardoni, 2008). These scaffolding proteins are highly enriched in the postsynaptic density and have been shown to interact with the NR2 subunits by binding on their C-terminal PDZ domain, at least in vitro (Kim and Sheng, 2004; Cousins et al., 2008b). Thus, by tethering various molecules to the intracellular mouth of the NMDAR channel, the PSD-95 MAGUKs can form calcium-dependent signalling clusters; these signalling complexes can be efficiently activated following NMDAR activation due to the vicinity of the Ca\(^{2+}\) influx (Hardingham and Bading, 2003; Kim and Sheng, 2004).

Apart from coupling NMDAR activity to downstream signals, it has also been suggested that the PSD-95-MAGUKs interaction with NR2 can modulate NMDAR trafficking. Albeit the link between MAGUKs and NMDAR trafficking lacks definitive evidence, in various paradigms PSD-95-MAGUKs enhance surface NMDAR expression and increase their synaptic localization, possibly by reducing NMDAR lateral mobility (Wenthold et al., 2003; Groc et al., 2006; Elias and Nicoll, 2007).

Because of the dissimilarity between the C-terminal portion of NR2A and NR2B, the type of interactions and signalling complexes present at the intracellular side of the receptors may depend on the NR2 subunit composition of the receptor. Indeed, PSD-95 MAGUKs interact differentially with NR2A and NR2B: NR2A has a higher affinity for SAP102 whereas NR2B interacts preferentially with PSD-95 (Sans et al., 2000; Cousins et al., 2008b); the association between the proteins involves domains of the NR2 C-terminus (amino acid 1382-1420 on NR2A and 1086-1157 on NR2B; Cousins et al., 2008a) in addition to the PDZ binding motif – an identical ESDV ending both of NR2A and NR2B sequences.
Therefore, the NR2 subunit composition may play a crucial role in determining the signals downstream of NMDAR activation, due to the identity of the intracellular binding partners associating with each subtype of NR2.

3. Physiological and pathophysiological signalling downstream of NMDARs

3.1 NMDAR-dependent plasticity
The capacity of NMDARs to induce cytoplasmic calcium transients gives to these glutamate receptors a pivotal role in intracellular physiological events. Of these, it has long been recognized that NMDAR activation can induce synaptic plasticity, synapse-specific modifications that are thought to be the cellular bases of learning and memory (Bliss and Lomo, 1973; Morris et al., 1986). NMDAR-dependent synaptic plasticity has been observed in diverse brain regions; two well-documented paradigms are the long-term potentiation (LTP) and long-term depression (LTD) of synaptic contacts between the Schaffer collateral axons and the dendritic arborisation of the pyramidal neurons of the CA1 region of the hippocampus (Malenka and Bear, 2004). These two paradigms of synaptic plasticity can be triggered at the same synapses, seemingly depending on the strength and duration of the Ca\(^{2+}\) influx through NMDARs.

3.1.1 Long-term potentiation and depression
Upon high frequency stimulation from the axons originating from the CA3 region, synapses from the CA1 hippocampal neurons potentiate rapidly and durably. This phenomenon called LTP requires postsynaptic NMDAR-induced calcium transients, and the subsequent activation the calcium-calmodulin kinase II (CamKII) enzyme (Lisman et al., 2002). Several other kinases are also thought to be playing a role at this stage; among those are cAMP-dependent protein kinase A (PKA), phosphatidylinositol 3-kinase (PI3K) and Src kinase (Malenka and Bear, 2004). The result of these intracellular cascades includes rapid phosphorylation of surface AMPARs, which potentiates their functions, and the insertion of additional AMPAR from a readily accessible vesicular pool (Malinow and Malenka, 2002). Following this early phase of LTP (around 1h), this
postsynaptic potentiation is consolidated through new protein synthesis and gene transcription that are thought to involve signalling to the nucleus by PKA, CamKIV and mitogen-activated protein kinases (MAPKs), and the consequent activation of the transcription factor CREB (cAMP-response element binding protein; Malenka and Bear, 2004; Vickers et al., 2005).

Hippocampal LTD, in contrast to LTP, is evoked by a low and sustained cytoplasmic calcium elevation –whereas short and intense transients arise from the LTP-inducing high frequency stimulus. The calcium entry through NMDAR can recruit various phosphatases like calcineurin and protein phosphatase 1 (PP1), which dephosphorylate AMPARs and cause their internalization, effectively reducing the excitability of the glutamatergic synapse (Lee et al., 1998; Malenka and Bear, 2004).

Note that a multitude of synaptic plasticity processes have been described throughout the CNS and in many cell types, exhibiting a remarkable variety of mechanisms that will not be described further here (presynaptic/postsynaptic; NMDAR/non-NMDAR-dependent; for review see Malenka and Bear, 2004).

Despite extensive investigation and perhaps due to the multiple types of synaptic plasticity, knowledge of the molecular details leading to NMDAR-dependent potentiation and depression is still incomplete, as well as the identity of the Ca\(^{2+}\)-sensors receiving the NMDAR-mediated ionic signal.

3.2 The role of NR2 subunit composition in NMDAR-dependent plasticity
As mentioned, the signal governing the direction of the synaptic modification is thought to be the shape of the postsynaptic calcium transient mediated by the NMDARs. In recent years an intriguing prospect has come into play: the possibility that the subunit composition of the NMDAR could dictate, or at least influence the type of synaptic plasticity neurons undergo (Yashiro and Philpot, 2008). NR2A- and NR2B-containing NMDARs exhibit different channel kinetics. Thus, the subunit composition of NMDARs can alter the temporal and quantitative characteristics of the calcium influx under a given stimulus. Concurrently, the distinct signalling complexes attached to each NR2 subunit can potentially influence synaptic plasticity induction, via NR2-specific intracellular
cascades or by modifying the subcellular localization of the NMDAR (Yashiro and Philpot, 2008).

3.2.1 Studies from transgenic animal models

The possibility of a link between the NR2 subunit composition of NMDARs and the direction of synaptic plasticity was first studied on transgenic mice lacking a particular subtype of NR. The NR2B knockout mouse (NR2B\(^{-/-}\)) dies shortly after birth and shows heavy developmental defects in several parts of the brain (Kutsuwada et al., 1996). In NR2B\(^{-/-}\) slices from immature tissue LTD cannot be triggered, suggesting a role for NR2B in LTD induction (Kutsuwada et al., 1996). Conversely the NR2B subunit has also been shown to participate in LTP; another mouse line, genetically driven to overexpress the NR2B subunit, demonstrates superior learning capacities and facilitated hippocampal LTP (Tang et al., 1999).

As opposed to the NR2B\(^{-/-}\) animal model, the NR2A-deficient mouse does not exhibit any morphological abnormalities, develops and breeds normally (Sakimura et al., 1995). This lack of developmental consequences is not surprising, as the NR2A subunit only appears after the first postnatal week. On the other hand, the absence of NR2A subunit causes a reduction of the hippocampal LTP magnitude, concomitant with a deficiency in spatial learning. However, the impairment of LTP in NR2A\(^{-/-}\) mice has later been shown to be rescued by compensating the loss of NR2A with an increase in NR2B subunit contribution (Kohr et al., 2003; Berberich et al., 2007). The ability of the NR2B subunit to functionally replace NR2A suggests that the lack of a particular subtype can be compensated for by another, regarding plasticity signals. Thus, studies using transgenic animal models did not provide a clear indication that a subtype of NR2 could be preferentially coupled to potentiation or depression.

However, several transgenic mice genetically altered to produce NR2 subunits lacking only the intracellular C-terminal portion develop phenotypes similar to the full knockout of the same subunit (Sprengel et al., 1998). Indeed, this finding implies that the C-terminus tail of each subunit plays a crucial role in NMDAR functions, as the lack
of the C-terminal portion of both NR2A and NR2B causes the same developmental and plasticity deficits observed in NR2B^{−/−} and NR2A^{−/−} mice.

3.2.2 Studies using pharmacological agents

As opposed to genetic deletion, the development of various subunit-specific antagonists permitted a more acute pharmacological approach to investigate the role of NR2A and NR2B in synaptic plasticity.

Ifenprodil (4-[2-[4-(cyclohexylmethyl)-1-piperidinyl]-1-hydroxypropyl]phenol) is a compound which exerts a remarkable selectivity to NR2B-containing NMDARs. This antagonist affects NR2B-containing receptors in a non-competitive, voltage-independent and activity-dependent manner; although from recombinant studies it was shown to inhibit maximally around 80% of NR2B-mediated currents (Williams, 1993; Chenard and Menniti, 1999). Whilst it fails at blocking the totality of the NR2B-containing NMDAR activity, it does so with no significant effect on di-heteromeric receptors comprised of NR2A subunits (Williams, 1993). While little is known about its inhibition of the tri-heteromeric (NR1/NR2A/NR2B) receptors, Hatton and Paoletti (2005) estimated the antagonism to be only partial - roughly 20% of inhibition.

Another compound, (R)-[[(S)-1-(4-bromo-phenyl)ethylaminol]-2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl]-methyl]-phosphonic acid (NVP-AAM077), is a competitive antagonist preferentially targeting NR2A-containing NMDARs. Its selectivity is however limited: about 5 fold more selective compared to NR2B when comparing antagonist binding. Indeed, early publications employing this drug used concentrations (0.4-1 µM) at which NVP-AAM077 also inhibits a significant proportion of NR2B-containing NMDARs (Berberich et al., 2005; Frizelle et al., 2006). At 30 nM, NVP-AAM077 antagonises around 70% of NR2A- and only 10% of NR2B-containing NMDARs; albeit being a competitive antagonist these proportions may change with the agonist concentration (Frizelle et al., 2006).

Other NMDAR antagonists include APV ((2R)-amino-5-phosphonovaleric acid) and MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine). APV is a competitive and reversible NMDAR antagonist; whereas MK-801 is a use-
dependent, open-channel blocker that has a high affinity for NMDARs and thus can be considered to be irreversible in experiments lasting up to an hour (Huettner and Bean, 1988; Tovar and Westbrook, 2002).

Thus, with the help of these pharmacological tools, other groups investigated the effect of NMDAR subunit-specific blockade and the contribution of subcellular pools of NMDARs on various plasticity paradigms. A study by Liu et al. (2004) found using hippocampal slice preparations that NR2B-containing NMDARs are required for the induction of LTD, whereas LTP necessitates the NR2A-containing NMDAR activity. Similar results were reported in cortical tissue by Massey et al. (2004), supporting the notion that the NR2 subunit composition bi-directionally governs the direction of NMDAR-dependent plasticity signals.

However, these reports were followed by contrary findings in which it was demonstrated that the NR2B subunit was necessary or at least contributing to LTP induction (Tang et al., 1999; Barria and Malinow, 2005). Indeed CaMKII, an essential mediator of LTP, associates preferentially with the NR2B C-terminus in vitro, as opposed to NR2A; implying that this subtype could facilitate CaMKII activation during LTP induction (Barria and Malinow, 2005; Park et al., 2008). Several other publications also reported a lack of NR2 subunit-dependence in various plasticity protocols and cellular models (Berberich et al., 2005; Weitlauf et al., 2005; Bartlett et al., 2007; Le Roux et al., 2007; Morishita et al., 2007; Harris and Pettit, 2008).

The difference in conclusions between studies can be due to several factors. The heterogeneity of the cellular models (e.g. slice preparations vs. dissociated neuronal cultures) and plasticity induction protocols (e.g. 100Hz stimulation vs. theta-burst stimulation for LTP induction) may explain the discrepancies within results obtained by different groups. Secondly, many studies aiming at targeting specifically NR2A-containing NMDAR used the antagonist NVP-AAM077 at concentrations (0.4-1 µM) were it lacks specificity and affects a significant proportion of NR2B-containing NMDAR currents (Berberich et al., 2005; Frizelle et al., 2006). In these cases, the plasticity-inhibiting effects observed using this compound can be due to a general
decrease of the NMDAR currents or even by off-target NR2B-containing NMDAR antagonism.

Thus, there is evidence supporting and discrediting the hypothesis of a role for NR2A/NR2B in governing the direction of synaptic plasticity signalling (Kohr, 2006) and the issue remains unresolved.

3.3 NMDAR-dependent pro-survival and pro-death signalling

Glutamate can induce brain damage (Olney, 1969), a process involving overactivation of glutamate receptors - particularly NMDARs. This phenomenon has been labelled “excitotoxicity”, and this type of cell death includes both necrotic and apoptotic features like chromatin condensation of the nuclei (Martin et al., 1998; Fujikawa et al., 2000).

Interestingly, in addition to excessive activation of NMDARs, lack of activity from this subclass of glutamate receptor also has detrimental effects on neurons (Hardingham and Bading, 2003). In vivo blockade of NMDAR activity with MK-801 (open-channel antagonist) during late embryonic or early postnatal stages triggers widespread apoptosis of developing neurons (Gould et al., 1994; Ikonomidou et al., 1999). In mature neurons, NMDAR antagonism renders neurons more susceptible to a toxic insult: in the adult rat brain, neurodegeneration caused by a mitochondrial toxin or by traumatic brain injury is enhanced by blockade of NMDARs (in the basal ganglia and the hippocampus, respectively; Ikonomidou et al., 2000). In vitro, induction of neuronal apoptosis by various chemical inducers including staurosporine is exacerbated by NMDARs antagonism (Hardingham et al., 2002; Papadia et al., 2005; Papadia et al., 2008).

Thus, too little and too high NMDAR activation promotes pro-death signals in glutamatergic neurons. In many pathophysiological conditions, extracellular glutamate concentration rises and triggers excitotoxicity via NMDARs (figure 1.2 from Papadia and Hardingham, 2007). Finding a way to effectively disrupt the excitotoxic cascades is the focus of intense research efforts, and of the work presented in this thesis. The cascades involved in excitotoxic and pro-survival signalling will be detailed in the next section.
Figure 1.2: Pro-survival and pro-death signalling from the NMDAR. Schematic including some of the molecular pathways involved in NMDAR-mediated fate signalling, from Papadia and Hardingham (2007).
3.3.1 Pro-survival NMDAR signalling
Between the two detrimental extremes (too much or too little activation), it has been demonstrated that normal, physiological levels of NMDAR activity actually recruit neuroprotective cascades.

3.3.1a The CREB pathway is induced by synaptic NMDAR activity
NMDARs can be localized both in synaptic and extrasynaptic regions in neurons (Cottrell et al., 2000). Synaptic NMDAR stimulation by neuronal activity renders neurons more resistant to excitotoxic and apoptotic insults (Hardingham and Bading, 2003). A mediator of this neuroprotection is the transcription factor CREB (Lee et al., 2005; Papadia et al., 2005). CREB, a nuclear protein activated by phosphorylation on its serine 133, can trigger the expression or silencing of various gene products; it is known to induce brain-derived neurotrophic factor (BDNF) and c-Fos expression, and is overall beneficial for neuronal survival (Hardingham et al., 2002; Dragunow, 2004; Lee et al., 2005; Papadia et al., 2005; Greer and Greenberg, 2008).

Synaptic activity-dependent CREB activation occurs following calcium entry in the cell nucleus. This calcium, released from intracellular store or entering the cell body through Ca\(^{2+}\)-permeable channels, activates the nuclear protein CaMKIV (Chawla et al., 1998; Hardingham et al., 2001a). CaMKIV then phosphorylates CREB on serine 133 (Hardingham et al., 2001a; Wu et al., 2001). In addition, the CREB pro-survival pathway can be recruited by activation of the extracellular signal-regulated kinases 1/2 (ERK1/2), a subclass of MAPKs also upregulated by synaptic NMDAR activity (Hardingham et al., 2001b; Ivanov et al., 2006). Apart from being able to promote CREB activation, the ERK1/2 pathway can inactivate pro-apoptotic Bcl-2 family member BAD (Bcl-2/Bcl-X\(_L\)–antagonist causing cell death; see Figure 1.2), but its involvement in neuronal viability remains uncertain (Ivanov et al., 2006; Papadia and Hardingham, 2007).

3.3.1b The PI3K/Akt pathway
The synaptic NMDAR activation of the CREB pathway induces neuroprotection through gene expression. However, ongoing synaptic activity is also “instantly” neuroprotective for neurons, a NMDAR-dependent process that does not require CREB-mediated gene transcription/translation (Papadia et al., 2005). Instead, the kinases PI3K/Akt pathway mediates these acute activity-induced pro-survival signals. The inhibition PI3K/Akt abolishes activity-dependent protection of trophically-deprived neurons and even enhances death in control cell (Papadia et al., 2005), highlighting its basal requirement for cell survival.

The PI3K/Akt pathway targets several anti-apoptotic pathways (Figure 1.2). Activated Akt –by PI3K- inhibits glycogen synthase kinase 3β (GSK3β), a kinase promoting the translocation of apoptotic factor Bax to the mitochondria (Papadia et al., 2005; Soriano et al., 2006). Akt also induces phosphorylation and nuclear export of pro-death transcription factor FOXO (Forkhead box subgroup O) as well as inhibiting transcription factor p53, Bcl-associated death promoter (BAD) and the stress-activated protein kinase (SAPK) subtype c-Jun N-terminal kinase (JNK); all known apoptosis inducers (Hardingham, 2006; Papadia and Hardingham, 2007).

The molecular link between NMDAR activation and PI3K/Akt induction is not well defined. It has been reported that PI3K binds directly to the NR2B subunit C-terminal tail when Tyr1336 is phosphorylated (Hisatsune et al., 1999; Waxman and Lynch, 2005b) but others have failed to coimmunoprecipitate both proteins. This is possibly due to low basal phosphorylation levels on Tyr1336 of NR2B (Man et al., 2003), as Tyr1336 is not the preferential phosphorylation site on NR2B (Waxman and Lynch, 2005b). Thus, it is possible that direct interaction of PI3K and NR2B only occurs in ischemic/excitotoxic conditions, when phosphorylation at Tyr1336 is increased.

3.3.2 Pro-death NMDAR signalling
Overactivation of NMDAR triggers neuronal death, but since this subclass of receptors is required for the neuronal survival and controls the activity-dependent modifications of their synapses, complete blockade of the NMDARs can have deleterious consequences (Ikonomidou and Turski, 2002; Hardingham and Bading, 2003). Thus, several NMDAR-
dependent pro-death signals have been identified in the hope that disruption of these pathways, rather than NMDAR activity, could be a better tolerated anti-excitotoxic approach.

Excitotoxicity is more than simple cytoplasmic Ca\(^{2+}\) overload as it was once thought (Hartley et al., 1993), as it has been shown to depend on the route of entry and on Ca\(^{2+}\) microdomains (Hardingham and Bading, 2003). Indeed, calcium fluxes through NMDARs are more toxic than through L-type voltage-gated calcium channels (VGCCs; Sattler et al., 1998), via several transduction pathways downstream of NMDAR activation. The work presented in this thesis is focussed on the stress-activated protein kinases (SAPKs) JNK and p38, two key mediators of excitotoxicity, but the following section will also provide a brief overview of other known pro-death cascades downstream of NMDARs.

### 3.3.2a The CREB and ERK1/2 pathways

As mentioned previously, activation of synaptic NMDARs induces pro-survival CREB phosphorylation and CREB-dependent gene expression (Hardingham et al., 2002; Lee et al., 2005; Papadia et al., 2005). On the other hand, extrasynaptic NMDAR stimulation turns off of the CREB pathway (Hardingham et al., 2002; Lee et al., 2005), as well as the ERK1/2 cascade (Ivanov et al., 2006). The stimulation of extrasynaptic NMDARs can be achieved by exposing neuronal cultures to bath-applied NMDAR agonist following synaptic NMDAR blockade (by a period of action potential firing in presence of use-dependent antagonist MK-801). With the synaptic NMDARs blocked, bath-applied agonists then exclusively activate the extrasynaptic pool of receptors. Rather than being *inefficient* at triggering CREB phosphorylation, stimulation of extrasynaptic NMDARs actively inactivate these pathways, which renders neurons more vulnerable to insults (Hardingham et al., 2002; Ivanov et al., 2006). The identity of the extrasynaptic NMDAR-coupled molecular pathways mediating CREB dephosphorylation remains to be elucidated. However, both PP1 and protein phosphatase 3 (PP3) can induce CREB dephosphorylation following stimulation with an excitotoxic concentration of NMDA (Hardingham et al., 2002; Lee et al., 2005).
3.3.2b Calpain-mediated proteolysis

Calcium-activated cysteine proteases calpains have also been shown to contribute to excitotoxicity. These enzymes cleave many different substrates and their overactivation following excessive NMDAR stimulation can prove deleterious. Following an *in vivo* ischemic episode or a toxic glutamate application on cultured neurons, the Na\(^+\)/Ca\(^{2+}\) exchanger 3 (NCX3) is cleaved by calpains. This cleavage of NCX3, normally operating cytoplasmic Ca\(^{2+}\) extrusion, can provoke a delayed second calcium overload that causes lysis of the neurons (Bano et al., 2005). It has also been suggested that calpain-mediated truncation of mGluR1 may contribute to this delayed calcium deregulation (Xu et al., 2007). The substrate consensus for calpain cleavage is not fully understood, and the role of the different calpain isoforms as yet to be elucidated. However, Bevers et al. (2008) discovered that knock down of the m-calpain isoform increases neuronal survival after applications of toxic doses of NMDAR agonist.

Intriguingly, NMDAR subunit composition has been proposed to play a role in excitotoxic calpain activation, following the observation that calpain-mediated spectrin degradation was linked to NR2B-containing NMDAR activity (Zhou and Baudry, 2006).

3.3.2c Mitochondrial damage

Mitochondria generate most of the cellular energy (ATP). This energy production is due to the conversion of the inner mitochondrial membrane potential into ATP by the ATP synthase. In addition, mitochondria are able to sequester large quantities of cytosolic calcium, the cation being driven in by the mitochondrial membrane potential and through a calcium uniporter. During excitotoxicity this membrane potential inevitably collapses, followed by the depletion of cellular ATP and mitochondria breakdown (Bano et al., 2005; Greenwood et al., 2007; Mattson, 2007). Whilst it is difficult to determine if this mitochondrial breakdown cascade is the cause or the consequence of neuronal excitotoxicity, it can be the result of different cellular events.

During excessive NMDAR activation, Ca\(^{2+}\) overload may lead to mitochondrial permeabilization through the activation of the permeability transition pore (Greenwood
et al., 2007). This can then provoke the sequestered Ca\(^{2+}\) to release back in the cytoplasm and create the delayed calcium deregulation. However, recent reports suggest that the delayed calcium deregulation instead occurs when the calcium intake overwhelms the mitochondria and depolarizes the organelle, which then fails to produce ATP or even depletes the cellular ATP in an attempt to restore its negative potential. The subsequent bioenergetic insufficiency then causes failure of the plasma membrane pumps, such as the Na\(^+\)/Ca\(^{2+}\) exchanger, which results in further calcium entering the neuron (Bano et al., 2005; Greenwood et al., 2007).

This model of mitochondrial damage-induced neurotoxicity have been supported by substantial experimental evidence and strongly suggest that mitochondrial dysfunction is an important contributor to NMDAR-dependent excitotoxicity (Ward et al., 2005; Greenwood et al., 2007; Mattson, 2007; Nicholls, 2008).

### 3.3.2d The JNK pathway

As mentioned above, the experiments contained in this thesis have focussed on two key pro-death signals downstream of NMDAR overactivation: the JNK and p38 SAPK pathways. The JNK SAPK pathway is induced in in vivo ischemic models and in cortical neuron cultures undergoing excitotoxicity. Inhibition of this cascade by D-JNKI1, a cell-permeable peptide which prevents access of JNK to many of its targets, is strongly neuroprotective (Borsello et al., 2003). There are several JNK isoforms (JNK1-3) and not all are implicated in excitotoxicity. In mice genetically altered to be lacking specific isoforms, the JNK3 isoform appears to contribute to ischemic damage (Kuan et al., 2003; Brecht et al., 2005). On the contrary, mice lacking the JNK1 isoform have enhanced apoptosis and enlarged ischemic infarcts (Kuan et al., 2003; Brecht et al., 2005), which indicates that this isoform may be required in the neuron for physiological processes.

The downstream targets and upstream activators of JNK in NMDAR-mediated excitotoxicity are still under investigation. Although a known JNK activator, the MAPK kinase 7 (MKK7) was shown to be upregulated by NMDA (Centeno et al., 2007), how NMDAR activity recruits pro-death JNK activation is still unknown.
3.3.2e The p38 pathway

The p38 SAPK pathway has also been implicated in NMDAR-induced neuronal death. In cerebellar granule cells (CGCs) it has been demonstrated that glutamate-induced cell death relies on activation of the p38 cascade (Kawasaki et al., 1997; Cao et al., 2005), a chain of events dependent on nitric oxide (NO) production (Cao et al., 2005).

In cortical and hippocampal neurons the p38 pathway is strongly activated, by phosphorylation, when neurons are treated with toxic concentrations of glutamate (Rivera-Cervantes et al., 2004; Molz et al., 2008). Similar to NMDAR blockade, inhibition of p38 SAPKs reduces the excitotoxic damage in these regions, identifying the p38 pathway as a NMDAR-dependent pro-death signal (Rivera-Cervantes et al., 2004; Segura Torres et al., 2006; Molz et al., 2008). As for the JNK pathway, the identity of the molecular link between NMDARs and the p38 SAPKs is still undiscovered.

SAPKs are ubiquitous in various cell types and can be activated by stress signals to trigger apoptosis. However, both JNK and p38 SAPKs are comprised of multiple isoforms which can mediate diverse cellular functions. As examples, the p38 cascade has a well characterized role in synaptic plasticity (Zhu et al., 2002; Waxman and Lynch, 2005b) and the JNK1 isoform is needed for neuronal development and survival (Brecht et al., 2005). Thus, it is crucial to identify the pro-death signals leading to the activation of these molecules following NMDAR activity.

3.3.2f The PSD-95 MAGUK

As mentioned previously, MAGUKs are a superfamily of submembranous proteins that can interact with a variety of proteins, multimerize and form signalling clusters (Gardoni, 2008). The PSD-95-like subfamily of MAGUKs is an attractive molecular link between NMDARs and their downstream signals (Figure 1.3 from Sheng and Kim, 2002), due to the high expression levels of these scaffolding proteins in neurons (Sheng and Pak, 1999; Kim and Sheng, 2004). In particular, PSD-95 has been the focus of most research efforts due to its enrichment at the postsynaptic density and the dendritic spines (Kim and Sheng, 2004; Gardoni, 2008). Indeed, PSD-95 has been found to interact with
Figure 1.3: A schematic of the NMDAR-associated protein complex. The PSD-95 MAGUK is can tether several PDZ-domain-containing proteins to NMDARs, including neuronal nitric oxide synthase (nNOS). AKAP: A-kinase anchor protein; CaMKII: Ca$^{2+}$/calmodulin-dependent protein kinase II; ERK: extracellular regulated kinase; Gap: GTPase-activating protein; GKAP: glucokinase-associated phosphatase; H: Homer; IP3R: IP$_3$ receptor; MEK: mitogen-activated protein kinase; mGluR: metabotropic glutamate receptor; PKA: protein kinase A; PP1: protein phosphatase 1; PYK2: proline-rich tyrosine kinase 2; RTK: receptor tyrosine kinase; SER: smooth endoplasmic reticulum; SPAR: spine-associated RapGAP. Schematic from Sheng and Kim (2002).
the PDZ, “ESDV” motif ending the sequence of the NR2A and NR2B subunits (Rutter et al., 2002; Stephenson, 2006; Cousins et al., 2008b). This interaction is proposed to play a role in the modulation of NMDAR kinetics and trafficking (Roche et al., 2001; Gray et al., 2006; Stephenson, 2006; Sornarajah et al., 2008), but is also thought to be a key element of NMDAR-coupled signalling complexes (Sheng and Pak, 1999; Kim and Sheng, 2004; Cui et al., 2007; Gardoni, 2008).

Other than by the tethering of signalling complexes to the mouth of the NMDAR channel, the binding of PSD-95 to the NR2 subunit might influence the downstream NMDAR-mediated signals in an indirect manner: by protecting the C-terminus cleavage of NR2 subunits by calpains (Dong et al., 2004; Yuen et al., 2008). The implications of this mechanism on NMDAR-mediated signal transduction during excitotoxicity remain unclear, especially considering the role of calpains in excitotoxicity (Bano et al., 2005; Xu et al., 2007), as described in Section 3.3.2b.

Otherwise, PSD-95 has been found to couple NMDAR with neuronal nitric oxide synthase (nNOS), and several studies found that disrupting PSD-95 from forming PDZ associations is neuroprotective (Sattler et al., 1999; Aarts et al., 2002; Hou et al., 2005; Cui et al., 2007; Sun et al., 2008). nNOS, tethered to the NMDAR/PSD-95 complex, is activated by Ca\(^{2+}\) influx through NMDARs and may, in an excitotoxic context produce an excess of NO that will trigger neuronal death (Rameau et al., 2007). Indeed, excitotoxic death can be blocked \textit{in vitro} by nNOS inhibitors and cerebral ischemia is reduced in mice lacking nNOS (Moro et al., 2004). NO overproduction leads to the formation of peroxynitrites, a reactive agent implicated in excitotoxicity. Peroxynitrites are powerful oxidants resulting from the interaction of NO and superoxides (Moro et al., 2004).

Disruption of the interaction between the PDZ binding domain of the NMDAR (the last four amino acids of the NR2 C-terminus) and PSD-95 shows remarkable neuroprotection against ischemia \textit{in vivo} and excitotoxicity \textit{in vitro} (Sattler et al., 1999; Aarts et al., 2002). This disruption is achieved using an oligopeptide mimicking the last nine amino acids of the NR2B sequence fused to a TAT sequence (the cell-membrane transduction domain of the human immunodeficiency virus-type 1) to make it cell-
permeable. This synthetic peptide, TAT-NR2B9c, can technically disrupt any PDZ interactions but appears to target preferentially the NR2A-B/PSD-95 and nNOS/PSD-95 interface (Cui et al., 2007). Moreover siRNA knock down of other member of the PSD-95-like MAGUKs (PSD-93, SAP97 and SAP102) does not confer any neuroprotective effect to neurons treated with toxic doses of NMDA, whereas knocking down of PSD-95 or nNOS does (Cui et al., 2007). Thus, PSD-95 and nNOS are key mediators in NMDAR-mediated toxic signals, above other PDZ domain-containing proteins.

On the other hand PSD-95 has potential roles in the trafficking and synaptic localization of NMDARs, therefore preventing its association with the NR2 subunits could have undesirable consequences on the normal physiological roles and signals emanating from the NMDARs. Whilst the presence of the TAT-NR2B9c peptide does not alter NMDAR currents (Aarts et al., 2002; Lim et al., 2003), it remains uncertain if it spares pro-survival and plasticity signals at the concentration shown to protect neurons against ischemia/excitotoxicity.

3.4 The role of NR2 subunit composition of NMDARs in pro-death and pro-survival signals

Synaptic NMDAR stimulation triggers pro-survival pathways, whereas extrasynaptic NMDARs preferentially activate pro-death signals (Hardingham and Bading, 2003). Interestingly, it has been reported that the postnatal upregulation of the NR2A subunit occurs specifically at the synapse, gradually excluding the NR2B subunit to extrasynaptic sites (Li et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). However, this differential subunit distribution has been challenged by several publications and needs further investigation (Mohrmann et al., 2000; Thomas et al., 2006; Harris and Pettit, 2007). Thus, the possibility that synaptic NMDARs are composed of NR2A subunits whereas NR2B-containing NMDARs are extrasynaptic implies that pro-survival signalling complexes are coupled to the NR2A C-terminus and pro-death signals to the NR2B C-terminus.

Indeed, recent studies have sought to address if the NR2 subunit composition could determine the type of signal (pro-survival or pro-death) downstream of NMDAR
activation. Liu et al. (2007) found that in immature cortical cultures, inhibition of NR2B-containing NMDARs is neuroprotective and does not affect NMDAR-dependent pro-survival signals. On the other hand, blockade of NR2A-containing NMDARs enhances excitotoxicity and abolishes the survival pathways downstream of NMDARs. Intriguingly, Liu et al. (2007) reported that the consequences of the subunit-specific blockades on neuronal viability remain unchanged when synaptic NMDARs are blocked, which implies that the subunit composition rather than the localization of NMDARs governs the type of signal triggered.

The dual role for NR2 subunit composition on neuronal viability is also supported by results from Chen et al. (2008), studying ischemic neuronal cell death and ischemic tolerance in the hippocampus. Ischemic tolerance consists in a non-toxic ischemic episode that renders neurons more resistant to a subsequent lethal insult. This preconditioning is thought to trigger protective cellular programs, which includes NMDAR-mediated CREB-dependent gene transcription (Chen et al., 2008). Thus, NR2B-specific blockade of NMDARs protects the rodent’s brain against an ischemic insult without affecting NMDAR-dependent ischemic pre-conditioning. Conversely, NR2A-containing NMDAR inhibition exacerbate brain damage, abolishes pre-conditioning and pre-conditioning-induced CREB phosphorylation (Chen et al., 2008).

In disagreement with these reports, another study failed to observe a link between NR2 subunit composition and the ability of the NMDAR to induce excitotoxicity (von Engelhardt et al., 2007). Indeed, von Engelhardt et al. (2007) suggest that both NR2A and NR2B subunits are able to induce excitotoxicity; any differential signalling observed when a particular subtype is inhibited could be due to the decreased NMDAR activity, regardless of the subunit composition (von Engelhardt et al., 2007).

Thus, the rules of engagement of the NR2 subunits in NMDAR-dependent pro-survival and pro-death signalling still remain unclear, as well as the subunit composition of NMDAR in synaptic/extrasynaptic loci.

NMDAR activation and consequent \( \text{Ca}^{2+} \) influx exert tremendously variable influences on neurons: these glutamate receptors are implicated in the molecular cascades leading to synaptic modifications underlying learning and memory processes;
their physiological activity ensures the survival and resistance of neurons to different types of insults; as well as being damageable to neurons in cases of abnormal activity. The ability of NMDARs to signal to these different outcomes potentially depends on their NR2 subunit composition: the cytoplasmic tail of said subunit can form signalling complexes –such as the PDZ domain interaction with PSD-95- which can trigger the induction of the different molecular cascades. Ultimately, the reason for investigating the NMDAR-dependent downstream signalling is to achieve a selective disruption of the excitotoxic pro-death signalling pathways, as excitotoxicity has been shown to contribute to several neuropathologies.

3.5 A brief overview of NMDAR-dependent processes in diseases

Huntington’s disease is thought to result from progressive neuronal loss, mainly in the striatum and caused by an aberrant form of the huntingtin protein. The mutant huntingtin protein appears to augment NMDAR-mediated currents and excitotoxicity in animal models, as well as altering downstream signalling events dependent on NMDARs (Waxman and Lynch, 2005a). The role of NMDAR-mediated excitotoxicity in Huntington’s disease is not fully understood, but certain NMDAR antagonists were found to slow the onset of the illness in preclinical studies (Estrada Sanchez et al., 2008).

NMDAR misregulation has also been strongly linked to epilepsy. Epileptical activity involves imbalances in the glutamatergic transmission, and aberrant NMDAR-mediated plasticity might be underlying the increased, pathological excitability of epileptic tissue. In fact, glutamate receptor inhibitors have proven to be anticonvulsant and blockade of NMDARs prevents neuronal damage following strong seizures. Moreover, abnormal NR2 subunit quantities have been found in different subtype of epileptic tissues (Waxman and Lynch, 2005a).

Another neuropathology, Alzheimer’s disease, is now widely recognized to involve excessive NMDAR activation and excitotoxicity. Soluble amyloid β oligomers are thought to be responsible for the impairment of synaptic function and neurodegeneration. Amyloid β has been reported to disrupt LTP and increase
glutamatergic activity which can result in NMDAR overstimulation and trigger neuronal death. Memantine, a moderate affinity, uncompetitive, voltage-dependent NMDAR antagonist with fast unblocking kinetics is being used for treatment of moderate to severe Alzheimer’s disease cases, highlighting the pivotal role of NMDARs in the pathology (Parsons et al., 2007). Additionally, NMDAR dysfunction –not necessarily excitotoxicity per se- may be implicated in Parkinson’s disease, depression, anxiety, addiction, migraine, and schizophrenia (Cull-Candy et al., 2001; Waxman and Lynch, 2005a; Muir, 2006; Koutsilieri and Riederer, 2007; Estrada Sanchez et al., 2008).

Lastly, ischemic stroke-induced brain damage is thought to be largely due to NMDAR-mediated excitotoxicity. An ischemic episode leads to the release of glutamate from the neuron terminals. Glutamate transporters from neurons and glial cells are responsible for the glutamate uptake and necessitate external/internal Na$^+$ concentration gradient for function. In absence of the blood-supplied nutrients (oxygen and glucose), the lack of cellular energy (ATP) causes the Na$^+$ gradient, maintained by the Na$^+$/K$^+$-ATPase, to collapse. Thus, glutamate uptake from the extracellular medium is impaired or even reversed in an ischemic episode (Camacho and Massieu, 2006). This rise in extracellular glutamate levels then leads to overactivation of NMDARs.

Whilst neurons in the centre of the ischemic area, and subject to an intense insult, die rapidly in a necrotic manner, the surrounding cells in the penumbra undergo a delayed excitotoxic demise that could potentially be rescued. The use of NMDAR antagonists have had considerable success in protecting neurons against focal ischemia in animal models, however clinical trials for stroke and traumatic brain injury with NMDAR antagonists failed to show any benefits (Ikonomidou and Turski, 2002; Muir, 2006). Moreover, administration of NMDAR antagonists is poorly tolerated and exerts many adverse side-effects such as nausea, agitation, hallucination, memory impairment, hypertension and neuronal cell death (Ikonomidou and Turski, 2002; Lynch and Guttmann, 2002; Muir, 2006). These disappointing outcomes are likely the consequence of chronic and/or complete blockade of the NMDAR itself: as described in this introduction, this class of glutamate receptors is also crucial for synaptic transmission and plasticity in addition to mediate pro-survival signals. Thus, inhibiting their activity
is ultimately detrimental and therapeutically unviable (Ikonomidou and Turski, 2002; Hardingham and Bading, 2003). Efforts to find an efficient anti-excitotoxic strategy are increasingly focussing on the characteristics of the NMDARs (subunit composition, subcellular localization) and associated protein complexes. Indeed, refining our understanding of how this single class of calcium-permeable glutamate receptors can signal to such a wide array of cellular events could eventually lead to the identification of therapeutic targets selectively disrupting the pro-death signals emanating from NMDARs.

3.6 Overview, research aims and chapters content
As discussed in this introduction, the NMDA subtype of glutamate receptor has a pivotal role in the excitatory glutamatergic neuronal network. Postsynaptic NMDAR activation, triggered by the binding of glutamate and the co-agonist glycine leads to a depolarization-driven unblock of their channel by Mg$^{2+}$ and permits an influx of Ca$^{2+}$ into the neurons. This in turn can induce cascades that modify synaptic excitability, as well as survival cascades and gene transcription. However, complete absence of NMDAR activity can lead to neuronal death and exacerbates death following an insult. Even more so, overactivation of NMDARs is neurotoxic; a phenomenon termed excitotoxicity and thought to contribute to several neuropathologies. The series of experiments presented in the following chapters have a general goal in attempting to define the role of the NR2 subunit composition and cytoplasmic tail in shaping the type of signalling induced by NMDARs, with an emphasis on the pro-death signals. The aims of the research presented here are summarized below.

3.6.1 Chapter 3
It has been suggested that NR2A signal to LTP and pro-survival pathways whereas NR2B specifically induces LTD and pro-death mechanisms. However, the ability of the NR2 subunit composition to determine the type of signalling downstream of NMDAR activation is still unclear due to contradicting reports.
Is it possible for a single subtype of receptors, NR2B-containing NMDARs, and within a single cell type to mediate the different types of NMDAR-dependent signals?

To answer this question, the role of the NR2B subunit in mediating signalling to survival, death and plasticity was investigated in developing hippocampal neurons. At the early stage where neurons were studied, the NMDAR subunit composition was determined to contain predominantly NR2B both at synaptic and extrasynaptic sites. Using various stimulation protocols, it was then found that pro-survival signalling, pro-death signalling as well as synaptic potentiation and depression could be induced in these neurons; the induction was abolished in each case by antagonizing the NR2B-containing NMDARs. From this study it can be concluded that in this particular cellular model, the subunit composition cannot account for the dichotomous nature of the NMDAR signals.

### 3.6.2 Chapter 4

In neurons, many pro-death signalling pathways have been identified downstream of NMDAR activation, which include the JNK and p38 SAPks. However, it is not known what the activating trigger for these pathways is and if their recruitment necessitates a NMDAR-bound signalling complex. Expression of NMDAR in non-neuronal cells is also toxic, but it is unclear if this reconstituted excitotoxicity triggers the same pro-death signals as in neurons. Studying the pro-death signals evoked by NMDARs in a non-neuronal context might be informative of their induction requirements in neurons.

-Does the NMDAR-mediated induction of the pro-death p38 and JNK pathways require a neuronal context?

Thus, in Chapter 4, the NMDAR-mediated pro-death signals were studied in a heterologous context. The reconstitution of NMDAR-mediated cell death in a non-neuronal cell line was achieved by inducing expression of NR1/NR2B subunits, which induced non-neuronal death following NMDAR agonist applications. However, unlike in neurons where excitotoxicity depends on the JNK and p38 SAPK cascades, the
toxicity was solely mediated by JNK signalling in the heterologous system. Thus, the lack of p38 SAPK activation suggests that this pro-death signal requires a neuronal induction context, whereas it is not the case for the JNK toxic signal.

3.6.3 Chapter 5
The disruption of the interaction between PSD-95 and the NR2 subunits of NMDAR protects neurons against excitotoxicity by uncoupling nNOS activation and the subsequent p38 SAPK cascade. The disruption can be achieved by applying a cell-permeable peptide mimicking the last 9 amino acids of the NR2B sequence, TAT-NR2B9c. However, PSD-95 may cluster many other proteins to the NMDAR, and disrupting its PDZ interaction with NR2 may affect other types of physiological signals. Concurrently, the ability of the PDZ ligand to activate the p38 pro-death pathway may be neuronal-specific since excitotoxicity in non-neuronal cells was independent of p38. These observations raise two questions about the signalling ability of the NR2 PDZ domains:
- Can the NMDAR pro-death signalling be disrupted without altering the properties of synaptic transmission, NMDAR-dependent plasticity or pro-survival signals?
- Does the NR2 PDZ domain-dependent pro-death signalling require a neuronal context?

The answers to these questions are described in Chapter 5, in which the pro-death signalling properties of the PDZ ligand were investigated. Disruption of the PSD-95 interaction with the NR2B subunit, whilst being neuroprotective, did not affect the basic properties of synaptic transmission and network excitability of neurons, even after surviving an excitotoxic insult. Moreover, synaptic potentiation induction was not altered by this disruption, suggesting that only the pro-death pathway was targeted by this approach. Indeed in a cell line, where NMDAR-induced toxicity does not depend on the p38 SAPK cascade, the neuroprotection afforded by displacing PSD-95 in neurons was non-existent; truncation of the NR2B PDZ domain did not change the profile of toxicity in non-neuronal cells, consistent with the absence of the PSD-95/nNOS in this model. Estimation of the potency of Ca\(^{2+}\) to trigger death in both cell types showed that,
likely due to the presence of the p38-inducing signalling complex, neuronal toxicity required around four times less calcium influx than in the cell line expressing NMDARs. Thus, the targeting of the PDZ domain of the NR2B subunit is an attractive strategy since it appears to spare the normal NMDAR-mediated functions whilst being efficient at disrupting an important pro-death cascade (p38).

3.6.4 Chapter 6

The ability of a single subtype of NR2 subunit to mediate pro-survival and pro-death signals was shown in Chapter 3. However, the divergent sequences of the NR2A/B C-terminus suggest that it may be possible for each subunit to show differential signalling properties. Indeed, PSD-95 associates preferentially to NR2B, which can imply that different signalling complexes associate with NR2A- and NR2B-containing NMDARs.

- Are NR2A- and NR2B-containing NMDARs equally efficient at triggering excitotoxicity in neurons?
- If not, do the pro-death inducing properties of each subunit depend on their intracellular C-terminal domain?

Thus, the relative efficacy of the NR2A and NR2B subunit and their C-terminal domain to trigger excitotoxicity was studied in Chapter 6. By overexpressing NR2 subunits, differential amounts of neuronal death were observed between NR2A and NR2B. To determine if this was due solely to the difference in channel properties and agonist affinities, the C-terminus of the NR2 subunit were interchanged. This modification did not change the current properties of the subunit, but had an influence on the amount of NMDA-induced toxicity in the cultures. This indicates that characteristics of the C-terminal sequence of the NR2 subunits exert an influence on the pro-death signals downstream of each subtype, with the NR2A C-terminus being less “toxic” than NR2B’s. Although the specific domains and interactions behind this difference remain unclear, this has repercussions for a putative neuroprotective approach -as targeting NR2B-containing NMDARs might be preferable.
Altogether, the results presented in this thesis expand our understanding of the NMDAR-mediated events in neurons, and of the role of the NMDAR subunit composition and their cytoplasmic domains in the activation of the variety of signalling pathways downstream of NMDARs.
Chapter 2
Material and methods
1. Neuronal culture

Neuronal cultures used in all experiments presented here were obtained by the following protocol. Coverslips or wells of 24-well plates were coated with poly-D-lysine (1.33% w/v in H₂O; from BD Bioscience, Oxford, UK) and laminin (0.5% w/v) at 37°C for 2 hours prior to plating of the cells. During this incubation period, Sprague-Dawley rat embryos, age E21, were anaesthetized with an intraperitoneal injection of pentobarbital (Ceva Sante Animale, La Ballastiere, France) then decapitated. Brains were removed and the cortex and hippocampus quickly dissected and put in dissociation medium (81.8 mM Na₂SO₄, 30 mM K₂SO₄, 5.84 mM MgCl₂, 0.252 mM CaCl₂, 1 mM HEPES, 0.001% Phenol Red, 20 mM D-glucose) supplemented with kyurenic acid (1 mM) and MgCl₂ (10 mM) -to prevent Ca²⁺ overload via NMDAR activation causing damage to neurons during the dissection and enzymatic digestion period. Once the tissue was isolated, excess medium was removed and replaced with 2 ml of pre-heated dissociated medium supplemented with kyurenate/MgCl₂ containing papain (10 enzymatic units/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA).

Cortical hemispheres and hippocampi were digested with papain for 20 minutes at 37°C, after which the enzymatic preparation was removed and replaced for a second 20 minute incubation period. Papain was then removed and the tissue washed twice with warm dissociation medium again containing kynurenate/MgCl₂, and particular care was taken to remove any membranous or blood-contaminated tissue from the distinct white neocortex matter. This step was repeated again with growth medium which consists of NeuroBasal-A Medium (Invitrogen, Paisley, UK) supplemented with 1% rat serum (Harlan UK Ltd, Oxon, UK), B-27 Supplement (1:50 of 50X stock; Invitrogen, Paisley, UK), 1 mM glutamine and 1:200 penicillin/streptomycin 100X antibiotic mixture. After these washing steps, the cortices and hippocampi were triturated with rapid suction/expulsion from a thin 1 ml plastic pipette in 10 ml of pre-heated growth medium, resulting in an opaque suspension of single cells. These isolated cells were diluted with Opti-MEM (Invitrogen, Paisley, UK) supplemented with 20 mM D-glucose to obtain a final volume of 14 ml/cortical hemisphere and 2.85 ml/hippocampus. Following this dilution, 0.5 ml of these suspensions was plated in 24-wells cell culture plates (Greiner
Bio-One, Stonehouse, UK). For electrophysiology experiments, 13 mm glass coverslips (VWR, Lutterworth, UK) were placed in each well so that cultured neurons could be transferred from 24-well plates to the electrophysiological a recording chamber. Cell density at the time of plating was around 800 cells/mm² for cortical and 300 cells/mm² for hippocampal cultures.

Culture plates were subsequently put at 37°C in a humidified 5% CO₂ atmosphere for two hours, after which the medium was replaced with 1 ml growth medium. Neurons were incubated at 37°C until the day of the experiment (DIV 8-16; Days In Vitro); only at DIV 4, 1 ml of growth medium containing 9.6 μM cytosine β-D-arabinofuranoside hydrochloride (AraC) was added to each well to prevent proliferation of glial cells. Prior to experimentation (2-12 hours), the growth medium was changed to TMo, a medium containing 10% MEM (Invitrogen, Paisley, UK), 90% salt-glucose-glycine (SGG) medium (114 mM, 0.219% NaHCO₃, 5.292 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 1 mM glycine, 30 mM glucose, 0.5 mM Na-pyruvate and 0.1% phenol red; osmolarity 325 mOsm/L). A variant of this trophic deprivation medium was used in some cases to increase the basal survival rate of neurons over long periods of time; in such case, TMits was used instead (TMo supplemented with 7.5 μg/L insulin, 7.5 μg/L transferrin and 43 nM sodium selenite). All reagents, unless otherwise specified were purchased from Sigma-Aldrich (Dorset, UK).

2. AtT20 cells culture
Mouse pituitary cell line AtT20 (ECACC number 87021902) were cultured in a 75 cm² flask filled with 15 ml of medium composed of 90% D-MEM, 10% fetal bovine serum (both from Invitrogen) and 1:100 penicillin/streptomycin. When cells reached around 90% confluency, medium was aspirated and the flask was washed once with 1 ml Trypsin-0.05% EDTA (Invitrogen) and another 1 ml added for approximately 5 minutes—until gentle tapping of the flask dissociates the cells from the surface. Once cells were detached, 5 ml of pre-warmed medium was put back in the flask and cells were triturated by aspirating/ejecting the medium with a 10 ml pipette. More medium was then added, generally 3 times more than the initial volume (45 ml), and 15 ml were used to re-seed
the cell line in a new 75 cm² flask whereas the rest was plated onto coverslips or 24-well plates for subsequent experiments (16-36 hours after plating). Culture medium was changed to serum- and antibiotic-free medium OPTI-MEM at least 2 hours before transfection. A very small subset of experiments were done using human embryonic kidney 293 cells (HEK293; ECACC number 85120602), for which the methodology for culture, transfection and electrophysiology were kept the same as for AtT20 cells.

3. Transfection procedure

For transfecting neurons (on DIV 8) HEK293 and AtT20 cells, a total of 0.5-0.65 µg of plasmid DNA (pDNA) was diluted with 33 µl of medium from the well to be transfected. Concurrently, 2.33 µl of Lipofectamine or Lipofectamine 2000 (Invitrogen) was prepared and left to stand for 5 minutes. The two preparations were mixed and left at room temperature for 20 minutes. After this period, a further 285 µl of medium from the wells was added to the mix with gentle agitation. Medium was aspirated from the well and 333 µl of the transfection mixture was put in. Cells were then placed at 37 °C for 2-4 hours (Lipofectamine 2000) or 4-6 hours (Lipofectamine). Note that the quantities cited here apply for 1 well, therefore numbers were scaled up for the total amount needed. At the end of the transfection, wells were transferred back to TMo or TMits in the case of neurons and Opti-MEM for AtT20 cells; the latter being supplemented with 50 µM D-APV (Tocris Bioscience, Bristol, UK) to prevent spontaneous NMDAR activation and cell death. Transfection using this method typically yielded 5-10% efficiency. The various transfections always included a reporter plasmid (0.1-0.3 µg of total); either pGL3-Luciferase (Promega) or pCMV-eGFP (enhanced, Green Fluorescent Protein; gift from Dr Martin Privalsky, University of California Davis, US) was co-transfected with plasmids encoding for NMDAR subunits, which were replaced by equal amount of pCMV-globin (a small globular protein) for control cells.
4. Stimulation protocols

4.1 Neuronal cell death

To induce neuronal excitotoxicity, neurons DIV 8-11 were exposed to NMDA or glutamate (0 -100 µM, as indicated for each individual experiment) in TMo or TMits for 1 hour, after which neurons were washed once and returned to fresh medium. Neurons that die in response to excitotoxic levels of agonist exhibit swollen cell bodies and pyknotic nuclei with small irregular chromatin clumps. Cell death was determined 16-24 hours after the end of the stimulation by fixing cultures with paraformaldehyde (3%w/v in phosphate buffer saline; PBS) for 30 minutes, washed once with PBS then permeabilized for 5 minutes with NP40 detergent (0.5% v/v in PBS). Wells were washed twice with PBS, after which a drop of Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Labs, Peterborough, UK) was added. DAPI emits blue fluorescence when bound to DNA, and thus effectively stains the nuclei. A thin glass coverslip was then deposited on the mounted cells to prevent evaporation - allowing fluorescence to be visualized for several weeks. Thus, the percentage of cell death was measured by counting the pyknotic nuclei as a percentage of total nuclei within a field; 2 pictures were usually taken from each well and 2 duplicate wells per experiment.

Staurosporine, a broad protein kinase inhibitor, was also used to induce neuronal apoptosis. Staurosporine (50 or 100 nM) was added to the culture medium for 24 hours, after which cells were fixed and DAPI-stained as above.

In experiments where the fate of individual GFP-expressing neurons was followed, transfected cells were visualized, their position marked and images taken using Leica AF6000 Microscope Suite and Software (Leica Mircosystem Ltd, Bucks, UK). 12-15 positions were marked per well, with an average of 20 neurons in total. Plates were allowed to re-equilibrate for a least 1 hour at 37°C and 5% CO₂, then toxicity was evoked by NMDA (20 µM) for 1 hour. Instead of changing the medium, stimulations were ended by adding 20 µM MK-801 to block further NMDAR activity and to minimize the disturbance of previously marked GFP-positive cells. 16-24 hours
later, a second set of pictures was taken at the identified positions to determine the fate of the transfected neurons. Additionally, the cells were subsequently fixed and DAPI-stained as for regular excitotoxicity experiments, and a last set of fluorescence images were taken (both for GFP and DAPI fluorescence) to allow for a supplementary method of assessing neuronal survival in the case of an ambiguous GFP signal.

4.2 Enhancement of synaptic NMDAR activity in neuronal cultures
To enhance the neuronal network activity in our cultures, 50 µM bicuculline was applied to cultures 16-24 hours before additional stimulation. Bicuculline is a gamma-amino butyric acid A (GABA_A) receptor inhibitor; GABAergic cells comprise approximately 10% of all neurons in our cultures (Papadia et al., 2008) and provide a tonic inhibition of the excitatory neuronal network. By relieving this tonic inhibition, bicuculline induces large bursts of action potential firing in principal neurons and augments synaptic NMDAR-mediated calcium transients (Hardingham et al., 2002; Arnold et al., 2005).

5. Luciferase assay
As an indicator of cell viability, a plasmid encoding for the firefly luciferase enzyme was transfected as described. For AtT20 cell transfection, the DNA ratio was 2:2:1 for NR1:NR2:Luciferase respectively, whereas for neuronal cultures the NMDAR subunit plasmids were replaced by a plasmid encoding for globin to keep the DNA ratios equal. 16-24 hours after transfection, drugs were added to the cultures for 1-2 hours then cells were stimulated with glutamate (0-100 µM for neurons and 0-1 mM for AtT20 cells) for 5-7 hours. After this incubation period, medium was aspirated and 90 µl of Steady-Glo Luciferase Assay Kit (Promega, Southampton, UK) was added with an equal amount of fresh medium. Cells were allowed to lyse at room temperature which was facilitated by gentle rocking for 10 minutes, during which the cytosolic luciferase enzyme contained in the surviving transfected cells was released in the mixture. 160 µl of the mixture was then transferred to opaque white 96-well plates (Greiner Bio-One). The firefly luciferase enzyme converts luciferin (contained in the Steady-Glo mixture) to oxyluciferin, which is a luminescent reaction. Thus, luciferase activity was measured by assaying the
chemiluminescence using a FLUOstar Optima plate reader (BMG Labtech, Aylesbury, UK). The relative light units were converted to percentage of cell death compared with the no agonist-control (100%) and the minimum luminescence value (the maximal agonist concentration) was normalized to 0% to account for transfection efficiency variations.

Figure 2.1: Luciferase chemiluminescence reaction. The luciferase enzyme expressed in transfected cells transforms luciferin, contained in the Steady-Glo mixture, in oxyluciferin; the reaction produces luminescence proportional to the amount of luciferase enzyme and to the number of transfected cells surviving at the beginning of the experiment. Schematic adapted from the Steady-Glo Luciferase assay kit guidebook (from Promega).
6. Immunohistochemistry
To identify the number of transfected AtT20 cells expressing the NMDAR subunits, immunostaining was performed 24 hours post-transfection. Cells in 24-well plates were fixed for 20 minutes with PAF, washed once with PBS and permeabilized with NP40 for 5 minutes. Subsequently, wells were rinsed twice with PBS then 0.5 ml of PBS containing 1:500 of rabbit polyclonal NR1 antibody (Cell Signaling Technology, Danvers, USA) and 0.02% sodium azide. The plate was left incubating with the primary antibody, gently rotating at 4 °C overnight. The following day, the NR1 antibody was recovered and cells were washed twice with PBS. 0.5 ml of PBS containing 1:200 biotinylated anti-rabbit secondary antibody (Vector Labs). After 1 hour incubation, the antibody mixture was aspirated and the well was washed twice with PBS. A final immunoreaction was done with a Cy3-streptavidin-conjugated antibody (1 hour; Stratech Scientific Inc., Suffolk, UK). Finally, the AtT20 cells were washed twice and mounted with Vectashield + DAPI covered with a glass coverslip.

7. 45Calcium assay (cell line and neurons)
To measure the calcium influx through NMDARs in neurons and transfected AtT20 cells, cultures were simultaneously subjected to a 45Ca²⁺ assay 16-24 hours post-transfection of AtT20 cells. To block calcium entry through L-type VGCC, present in both neurons and the AtT20 cell line, nifedipine (5 µM; Tocris Bioscience) was added to the medium prior to the experiment. Each well was then stimulated for 10 minutes at 37 °C with glutamate (0-100 µM for neurons and 0-1 mM for AtT20 cells) in medium enriched with 30-50 MBq/L of 45Ca²⁺ (PerkinElmer, Bucks, UK). After the 10 minutes stimulation, cells were washed once with fresh medium (TMo and OptiMEM + APV for neurons and AtT20 cells) supplemented with kynurenate (10 mM) and magnesium (10 mM); the medium was then removed and cells were lysed with 1% Triton X-100 (in PBS, for 10 minutes) to allow the 45calcium intake to escape the cytosol of the cells. The lysate was put in scintillation vials in which 1 ml of scintillation fluid was added (VWR, Lutterworth, UK). Vials were vortexed then left to settle 5 minutes. The radioactivity in count per minute (CPM) was quantified using a Beckman LS6500 scintillation counter.
Data from each experiment were normalized for the amount of $^{45}\text{Ca}^{2+}$ activity used, and the basal radioactivity count from the nonstimulated wells was subtracted from each subsequent condition—as non-NMDAR-activity-mediated $\text{Ca}^{2+}$ entry.

8. Western blotting and antibodies

AtT20 cells were cultured to a confluence of around 90% in 24-well plates and, where noted, stimulated for two hours prior to sample preparation. Wells were then homogenised in 15 µl of sample buffer consisting of 1.5 M Tris (pH 6.8), 15% glycerol, 3% SDS, 7.5% β-mercaptoethanol and 0.0375% bromophenol blue. The contents of 2 wells were scraped and pooled, put in a 1.5 ml tube and boiled at 100 °C for 10 minutes. Gel electrophoresis and Western blotting were performed using the Xcell Surelock system (Invitrogen) with precast gradient gels (4-20%; Invitrogen). 20 µl of each sample was loaded per well and electrophoresis was done in running buffer (192 mM glycine, 24.8 mM Tris-base, 3.5 mM sodium dodecyl sulfate) at 125 V for 2-3 hours. Once the migration was satisfactory, proteins were transferred onto a PVDF membrane (Millipore, Watford, UK) in buffer containing 96 mM glycine, 12 mM Tris-base, 20% methanol at 25 V for 2 hours. Once the transferred was completed, the membrane was blocked for 1 hour in TBS solution (20 mM Tris-base, 137 mM NaCl and 0.1% Tween-20) supplemented with 5% dried semi-skimmed milk. The membrane was then incubated overnight at 4 °C in TBS + milk containing the appropriate dilution of primary antibody. On the following day, 3 washes (5 minutes) were done in TBS to remove unbound antibodies and 1 hour incubation was made in TBS + milk enriched with horse-radish peroxidase-conjugated secondary antibody. The membrane was washed for an additional 3 times (5 minutes) in TBS, then incubated in LumiGlo reagent and peroxide (for 1 minute; Cell Signaling Technology). Chemiluminescence was then detected by exposing a Kodak X-Omat film to the membrane; once developed the blots were scanned and the band densitometry was analysed using ImageJ software.

The densities were normalized to their loading control, which was obtained by stripping the membrane of antibodies using ReBlot Plus Strong Stripping Solution (15 minutes; Millipore). The membrane was then washed with TBS 3 times (each for 10
minutes) and subsequently incubated in primary antibody overnight, after which the protocol was identical to the first immunoreaction. The following antibodies and dilutions were used: phospho-p38 Thr180/Tyr182 (1:400, mouse; Cell Signaling Technology), p38 (1:1000, mouse; Santa Cruz Biotechnology, Heidelberg, Germany), PSD-95 (1:1000, goat; Abcam, Cambridge, USA), nNOS (1:500, goat; Abcam) and calmodulin (1:100000, rabbit; Cell Signaling Technology). The phospho-p38 bands were normalized to p38, whereas for PSD-95 and nNOS, calmodulin was used as a loading control. The horse-radish peroxidase-conjugated secondary antibodies for goat (1:2000), rabbit (1:2000) and mouse (1:1000) were from Dako UK Ltd (Ely, UK).

To compare similar amounts of cell homogenate between neuronal and AtT20 cultures, the samples were prepared as followed: cells were solubilized in 0.1% Triton X-100, scraped and put into tubes. The protein concentration was obtained by using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Cramlington, UK) according to manufacturer’s instructions and by reading absorbance of each sample at 562 nm. The volume of each sample to load the same amount of protein was then defined (10-20 µl per well). Ultimately, the loading quantities were also verified by calmodulin immunoreactivity as a loading control, as described earlier.

9. Electrophysiology

All electrophysiology experiments were performed on cells cultured on glass coverslips. Coverslips were transferred to a recording chamber perfused with an external recording solution composed of 150 mM NaCl, 2.8 mM KCl, 10 mM Hepes, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 0.1 mM glycine and 2 µM strychnine, pH 7.3 (320-330 mOsm). Patch pipettes were made from thick-walled borosilicate glass (Harvard Apparatus, UK) and filled with internal solution (155 mM K-gluconate, 2 mM MgCl₂, 10 Na-Hepes, 10 mM Na-phosphocreatine, 2 mM Mg₂-ATP and 0.3 mM Na₃-GTP, pH 7.3, 300 mOsm). Electrode tips were fire-polished for a final resistance ranging between 5 and 10 MΩ. For recordings of miniature excitatory postsynaptic currents (mEPSCs), which require a high signal-to-noise ratio, electrodes were coated with Sylgard 184 resin (Dow Corning, Midland, USA) to reduce the pipette capacitance. Currents were recorded at room
temperature of 21 ± 2 °C, using an Axopatch-1C amplifier (Molecular Device, Union City, USA) and stored on digital audiotape. Data were subsequently digitized using WinEDR v6.1 software (John Dempster, University of Strathclyde, UK) and analyzed offline.

9.1 Measurements of NMDAR-mediated currents

Hippocampal neurons or AtT20 cells were voltage-clamped at -60 or -70 mV (as stated in each Results section), and recordings were rejected if the holding current changed by more than ±100 pA during the recording or if the series resistance drifted by more than 20% of its initial value (<35 MΩ). Series resistance compensation was not used. Whole-cell NMDAR-mediated currents were measured in external recording solution in which the MgCl₂ (1 mM) was replaced by NaCl (2 mM), supplemented with 300 nM tetrodotoxin (TTX; voltage-gated sodium channel blocker; Tocris) and 50 µM picrotoxin (PTX; GABA<sub>A</sub> receptor inhibitor; Tocris), flowing in the recording chamber at a rate of 3-5 ml/min. NMDAR-mediated currents were elicited by switching from the external recording solution line to one containing NMDA for 5-10s, until the current reached a steady-state plateau, then back to agonist-free solution. Currents reported were measured as the average value of the steady-state, plateaued currents over 1-2 seconds. Because cells were difficult to maintain after too several high concentration-agonist applications, each agonist application was followed by at least 1 minute washout and limited to 2 or 3 repeats. The NMDAR antagonists NVP-AAM077 and ifenprodil were bath-applied for 3 minutes before currents measurements. All currents were normalized to their respective initial whole-cell agonist-elicited current and are shown as percentage of basal. Alternatively, for comparison of NMDAR-mediated currents in NR2-transfected neurons the values (in pA) were divided by the whole-cell capacitance (in pF) to obtain a current density (in pA/pF). For measuring the sensitivity of NMDAR currents to NVP-AAM077, increasing concentrations of the antagonist were applied to glutamate-evoked currents (3 µM) which allowed the determination of an IC<sub>50</sub> value for NVP-AAM077 (Frizelle et al., 2006). For this set of experiment, the external recording solution was
supplemented with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 15 µM; Tocris) to block glutamate-evoked activation of AMPA and kainate receptors.

9.2 Measurement of spontaneous EPSC frequency
Recordings of spontaneous EPSCs (sEPSCs) from neuronal cultures were obtained in external recording solution, drug-free but with increased CaCl$_2$ (3 mM) to ensure a reliably high level of spontaneous events in the cultures. These spontaneous events, recorded in voltage-clamp, consist in large inward currents of various sizes originating from incoming action potentials from other neurons. Once the neuron was patch-clamped at -70 mV, sEPSCs were recorded for 3 minutes. Only sEPSCs > 200 pA and spaced by > 1 s were selected for frequency analysis.

9.3 Analysis of extrasynaptic NMDAR currents
To block synaptically-located NMDARs, we used a quantal activation-mediated blockade by MK-801 (Tovar and Westbrook, 2002; Nakayama et al., 2005). In Mg$^{2+}$-free external recording solution containing TTX, release of glutamate into the synaptic cleft can occur only via spontaneous release of synaptic vesicles of glutamate – independent of action potentials. In the presence of MK-801 (10 µM; Tocris), an irreversible (in this experimental time-frame) open-channel blocker, only the NMDARs experiencing this localized release –therefore defined as “synaptic”- were blocked by MK-801. Following incubation with MK-801 to allow sufficient blockade of synaptic NMDARs (defined experimentally in the Results section) and washout of the drug for 3 minutes, subsequent agonist-evoked NMDAR currents were then solely mediated by extrasynaptic receptors. When used, ifenprodil was never applied before the quantal block protocol due to the difficulty of washing the compound out and the possibility that it might lead to an underestimation of the NR2B-containing synaptic NMDAR population. Thus, ifenprodil sensitivity of the whole-cell and extrasynaptic current fractions were compared in an unpaired manner. Extrasynaptic NMDAR currents were normalized to the pre-MK-801 values and are shown as percentage of basal.
9.4 Models of NMDAR-dependent synaptic potentiation
To potentiate mEPSCs frequency, 50 µM bicuculline was added to culture medium for 15 minutes, after which coverslips were transferred to pre-equilibrated drug-free medium for approximately 1 hour before electrophysiological recordings. By adding GABAA receptor antagonist bicuculline, tonic inhibition of the neuronal network is relieved, inducing synchronous bursting of neurons which triggers a long-lasting increase in mEPSC frequency; due to conversion of silent synapses into functional, AMPAR-containing ones (Abegg et al., 2004; Arnold et al., 2005). Following this induction protocol, hippocampal neurons were transferred to the recording chamber containing external recording solution supplemented with TTX and PTX. mEPSCs were recorded for 5-10 minutes (minimum of 200 events) from neurons clamped at -70 mV. Data were analyzed offline with MiniAnalysis software (Synaptosoft, Fort Lee, USA) and as described in Baxter and Wyllie (2006). mEPSCs were manually selected with a minimum amplitude of 6 pA (approximately 2 times the baseline noise level). Cells where the mEPSC frequency was less than 0.3 Hz, or recordings where the noise level was in excess of 3.5 rms were rejected. For experiments where the effects of antagonists or inhibitors on the bicuculline-induced potentiation were studied, drugs were pre-applied to wells at least 20 minutes before bicuculline stimulation.

9.5 Model of NMDAR-dependent synaptic depression
To induce synaptic depression, 30 µM NMDA was applied to hippocampal cultures for 4 minutes. Following this, coverslips were transferred to agonist-free medium for 30 min to 1 hour. sEPSC were subsequently recorded for at least 3 minutes as described above and their frequency analysed. As for the potentiation protocol, drugs were applied 20-30 minutes before adding NMDA to the wells.

10. Plasmid preparation and chimera construction
Plasmids used for transfection were prepared by transformation of 10 ng of pDNA into 50 µl of JM109 competent cells (Promega), by transferring ice-cold bacteria to a 42 °C waterbath for 60 seconds then allowing growth overnight (37 °C) on petri dishes.
containing Luria-Bertani (LB) agar supplemented with 50 ng/ml of ampicillin (all plasmids possessed an ampicillin-resistance insert). The following day, colonies were picked and seeded into 50 ml of LB broth with ampicillin for 12-15 hours at 37 °C. Plasmids were then purified using the Qiagen Plasmid Midi kit (Qiagen UK, Crawley, UK) according to the manufacturer’s instructions. pCis plasmids encoding for the full-length mouse NR1-1a, NR2A and NR2B proteins were generously provided by Prof Anne Stephenson (School of Pharmacy, University College of London, UK).

10.1 Digestion of pDNA and mutagenesis

Digestion of plasmids with various restriction enzymes was accomplished by mixing on ice: 15-30 ng of pDNA with 5 Units of restriction enzyme, the relevant buffer supplied with the enzyme and water to a final volume of 25 µl. The mixture was then placed at the enzyme’s optimal temperature (commonly 37 °C) for 1 hour. Digestion products were then loaded on a 0.8% agarose gel containing 1 µM 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, along with a Kb DNA ladder (Agilent Technologies UK Ltd, Stockport, UK) and allowed to migrate for 45 minutes with a current of 90 mA. After the electrophoresis, digestion bands were visualized with a U:Genius UV illuminator (Syngene, Cambridge, UK). If fragments were required, bands were cut out from the gel and put at -20 °C for subsequent use.

A unique restriction site was inserted in the sequence of NR2A and NR2B subunits in order to create C-terminal chimeras. Both wild-type pCis-NR2A and pCis-NR2B plasmids were not digested by the NotI restriction endonuclease (Promega), which targets 5’ GC’GGCC GC 3’. Thus, mutagenesis of both plasmids was done using the following oligonucleotides (with the mutated nucleotides in bold) in order to insert a NotI site at nucleotide 2474(NR2A) and 2477(NR2B):

NR2A primer 1 = TTCTACATGCTGGCGGCGCTATGGCCCTCAGCCT
NR2A primer 2 = AGGCTGAGGGCCATAGCGCGCCGCGAGCATGTAAGA
NR2B primer 1 = TTCTATATGTGGCGGCGGCCATGGCTCTCAGCCT
NR2B primer 2 = AGGCTGAGAGCCATGGCGCGGCGCCAACATATAGAA

Plasmids underwent mutagenesis using Stratagene QuickChange XL Site-Directed Mutagenesis kit (Agilent Technologies UK Ltd) using the primers as described above and as detailed in the instructions provided by the manufacturer. Once finished, the reaction products were transformed in JM109 competent cells as described in the previous section. From the resulting colonies 15 individual colonies were picked and seeded in 10 ml of LB broth + ampicillin for growth (12-15 hours, 37 °C). Plasmids from each colony were then purified with QIAprep Spin MiniPrep Kit (Qiagen UK) and a diagnostic digestion with NotI restriction enzyme was made to verify the successful integration of the mutation (single plasmid bands at 9.8-10 kb). Bacteria from NotI-containing pCis-NR2A and NR2B colonies were thereafter used for further production of plasmids using a Qiagen Plasmid Midi kit (Qiagen UK).

10.2 Chimera construction

Plasmids encoding for NR2A with a NR2B-C-terminal domain (NR2A(2B<sup>CTD</sup>)) and of NR2B with a NR2A-C-terminal domain (NR2B(2A<sup>CTD</sup>)) were created as follows. For NR2A(2B<sup>CTD</sup>), fragment NR2A(1-824) was obtained by digestion of pCis-NR2A with EcoRI and NotI (Promega); concurrently, pCis-NR2B was also digested with EcoRI and NotI to obtain the complementary fragment pCis-NR2B(826-1482). The DNA fragments were purified from the agarose gel using QIAquick Gel Extraction kit (Qiagen UK). Relevant fragments were ligated overnight at 4 °C by mixing 6 µl of NR2A(1-824), 2 µl of pCis-NR2B(825-1482), 1 µl of T4 DNA ligase (Agilent Technologies UK Ltd) and 1 µl of T4 DNA ligase Buffer.

To construct NR2B(2A<sup>CTD</sup>): fragment NR2B(1-825) was isolated with ClaI (Promega) and NotI; fragment NR2A(825-1464) by restriction enzymes NotI and EcoRI; and the vector pCis was obtained with ClaI and EcoRI. The purified DNAs were then ligated using 3.5 µg NR2B(1-825), 3.5 µg NR2A(825-1464), 1 µl pCis, 1 µl T4 DNA ligase and 1 µl of T4 DNA ligase Buffer. The resulting ligation reactions were then
transformed in JM109 competent cells, and subsequent steps followed the same procedure as for the mutagenesis reaction. All constructs (pCis-NR2A/-NR2B with the NotI mutation, pCis-NR2A(2B<sup>CTD</sup>) and pCis-NR2B(2A<sup>CTD</sup>)) were then sent to sequencing (SBS sequencing service, University of Edinburgh) to confirm the correct identity of the final plasmid sequences.

11. Data and statistical analysis
All results are presented as mean ± standard error of the mean (SEM). Statistical analysis was done using unpaired two-tailed Student’s t-test, or paired in the cases where control values were obtained in the same sample set. When comparing several means, a one-way ANOVA with a subsequent Fisher’s PLSD post-hoc test were performed. Statistical significance was achieved at p < 0.05.
Chapter 3
NR2B-containing NMDARs can mediate signalling to neuronal survival, death and synaptic plasticity
1. Chapter introduction

NR2A and NR2B NMDAR subunits are the two major glutamate-binding subunits that are expressed in forebrain neurons (Cull-Candy et al., 2001). Therefore, in the mature hippocampus a large majority of neuronal NMDA receptors are diheteromeric (NR1/NR2A; NR1/NR2B) or triheteromeric channels (NR1/NR2A/NR2B). However, in the rodent brain, the NR2A subunit expression only appears in the second week of postnatal development. Prior to that developmental stage, the NR2B-containing NMDARs form the bulk of functional NMDA receptors. Indeed, the NR2A\(^{-/-}\) knockout mouse is viable and only develop phenotypes later in life (Sprengel et al., 1998; Zhao and Constantine-Paton, 2007), whereas the NR2B\(^{-/-}\) knockout proves to be lethal around birth (Sprengel et al., 1998).

Apart from the biophysical properties they confer to the receptor, such as channel deactivation and agonist affinity, the NR2A/B subunits also differ in their intracellular C-terminus sequences. Their unique C-terminus sequence allows them to interact differently with other intracellular proteins, which could affect the type of signalling they mediate or their localization in the neuronal membrane. It has been proposed that the NR2A subunit is selectively trafficked to the synapse, gradually replacing the NR2B-containing receptors, the latter subtype then being relegated to extrasynaptic sites (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Townsend et al., 2003). Building on this theory, some groups have observed that synaptic NR2A-containing NMDA receptors mediate long-term potentiation (LTP), while extrasynaptic NR2B-containing receptors trigger long-term depression (LTD) (Lu et al., 2001; Liu et al., 2004; Massey et al., 2004). However, several other groups have failed to observe similar outcomes, regarding both subunit segregation (Mohrmann et al., 2000; Thomas et al., 2006; Harris and Pettit, 2007) and plasticity (Berberich et al., 2005; Weitlauf et al., 2005; Zhao et al., 2005; Bartlett et al., 2007; Le Roux et al., 2007).

Synaptic and extrasynaptic NMDARs have a dual influence on pro-survival CREB activation and CREB-dependant gene expression (Hardingham and Bading, 2003). Thus, distinct subunit pools between synaptic and extrasynaptic NMDARs may also offer an explanation for the dichotomy in signalling: synaptic NMDARs could
preferentially promote pro-survival signalling due to their enrichment in NR2A subunits, whilst on the other hand extrasynaptic receptor could trigger pro-death signals more efficiently because of their NR2B composition. This hypothesis has been supported by some evidences (Liu et al., 2007), but not by others (von Engelhardt et al., 2007).

The discrepancies between studies regarding subunits partitioning at the synapses or subunits involvement in plasticity and viability signalling can be explained by the variety of cell culture paradigms and slice preparations, as well as the developmental stages at which said neurons are studied in a particular set of experiments. Also, the lack of consensus over the pharmacology of NVP-AAM077, a NR2A subunit-prefering antagonist, can explain some contradictory results. Recent studies measured rather modest NR2A specificity values over NR2B (Berberich et al., 2005; Frizelle et al., 2006), suggesting that some studies using NVP-AAM077 might have had significant inhibition of NR2B-containing NMDARs in addition to the NR2A-NMDARs blockade.

Therefore, the role of NMDAR subunit composition in determining the type of signalling downstream of NMDAR activity is still unclear. Thus, we sought to verify if a single subtype of NR2-NMDAR could mediate the different types of NMDAR-dependent signals.

We took advantage of the developmental regulation of NR2A to NR2B subunit and investigated the signalling capacities of NR2B-containing NMDARs. Prior to their second week in vitro, dissociated hippocampal neurons only express very little NR2A (Monyer et al., 1994). Then, considering that the majority of the synaptic and extrasynaptic NMDARs are NR2B-containing, we assessed the ability of receptor composed of the NR2B subtype to signal to death, survival, synaptic potentiation and synaptic depression in developing hippocampal neurons. The following experimental findings are presented in this Chapter:

- The NMDARs in hippocampal neurons DIV 7-11 are overwhelmingly composed of NR2B subunit;
- The synaptic and extrasynaptic subunit composition is equally dominated by NR2B at DIV 7-11;
- Later in development (DIV 12-18), the amount of ifenprodil-insensitive NMDARs increases, indicating a rise in NR2A subunit expression;
  - At DIV 12-18, the proportion of NR2A-containing NMDARs increases preferentially but not exclusively at the synapse;
  - In DIV 8-11 neurons, NR2B-containing NMDARs mediate excitotoxicity;
  - At the same developmental time, synaptic NMDAR-activity induced neuroprotection requires NR2B-containing NMDAR stimulation;
  - In DIV 8-11 neurons, NR2B-containing NMDARs mediate signals to synaptic potentiation and depression.

In conclusion we found that at this developmental time, NMDARs composed of NR2B subunits are able to initiate signalling to pro-death, pro-survival and bidirectional synaptic plasticity in hippocampal neurons. Therefore, the NR2 subunit composition cannot account for the dichotomous nature of NMDAR signalling in these neurons. The majority of the work presented in this Chapter has been published in Martel et al. (2009a) and Papadia et al. (2008).
2. Results

2.1 Ifenprodil sensitivity of whole-cell NMDAR currents

Hippocampal neurons used in this study are cultured from embryonic day 21 Sprague-Dawley rats, or 1 to 2 days pre-natal. The increase in expression of the NR2A subunit begins in the second week postnatal, which means that the cultured neurons used in this study should show a similar switch during the second week in vitro. To assess the subunit composition of NMDARs of these dissociated hippocampal neurons, we measured the sensitivity of NMDA-evoked currents to ifenprodil, a non-competitive, voltage-independent and activity-dependent NR2B-specific antagonist (Williams, 1993; Chenard and Menniti, 1999). According to data from recombinant NMDAR expressed in *Xenopus* oocytes, ifenprodil at a concentration of 3 µM inhibits around 80% of NR1/NR2B receptors current without affecting NR1/NR2A receptors current (Williams, 1993).

Thus, neurons were patched-clamped and maintained at -70 mV in presence of TTX and PTX. Cells were subjected to pulses of 150 µM NMDA, long enough to observe the steady-state current component –as opposed to the peak current, before receptor desensitization. At that concentration, NMDA elicits a maximal response; a small number of recordings were made with 100 µM NMDA. Due to a rundown of NMDAR currents at each application of agonist (Li et al., 2002), steady-state currents were always measured from the second NMDA application.

Currents measured at a saturating NMDA concentration give an indication of NMDAR expression. During their development from DIV 3 to DIV 18, cultured cells exhibited larger NMDAR currents (Figure 3.1; n = 66 neurons), indicating greater NMDAR expression with development. Although currents were highly variable between cells, currents tended to increase with development and ranged from as low as 123 pA at DIV 3 to 3479 pA at DIV 18.
Figure 3.1: Hippocampal neurons exhibit increasingly larger NMDAR-mediated currents with time *in vitro*. A. Currents evoked by a saturating NMDA application were increasing gradually from DIV 3 to DIV 18, representative of the gradual rise in NMDAR expression in hippocampal neurons. Currents are averages of the steady-state values during 5-10 seconds application of 150 µM NMDA. n = 66 cells. B. Example traces of NMDA-evoked currents. Currents exhibited a small rundown between each subsequent response (1, 2, 3x). All currents were measured from the second NMDA application and were obtained from the average value of 1-2 seconds of the steady-state plateau, as shown in the middle example trace (dotted lines).
Ifenprodil was then used to assess the sensitivity of NMDAR currents from these cells. After the initial measurement of the whole-cell NMDA-induced current, ifenprodil (3 µM) was applied, and then currents were re-measured. The steady-state current value of this current was then divided by the current recorded in the absence of ifenprodil to assess the ifenprodil insensitivity. As it can be seen in Figure 3.2, the ifenprodil-insensitive proportion stayed near its maximum from cells under DIV 11. Note that cells under one week in vitro will not be further considered due to the fact that they may not exhibit proper network activity at the stage; therefore only cells aged DIV 7-11 were included in the following experiments. From DIV 12, the insensitivity to the NR2B-specific blocker sharply started to increase, indicating that NR2A-containing NMDAR begin to integrate with the receptor pool at that stage.

Altogether, neurons at the developmental stage of DIV 7-11 showed an ifenprodil sensitivity of 74 ± 2% (n = 21 cells) whereas at DIV 12-18 the average dropped to 52 ± 4% (n = 19 cells; Figure 3.3A-B). In NR1/NR2B heteromeric receptors expressed in *Xenopus* oocytes the sensitivity is around 80% at an ifenprodil concentration of 3 µM (Williams, 1993). This strongly suggests that, at a sensitivity of 74 ± 2% to ifenprodil, NMDAR currents from neurons at DIV 7-11 are mostly mediated by NR2B-containing receptors. Moreover, the decrease in current sensitivity to the drug from DIV 12 is in agreement with the developmental switch in NMDAR subunits in the second postnatal week (Williams et al., 1993; Sheng et al., 1994).
Figure 3.2: The decrease of ifenprodil sensitivity of NMDAR currents starts from DIV 12. Currents evoked by 150 μM NMDA were highly sensitive to ifenprodil (3 μM) between DIV 7-11. Conversely, the ifenprodil-insensitive component rose in older hippocampal neurons (DIV 12-18), indicating an increased contribution from NR2A-containing NMDARs at that stage. Currents are expressed as percentage of current remaining with ifenprodil compared to the initial NMDAR current measurement. n = 49 cells.
Figure 3.3: DIV 12-18 hippocampal neurons contain a lower proportion of NR2B-containing NMDARs than DIV 7-11 neurons. A. Comparison of ifenprodil sensitivity of whole-cell NMDAR currents evoked by 150 µM NMDA in DIV 7-11 neurons (n = 21) with DIV 12-18 neurons (n = 19). B. Example traces used to generate the data in A. C. Example NMDAR-mediated currents recorded from a DIV 9 neuron in the absence and presence of NVP-AAM077 (0, 0.1 and 3 µM). D. Mean inhibition curve used to determine the IC50 of NVP-AAM077 acting at NMDAR currents of DIV 7-11 neurons (evoked by 3 µM glutamate). The value obtained (203 ± 3 nM) is consistent with a near-pure population of NR2B-containing NMDARs. For each data point, measurements were made from 3-7 cells (DIV 7-11). * p < 0.001.
To confirm our assessment of the NR2B-NMDAR proportion in hippocampal neurons at DIV 7-11, we measured the effect of NVP-AAM077 on glutamate-induced NMDAR currents, in presence of AMPA receptor antagonist CNQX (15 µM). NVP-AAM077 is a competitive NMDAR antagonist that targets preferentially NR2A-containing NMDARs (Berberich et al., 2005; Frizelle et al., 2006). To evaluate the IC$_{50}$ of NVP-AAM077 on currents (Wyllie and Chen, 2007), glutamate was used at its EC$_{50}$ concentration for NR2A-containing NMDARs (3 µM). Glutamate was bath-applied continuously to reach steady-state plateau, then increasing amounts of NVP-AAM077 were added to form a dose-response inhibition curve as seen in Figure 3.3C-D (n = 3 to 7 cells for each data point). The IC$_{50}$ value of NVP-AAM077 was 203 nM, which is comparable to the IC$_{50}$ value measured in NR1/NR2B-expressing oocytes of 215 nM –whereas for NR1/NR2A receptors the IC$_{50}$ is 31 nM (Frizelle et al., 2006). Overall, the data from ifenprodil and NVP-AAM077 inhibitions are both consistent with the notion that hippocampal neurons at DIV 7-11 overwhelmingly express NR2B-containing NMDARs.

2.2 Ifenprodil sensitivity of extrasynaptic NMDAR currents

Even at this stage where NMDARs were overwhelmingly composed of NR1/NR2B subunits, it remained a possibility that a small quantity of NR2A-containing NMDARs could be concentrated at synaptic sites and thus influence the downstream signalling following synaptic NMDAR stimulation (Liu et al., 2004; Liu et al., 2007; Yashiro and Philpot, 2008). To refute this possibility, we sought to evaluate if both the synaptic and extrasynaptic pools of NMDAR were equally dominated by NR2B-subunits.

To selectively block synaptic NMDARs, we used the open channel blocker MK-801 as explained in Material and methods. MK-801 selectively blocks activated NMDAR channel with high affinity and in an almost irreversible manner. Indeed, dissociation of MK-801 only occurs under repeated NMDAR stimulation and when the neuron is depolarized, returning NMDAR to a functioning, permeable state (Tovar and Westbrook, 2002). However in our experimental timescale (less than 30 minutes) and when maintaining the neuron in voltage-clamp, MK-801 antagonism can be considered irreversible.
Spontaneous release of single vesicles of neurotransmitter glutamate in the synaptic cleft i.e. “quantal” release, causes MK-801 to block the NMDARs facing the presynaptic terminal -by definition the synaptic NMDARs. After successful blockade of synaptic NMDARs, subsequent NMDA applications measured the remaining current, which is mediated exclusively by extrasynaptic NMDARs -spared by the “quantal blockade” with MK-801 (Figure 3.4; Tovar and Westbrook, 2002; Nakayama et al., 2005).

To determine the time needed for the quantal release to effectively activate and block all synaptic NMDARs, different incubation times with MK-801 were tested (n= 3 cells each, Figure 3.4A). The proportion of current remaining after quantal blockade showed a decrease with time but plateaued after 10 minutes, indicating that antagonism of synaptic receptors attained saturation at that time. To verify that the synaptic NMDARs were indeed blocked by this protocol, we studied the decay kinetics of miniature currents (mEPSCs), as illustrated in Figure 3.4C-D. These mEPSCs being a combination of the fast kinetics of AMPARs and slow kinetics of NMDARs, the slower component of the decay was lost when all NMDARs were blocked by bath-applying 100 µM NMDA and 10 µM MK-801 together. Similarly, quantal blockade with MK-801 for 10 minutes produced the same decrease in the decay kinetics, suggesting a total blockade of the synaptic NMDARs with this protocol (see Figure 3.4C-D for example of traces and change in cumulative distribution of the decay constant).

Using 10 minutes quantal blockade on DIV 7-11 and DIV 12-18 neurons, we observed that the proportion of extrasynaptic current between the two groups did not change significantly (59 ± 4% and 61 ± 4%, n = 23 and 17 cells respectively, Figure 3.5).

Then, to assess the NR2B-containing NMDAR composition in the extrasynaptic fraction, 3 µM ifenprodil was applied subsequently to the quantal block with MK-801. As seen in Figure 3.4, the responses to 150 µM NMDA + 3 µM ifenprodil remained similar to the whole-cell level in DIV 7-11 neurons, with 77 ± 2% of the current being inhibited by ifenprodil (n = 18 cells, -200 ± 40 pA remaining; as opposed to 74 ± 2% for whole-cell, -400 ± 70 pA, Figure 3.3). The magnitude of current inhibition indicates
that, like it is the case in the entire neuronal population of receptor, the extrasynaptic pool of NMDARs is almost exclusively composed of NR2B-NMDARs at this developmental stage.

It has been reported that the expression of NR2A-containing NMDARs occurs exclusively at the synapses (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Townsend et al., 2003), although this differential partitioning of NR2 subunits is still controversial (Mohrmann et al., 2000; Thomas et al., 2006; Harris and Pettit, 2007). To assess if the upregulation of NR2A at DIV 12-18 occurred specifically in the synaptic NMDAR fraction, we measured the ifenprodil sensitivity of whole-cell and extrasynaptic NMDAR currents in these more mature neurons.

In DIV 12-18 cells, the extrasynaptic current fraction showed a higher ifenprodil sensitivity (65 ± 3%, n = 16 cells, -420 ± 50 pA remaining) compared to the whole-cell currents (52 ± 4%, Figure 3.4E, -1000 ± 100 pA remaining), indicating that the contribution of NR2B-containing NMDAR is higher in extrasynaptic sites. However, the magnitude of the extrasynaptic current inhibition still appeared lower than what was observed in younger neurons (65 ± 3% for DIV 12-18 and 77 ± 2% for DIV 7-11 cells). Thus, the difference in ifenprodil inhibition between the whole-cell and extrasynaptic currents of DIV 12-18 suggests that the rise in NR2A-containing NMDARs proportion after DIV 12 occurs preferentially at the synapse. However this partitioning was not exclusive since the ifenprodil sensitivity of extrasynaptic currents was also diminished in older cells compared to DIV 7-11 neurons. Those observations are in agreement with other published work showing a segregation of the NR2A subunit to the synapse (Li et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Thomas et al., 2006). Moreover, our data show that the subunit partitioning is only partial (at least at this developmental stage) which can offer an explanation as to why others have failed to observe that NR2A partitions in the synaptic NMDAR fraction (Mohrmann et al., 2000; Harris and Pettit, 2007).
Figure 3.4: Developmental loss of ifenprodil sensitivity of NMDAR currents is not solely restricted to synaptic locations. A. Loss of whole-cell NMDAR-mediated current due to MK-801 (10 µM) exposure under quantal transmission plateaus after 10 minutes (n = 3), indicating saturation of the synaptic NMDAR blockade. Hippocampal neurons were placed in Mg$^{2+}$-free external recording solution for the indicated time to allow open-channel blockade of synaptic NMDARs following their activation by single vesicular (quantal) releases of glutamate. B. Example traces of whole-cell current in DIV 9 hippocampal neurons before and after 10 min application of MK-801 during which NMDARs are activated only by spontaneous release of glutamate vesicles (denoted “quantal block”). C-D. Confirmation that all synaptic NMDARs are
blocked by this procedure. C. Examples of mEPSC shape before and after 10 min of MK-801 block of NMDARs activated by spontaneous transmitter release. Also for comparison is a mEPSC recorded at the end of the experiment where all NMDARs were blocked by addition of a high concentration of agonist in the presence of MK-801. D. Example of cumulative distribution curve of the decay constant of mEPSCs recorded from a neuron before (n = 185 events) and after (n = 157) 10 minutes of quantal block to illustrate the change in decay kinetics. E. Comparison of the developmental loss of ifenprodil (3 µM) sensitivity of the whole-cell and extrasynaptic NMDAR current fractions. Whole-cell currents of DIV 7-11 neurons (n = 21) and DIV 12-18 neurons (n = 19) were analyzed; extrasynaptic currents of DIV 7-11 neurons (n = 21) and DIV 12-18 neurons (n = 17) were analyzed. * p < 0.05.
Figure 3.5: The proportion of synaptic and extrasynaptic NMDAR currents remains similar between DIV 7-11 and DIV 12-18 hippocampal neurons. Following 10 min quantal block by MK-801, extrasynaptic NMDAR currents evoked by 150 µM NMDA were 59 ± 4% of total (whole-cell) NMDAR current in DIV 7-11 neurons (n = 23). Older neurons (DIV 12-18) showed a comparable amount of extrasynaptic receptors with 61 ± 4% of the NMDAR-mediated current being spared by quantal blockade (n = 17).
2.3 NR2B-NMDARs can mediate pro-death signalling

To investigate the involvement of NR2B-containing NMDARs in pro-death signalling, we induced NMDAR-dependent neuronal death by bath applying NMDA on DIV 8-11 neurons. Stimulation with NMDA concentrations higher than 20-30 µM (for 1 hour) trigger excitotoxicity in trophically-deprived neurons, which can be observed 24h after the insult (Soriano et al., 2006).

As seen in Figure 3.6, 50 µM NMDA induced 68 ± 3% cell death in control condition (for all conditions n = 3 different experiments). This NMDAR-mediated death was completely abolished when NMDAR channel-blocker MK-801 was pre-applied in the well (12 ± 2%). Moreover, antagonism of NR2B-containing NMDARs with ifenprodil completely prevented excitotoxicity (12 ± 2%), indicating that the pro-death signal relies on NR2B-NMDAR activation. NVP-AAM077, at a concentration where it inhibits a large fraction (70%) of NR2A-containing NMDARs and only 10% of NR2B-NMDARs (Frizelle et al., 2006), did not alter the amount of cell death (30 nM NVP, 70 ± 3%). On the other hand, at a higher concentration (400 nM) the compound significantly reduced NMDA-induced cell death (18 ± 3%). Indeed, at this concentration, NVP-AAM077 is unspecific and inhibits both NR2A- and over 70% of NR2B-mediated currents (Frizelle et al., 2006), supporting the conclusion that NR2B-containing NMDARs are required for pro-death signalling using that stimulation protocol.
Figure 3.6: NR2B-containing NMDARs can mediate pro-death NMDAR signalling. 
A. Hippocampal neurons were pre-treated with MK-801 (MK; 10 µM), ifenprodil (ifen; 3 µM) or NVP-AAM077 (NVP; 30 and 400 nM) prior to exposure to 50 µM NMDA for 1 hour. Neurons were then returned to drug- and agonist-free medium and death was assayed 16-24 hours after by counting the number of pyknotic nuclei as percent of total. All antagonists targeting NR2B-containing NMDARs abolished NMDA-induced cell death. B. Example pictures of DAPI-stained fixed hippocampal cultures. Pyknotic nuclei show bright and dense spots of fluorescence (scale bar 20 µM). * p < 0.05.
2.4 NR2B-NMDARs can mediate pro-survival signalling

Pro-survival signalling from NMDAR occurs under physiological activation of synaptic receptors (Hardingham, 2006a). Synaptic NMDARs are able to induce CREB-dependent gene expression and activate PI3K/Akt and ERK1/2 pathways, as well as boosting antioxidant defenses (Hardingham et al., 2002; Papadia et al., 2005; Ivanov et al., 2006; Papadia et al., 2008). In adult tissues, blockade of normal synaptic NMDAR activity renders neurons more vulnerable to neurodegeneration (Ikonomidou et al., 2000). This beneficial role is particularly obvious during the development of cortical and hippocampal neurons in vitro: not only does synaptic NMDAR activity confer some resistance against neurotoxic insults (Papadia et al., 2005), but in some conditions is required for their very survival (Hardingham et al., 2002).

The induction of NMDAR-dependent pro-survival signals can be triggered by enhancing the spontaneous firing of the neuronal network, which is thought to recruit synaptic NMDAR activity (Hardingham et al., 2002). Thus, we first verified that action potential firing was indeed selectively activating synaptic NMDARs, by using the following variation of the “quantal blockade” protocol.

After the measurement of NMDAR currents (with 100 µM NMDA), the culture network was allowed to resume its axonal transmission and synaptic firing (by washing out TTX). Once neurons showed action potentials, the recording configuration was switched to current-clamp and a resting membrane potential of around -60 ± 2 mV was imposed via current injection. MK-801 was introduced in the external solution for 5 or 10 minutes. During that time, and because the cell was allowed to depolarize in current-clamp, MK-801 irreversibly blocked NMDARs recruited during spontaneous network activity. Once the incubation period ended, the cell was switched back to voltage-clamp and MK-801 was removed from the recording chamber. The remaining NMDA-induced current was finally measured and compared to the initial whole cell current.

Similar to what was observed with the quantal blockade protocol (Figure 3.4), current blockade by activity showed a plateau after 5 minutes (57 ± 4% at 5 minutes and 65 ± 7% at 10 minutes; n = 7 and 3 cells; Figure 3.7). This MK-801-induced reduction was axonal transmission-dependent since the same stimulation in presence of TTX did
not change the NMDAR current (103 ± 19%, n = 3 cells), since miniature events alone (quantal release) in the presence of magnesium ions are not making the receptor apt for blockade by MK-801.

The amount of current decrease using the activity blockade protocol was remarkably similar to the level of receptor inhibition by quantal blockade (57 ± 4% vs 67 ± 5%; n = 7 and 8 cells, respectively), suggesting that the network activity spontaneously occurring in the cultures only recruits synaptic NMDARs (Figure 3.7). To confirm this, cells were treated sequentially to both stimulation protocols to see if the MK-801 targeted the same population of receptors. Indeed, after 5 minutes of activity block and the cell being returned to Mg^{2+} and TTX-containing recording solution, a further 10 minutes of quantal block were performed. The total amount of NMDAR inhibition by MK-801 using both protocols decreased currents to 54 ± 4% of their initial values (n = 3 cells), a result that is not significantly different from either technique when performed alone. Therefore, the pattern of MK-801 blockade of NMDARs when cells are exhibiting action-potential-driven glutamate release indicates that only synaptic NMDARs are activated under basal condition.
Figure 3.7: Endogenous network firing recruits synaptic NMDAR activity. A. Hippocampal neurons (DIV 10-11) were left to fire normally in presence of MK-801 for 5 and 10 minutes (n = 7 and 3). 5 minutes of this “activity block” were sufficient to reach saturation of the blockade. B. The activity blockade targets synaptic NMDARs. When quantal blockade (+TTX in Mg$^{2+}$-free recording solution) was performed following 5 min of activity block, no further decrease of NMDAR currents was observed (57 ± 4% activity block vs 54 ± 4% both protocols; n = 7, 3). The NMDAR inhibition was indeed activity dependent since presence of TTX prevented the blockade (103 ± 19%; n = 3). * p < 0.05.
To study the pro-survival function of this ongoing synaptic NMDAR activity in neurons DIV 7-11, we used staurosporine to induce apoptosis. Staurosporine is a broad-spectrum kinase inhibitor and initiates apoptosis in a caspase-dependent manner in hippocampal neurons (Krohn et al., 1998). In control condition, the staurosporine treatment caused 45 ± 6% cell death (Figure 3.8A, n = 4 experiments). When the spontaneous synaptic NMDAR activity was blocked by MK-801, this number rose to 65 ± 5%. Inhibition of synaptic NR2B-NMDARs by ifenprodil also produced a similar increase in cell death (57 ± 4%). This exacerbation in staurosporine-induced apoptosis of hippocampal neurons after inhibition of NR2B-containing synaptic NMDARs indicates that these receptors mediate an ongoing pro-survival effect in neurons. Thus, the silencing of these NR2B-containing receptors renders cells more vulnerable to apoptosis.

The amount of spontaneous activity present in the hippocampal network can be quite variable between cultures, possibly because of variations in final plating density (Mangan and Kapur, 2004; Bauer et al., 2008). To increase synaptic NMDAR activation to a more reliable threshold, and by the same process enhance their pro-survival-inducing properties, 50 µM bicuculline was added to the culture medium (Hardingham et al., 2001b). Presence of bicuculline triggers bursts of action potentials, increases synaptic NMDAR activation and their associated calcium transients (Hardingham and Bading, 2002). A strong neuroprotection against the apoptotic insult was seen in neurons treated with bicuculline for 16 hours prior to 100 nM staurosporine stimulation (24 hours; from 52 ± 3% to 12 ± 2% cell death; n = 3 experiments; Figure 3.8B-C). However, when the enhancement of synaptic NMDAR activity was blocked by MK-801 or by NR2B-selective ifenprodil (10 µM MK-801 and 3 µM ifenprodil, added simultaneously to bicuculline), the apoptosis returned to higher levels (MK-801: 42 ± 3%; ifenprodil: 40 ± 3%). Whereas MK-801-treated cells showed similar amount of cell death to the control staurosporine-treated condition, apoptosis was still slightly reduced with ifenprodil (52 ± 2% vs 40 ± 3%). Since ifenprodil does not inhibit completely NR2B-containing NMDARs, a residual synaptic NMDAR recruitment could explain the partial protection seen under this condition.
Taken together, the results from Figure 3.8 show that NR2B-containing NMDARs can signal to pro-survival pathways, which protect against staurosporine-induced apoptosis of hippocampal neurons at DIV 8-10.
Figure 3.8: NR2B-containing NMDARs can mediate pro-survival signalling. A. Blockade of spontaneous NR2B-containing NMDAR activity exacerbates staurosporine-induced apoptosis. Hippocampal neurons were treated with MK-801 (10 µM) or ifenprodil (3 µM) for 16 hours prior to exposure to staurosporine (50 nM) for 24 hours (n = 4). B. Neurons treated where indicated with bicuculline (BiC, 50 µM) in the presence of absence of the indicated antagonists for 16 hours prior to exposure to staurosporine (100 nM) for 24 hours. Bicuculline treatment strongly protects against staurosporine-induced apoptosis. Blockade of NR2B-containing NMDARs reduces pro-survival signals emanating from the enhanced synaptic NMDAR activity. C. Example pictures from B (scale bar 20 µM). * p < 0.05.
2.5 NR2B-NMDAR activation can trigger synaptic potentiation

Some groups have reported that NR2A-containing NMDARs are required for induction of synaptic potentiation, whereas the NR2B-containing ones are linked to synaptic depression (Liu et al., 2004; Massey et al., 2004). Therefore, we investigated if our hippocampal cultures, being at a stage where they mostly possess NR2B-NMDARs, could exhibit NMDA-dependent synaptic potentiation.

We used a bicuculline-induced mEPSC frequency potentiation; in this paradigm the period of synchronous bursting of the neuronal network leads to a synaptic potentiation, measurable by an increase in miniature excitatory postsynaptic currents (mEPSCs) frequency (Arnold et al., 2005). This change in synaptic transmission is persistent for several days and caused by the postsynaptic insertion of AMPA receptors to silent synapses (Abegg et al., 2004; Arnold et al., 2005).

The bicuculline treatment induced a robust increase in mEPSC frequency in hippocampal cells at DIV 8-11, from 1.3 ± 0.2 Hz to 2.9 ± 0.4 Hz (n = 9 cells each; Figure 3.9). The mEPSC potentiation required NMDAR activity for its induction since pre-incubation with channel blocker MK-801 completely prevented the change in frequency (1.4 ± 0.4 Hz, n = 10 cells). To assess if this NMDAR-dependent potentiation model was mediated by NR2B-containing NMDARs, the stimulation was done in presence of 3 µM ifenprodil. Indeed, the potentiation failed to take place whilst NR2B-containing NMDARs were inhibited (1.3 ± 0.4 Hz, n = 9 cells). Moreover, using NR2A-prefering compound NVP-AAM077 at 30 nM, a concentration where it affects NR2A-NMDARs while sparing NR2B-NMDARs, the potentiation was not impaired (2.5 ± 0.5 Hz, n = 8 cells). On the other hand, when used at the non-discriminating concentration of 400 nM, NVP-AAM077 blocked the mEPSC frequency potentiation (1.4 ± 0.2 Hz, n = 8 cells).

Thus, the synaptic potentiation caused by 15 minutes of bicuculline-induced bursting is NMDAR-dependent. Also, in neurons at DIV 8-11, this potentiation specifically requires activation of NR2B-containing NMDARs, highlighting their ability to mediate this type of signalling.
Figure 3.9: NR2B-containing NMDARs mediate activity-dependent potentiation of mEPSC frequency. A. Hippocampal neurons pre-treated with antagonists as indicated (30 min) and in the presence of these compounds were treated with medium ± bicuculline (50 µM) for 15 min, then allowed to settle for 30 min. mEPSCs were then recorded for 5-10 min (minimum of 300 events) and the frequency of events was calculated (n = 8-10 for each condition). Bicuculline induces potentiation of the mEPSC frequency. Antagonists targeting NR2B-containing NMDARs (10 µM MK-801, 3 µM ifenprodil and 400 nM NVP-AAM077, but not 30 nM NVP-AAM077) prevent the induction of this synaptic potentiation model. B. Examples of traces used to generate the data shown in A. * p < 0.05.
2.6 NR2B-NMDARs are able to signal to synaptic depression at DIV 8-11

NMDARs containing the NR2B subunit have been linked to depression signalling by other reports (Liu et al., 2004; Massey et al., 2004; Zhao and Constantine-Paton, 2007). However, experiments in these publications were done using rodent hippocampal slices and at a different developmental time than our current dissociated neurons culture model. Therefore we investigated if, within a single cell type and at the same developmental stage, NR2B-NMDARs were able to signal to depression—in addition to death, survival and potentiation.

To induce synaptic depression, we adapted a protocol used on slices in which a short application of a low concentration of NMDA is producing an AMPA receptor internalization that occludes electrically-induced LTD (Lee et al., 1998).

We first sought to use a stimulation that wouldn’t prove lethal to the neuronal network in the first place. To do so 30 µM NMDA was added to the well of DIV 8-11 culture for 4 minutes or 1 hour and cell death was assessed. Unlike the 1 hour stimulation which induced nuclear pyknosis, cell death with the 4 minutes NMDA stimulation did not differ from control wells (Figure 3.10, from 2 wells each).
Figure 3.10: A short stimulation of agonist at low concentration does not induce cell death. A. Hippocampal neurons were treated to 30 µM NMDA for either 4 min or 1h then returned to NMDA-free medium. Nuclear pyknosis was assayed 24 hours after. As opposed to 1h stimulation, 4 min of 30 µM does trigger noticeable amount of cell death. B. Example pictures (scale bar 20 µM).
The same stimulation was therefore applied to wells containing neurons growing on coverslips. After the 4 minutes with 30 µM NMDA, the coverslip was transferred to a dish containing fresh, pre-equilibrated medium and put back at 37°C, 5% CO₂ for a least 30 minutes. Recordings were then taken from cells under voltage clamp at -60 mV, and in an external solution containing 3 mM CaCl₂ (instead of 2 mM) to elicit a higher frequency of spontaneous activity within the network. Control cells (DIV 8-11) showed a frequency of spontaneous events of 0.12 ± 0.02 Hz (n = 19 cells, Figure 3.11). In neurons treated with NMDA (4 minutes), the frequency was drastically decreased to 0.032 ± 0.005 Hz (n = 7 cells). This depression of synaptic transmission was completely inhibited when the stimulation was done in presence of the competitive NMDAR antagonist D-APV (50 µM). At a low dose of NVP-AAM077 (30 nM), the protocol still induced synaptic depression, indicating that NR2A-containing NMDARs were not required (0.042 ± 0.003 Hz, n = 6 cells). Even with 400 nM NVP-AAM077 the frequency of spontaneous currents was decreased to 0.044 ± 0.008 Hz (n = 6), suggesting that even a partial activation of NR2B-NMDARs was still enough to trigger the depression signalling. Only by using 1 µM of NVP-AAM077 did the level of NR2B-NMDAR inhibition was sufficient to prevent the change in network activity (0.14 ± 0.02 Hz, n =4 cells). Note that ifenprodil and MK-801 were not used in this depression paradigm, as application of the compounds decreased basal sEPSC frequency in unstimulated cells. Overall, inhibition of both NR2B- and NR2A-containing NMDARs was successful at disrupting the induction of this model of synaptic depression, whereas antagonism of NR2A-NMDARs alone was not. This indicates that NR2B-containing NMDAR activation can signal to synaptic depression in hippocampal neurons at DIV 8-11.
Figure 3.11: NR2B-containing NMDARs mediate agonist-induced depression of spontaneous EPSC (sEPSC) frequency. Hippocampal neurons DIV 8-11 were pretreated as indicated with antagonists for 30 min before NMDA (30 μM) was applied for 4 min. Coverslips were then quickly transferred to fresh medium and left idle for 30 min. sEPSC were recorded 4-8 minutes and the frequency of events bigger than 150 pA was measured. The short agonist application subsequently reduces sEPSC frequency. This synaptic depression model is not prevented by antagonists targeting NR2A-containing NMDARs, NVP-AAM077 at 30 nM and 400 nM [NVP(0.03) and NVP(0.4)]; but is abolished when both NR2A- and NR2B-containing NMDARs are blocked by 50 μM D-APV (APV) and 1 μM NVP-AAM077 [NVP(1)]. * p < 0.001
3. Chapter discussion

NMDAR antagonists show great efficacy in preventing neuronal death following excitotoxic concentration of glutamate in *in vitro* models, but clinical trials involving such a strategy have failed (Ikonomidou and Turski, 2002). With this realization came an increase in interest for the different NR2 subunits of the NMDAR and their downstream signalling pathways. Indeed, if a certain subtype of NMDAR is to be responsible for the pro-death signalling, whilst another mediates the normal physiological functions important for neuron survival and memory processing, the targeting of such receptor could potentially reduce the side effects of the NMDAR blockade when preventing cell death in ischemic conditions. Several publications reported differential signalling roles for NR2A/B subunits (Lu et al., 2001; Liu et al., 2004; Massey et al., 2004; Liu et al., 2007), but these findings remain controversial (Berberich et al., 2005; Weitlauf et al., 2005; Bartlett et al., 2007; von Engelhardt et al., 2007). These reports focussed on specific NMDAR-dependent signals and used various neuronal models, which render comparisons between studies difficult. Thus, the aim of the present study was to take a general and inclusive approach: to determine if, within the same neuron and at the same developmental stage, a single NR2 subtype could mediate the several NMDAR-dependent signalling events. More specifically, we investigated if NMDARs composed of NR2B subunits were able to signal to death, survival, synaptic potentiation and depression.

In dissociated hippocampal neuron cultures, we found that the developmental switch in NR2 subunit composition did not appear before DIV 12. After that point, the NR2A subunit expression rose and began to be integrated in the NMDAR population. Although the total amount of NMDAR-mediated current was stably rising within the hippocampal neurons, the proportion of ifenprodil-sensitive receptors was sharply falling from DIV 12. This agrees well with others’ documentation of the developmental patterns of subunit expression, pinning the NR2A mRNA appearance to around P7 in the rat brain (Monyer et al., 1994; Cull-Candy et al., 2001).

The dominance of NR2B-containing NMDARs was equally distributed in both synaptic and extrasynaptic populations in hippocampal neurons DIV 7-11, as evidenced
by the ifenprodil sensitivity reaching near maximal values for both whole-cell and extrasynaptic currents. The selective blockade of synaptic NMDARs by quantal blockade allowed us to estimate the proportion of extrasynaptic NMDARs to be more than half the total. This proportion seemed unchanged in older neurons expressing the NR2A subunit, but as pointed by others using a similar protocol, developing neurons should gradually exhibit a higher synaptic to extrasynaptic ratio (Tovar and Westbrook, 1999, 2002; Nakayama et al., 2005). However, this reported shift is apparent in neurons after 2-3 weeks in vitro and likely depends on culture types and conditions, as the connectivity, synapses strength and subunit composition can be influenced by the endogenous activity of the network (Quinlan et al., 1999; Nakayama et al., 2005).

In addition, we observed that the targeting of the NR2A subunit appears to be preferentially synaptic, as the ifenprodil sensitivity of extrasynaptic currents in older neurons was greater than for the whole population of receptors. This raises interesting questions about the trafficking, interactions and indeed signalling properties of NMDARs at a latter stage, but this was not the immediate goal of our study. Instead we focussed on neurons at DIV 7-11, so that most receptors would be di-heteromeric NR1/NR2B subunits, and study the NR2B-NMDAR-dependent signals.

The dichotomy of synaptic and extrasynaptic on pro-death and pro-survival signalling has been clarified previously (Hardingham and Bading, 2002; Hardingham et al., 2002; Hardingham and Bading, 2003; Lee et al., 2005), but the potential role of the NR2 subunit on this issue is unclear. The NR2B-containing NMDARs were able the signal to both death and survival pathways in our cultures. The advantage of our approach is that the virtual absence of the NR2A subunit prevents any confusion between subunit and localization. Indeed, since virtually every NMDAR are similarly composed of NR2B subunits, which outcome they will signal to depends on the stimulation type. As such, stimulation of synaptic and extrasynaptic receptors by bath application of NMDA triggers preferentially pro-death cascades, such as the inactivation of CREB via dephosphorylation and the subsequent BDNF expression (Hardingham and Bading, 2002; Ivanov et al., 2006). On the other hand activation of synaptic NMDARs, either by endogenous network activity or by enhancement of the action potential
bursting with bicuculline, is pro-survival. These pro-survival pathways include the PI3K-Akt cascade, the Ras-ERK1/2 cascade and the CRE-dependent gene transcription (Hardingham, 2006a; Papadia and Hardingham, 2007). The identity of the particular pathways involved in the pro-death and pro-survival signals evidenced in the present study has not been addressed, but the results obtained show that they share an absolute reliance on NR2B-containing NMDARs.

Liu et al. (2007) have observed a differential role of NMDAR subunits in mediating excitotoxicity in dissociated neurons. Their results suggest that, regardless of their subcellular localization, NR2A-NMDARs mediate survival signalling and NR2B-NMDARs death signalling. However their use of cortical neurons at DIV 11-14, in light of our observation that the NR2A subunit expression begins to rise exactly at that developmental time (in hippocampal cells at DIV 12), raises some issue about the actual composition of their NMDAR population—which can differ greatly from one day to another or between cultures. In addition, the conclusions of this study do not take into consideration the unspecific effect of NVP-AAM077 on NR2B-NMDARs, and the incomplete inhibition of NR2B-specific antagonists. Thus, the remaining activity during incomplete NR2B-blockade could be exerting an effect similar to what is seen with low concentration of NMDA; bath-applying low doses of agonist triggers an increase in network excitability, enhancing synaptic NMDAR activity and downstream pro-survival signals (Soriano et al., 2006).

Contrary to the conclusions of Liu et al. (2007), it was also reported that both NMDAR subtypes are able to mediate cell death (von Engelhardt et al., 2007). In this study, excitotoxicity in DIV 21 neurons remains unchanged when inhibiting either NR2A- or NR2B-containing NMDARs, but is abolished when both populations are blocked synergistically (von Engelhardt et al., 2007). Interestingly, von Engelhardt et al. (2007) also observed that blockade of NR2A-containing NMDARs exacerbated cell death following a moderate excitotoxic insult, suggesting that NR2A could be mediating neuroprotective signals in that context. It has been shown that the NR2A subunit can interact with the dopamine D1 receptor, and that this association can trigger PI3K-Akt
pro-survival signals in hippocampal neurons (Lee et al., 2002). Such interactions may affect differentially the downstream signals evoked by NMDARs of different subunit composition and need further investigation. Indeed the work presented here does not rule out a link between subunit composition and pro-death/survival signalling, but rather stresses the fact that it is not absolute: the NR2B subunit is perfectly capable of mediating both survival and death.

Enhancing the network activity by adding the GABA<sub>A</sub> blocker bicuculline induces a change in activity pattern observed in the whole network. When the tonic GABAergic inhibition is relieved, uncoordinated firing of neurons gradually becomes organised into synchronous bursting of the network (Arnold et al., 2005). This causes the synaptic efficacy to increase in a NMDAR-dependent manner, and this potentiation is measurable by an increase in mEPSC frequency. The frequency potentiation is likely due to insertion of AMPARs into silent synapses, synapses composed of NMDAR but lacking AMPARs (Lu et al., 2001; Man et al., 2003; Abegg et al., 2004; Baxter and Wyllie, 2006). An increase in neurotransmitter release probability would also augment mEPSCs frequency. We did not verify if it was the case, as the assessment of presynaptic properties is difficult in dissociated cultures. However, it was shown by others that paired-pulse facilitation is unchanged in bicuculline-potentiated slices, suggesting that this model of potentiation does not alter presynaptic properties (Abegg et al., 2004). In addition, this model of potentiation occludes classical LTP induced by electric stimulation and can be reversed by adequate stimuli, suggesting that both paradigms share common mechanisms (Debanne et al., 2006). Following NMDAR-mediated calcium influx into the postsynaptic cell, induction of this synaptic potentiation requires the PI3K and ERK1/2 pathways (Man et al., 2003; Arnold et al., 2005), which is consistent with the type of signalling downstream of synaptic NMDARs.

We obtained similar mEPSC frequencies in control conditions between cells and cultures, allowing us to realise the potentiation experiment with unpaired recordings. On the other hand, amplitudes of mEPSCs appeared highly variable between cells. Therefore, we did not consider mEPSC amplitude in our study, although others have shown that this bicuculline-induced long-term potentiation model does upregulate this
parameter—the fast peak of mEPSCs being mediated by AMPAR (Abegg et al., 2004; Arnold et al., 2005). Treatment of our cultures with bicuculline triggered a reliable, more than two-fold increase in mEPSC frequency, a level similar to what is reported by Arnold et al. (2005).

Our other model of synaptic plasticity, synaptic depression, was evoked by a brief application of NMDA at a low dose that was not toxic for hippocampal neurons. Several other groups have used a similar approach to induce synaptic depression (Lee et al., 1998; Carroll et al., 1999; Beattie et al., 2000) or potentiation (Broutman and Baudry, 2001; Martel et al., 2006). The depression observed after bath-applied agonist stimulation requires Ca$^{2+}$ influx through the NMDAR channel, calcineurin activation and results in AMPAR endocytosis (Beattie et al., 2000). Albeit others using this model measured its effect on mEPSCs (lower frequency and amplitude), we hypothesized that the decrease in synaptic efficacy would be easily assessable by recording spontaneous action potential-driven EPSCs. Indeed, we observed a striking decrease in sEPSC frequency in NMDA-treated cells compared to control condition.

Using these two models of synaptic plasticity, we studied their dependence on the NR2B-containing NMDARs in hippocampal neurons at DIV 8-11. We observed that the bicuculline-induced potentiation was NR2B-NMDARs-dependent, as ifenprodil, MK-801 and a high dose of NVP-AAM077 were all able to disrupt its appearance. Similarly, the NMDA-induced sEPSC depression was blocked by APV and by high dose of NVP-AAM077, but not by a low NR2A-specific NVP-AAM077 concentration. This indicates that inhibition of NR2A-NMDARs alone does not interfere with the depression, whereas inhibition of NR2A- and NR2B-NMDARs does. Considering that the ifenprodil sensitivity of the currents measured in these immature neurons shows that they predominantly express NR2B-containing NMDARs, this strongly suggests that NR2B-NMDARs are mediating the synaptic depression signalling in this model.

Therefore, the subunit composition of NMDARs does not seem to influence which direction takes the synaptic plasticity; it rather relies on the type of stimulation given to the neuronal network. Short calcium transients through the synaptic NMDARs
trigger potentiation signalling whereas long, low levels of both synaptic and extrasynaptic NMDAR activity push the network into depression.

The lack of subunit dependency, or instead the requirement for NR2B-NMDARs channel opening in both types of plasticity has to be put in context before direct comparison with other published data. Our culture model contains almost exclusively di-heterologous NR1/NR2B-NMDARs. Indeed, the absolute requirement of NR2B-NMDARs for the induction of potentiation and depression is hardly unexpected for NMDAR-driven events in such a situation. However, it provides strong evidence against reports that subunit-specific recruitment governs the type of plasticity synapses undergo (Liu et al., 2004; Massey et al., 2004).

Our results cannot predict the way the late NR2A expression may alter the nature of plasticity at hippocampal glutamatergic synapses. Given that this subunit appears to be preferentially integrated to synaptic sites, one might expect that the dichotomy of plasticity signalling would be influenced by this factor. NR2B-NMDAR kinetics are slower than the NR2A-containing ones and lead to a larger calcium influx in the postsynaptic cell (Erreger et al., 2005). Therefore, the threshold for LTP and LTD induction is undoubtedly shifting when the partitioning of subunit appears during neuronal development. Apart from the channel properties, the intracellular interactions each of the distinct subunit’s C-terminus tail also needs to be considered, such as its binding to CaMKII or PSD-95. Barria and Malinow (2005) have found that active CaMKII, which is central to establishment of LTP, associates strongly with the NR2B subunit intracellular tail as opposed to NR2A. By altering the NR2 subunits to decrease their ability to interact with the active kinase, the synaptic potentiation levels also deceased. Thus, they argue that the upregulation of NR2A subunits, which diminishes NMDAR association with CaMKII, correlates with the reduction of plasticity in the hippocampus. This goes beyond the scope of the present study on immature neurons, but highlights the intriguing possibility that each type of NR2 subunit can alter the plasticity, and indeed the pro-death/survival signalling in neurons at a later stage.

The discrepancies in publications studying the relation between the NR2 subunits and signalling (apart from the different cellular models) may possibly originate from the
unspecific nature of NVP-AAM077. The competitive nature of the antagonist renders its efficacy and specificity for NR2A dependent on the agonist concentration. Thus, the IC$_{50}$ of NVP-AAM077 can vary substantially depending on the stimulation, such as bath application of exogenous NMDA or synaptically-released glutamate (Frizelle et al., 2006). Most studies which found a reliance on NR2A-NMDARs for potentiation and pro-survival signalling used concentrations higher than 0.4 µM NVP-AAM077 (Liu et al., 2004; Massey et al., 2004; Zhou and Baudry, 2006; Liu et al., 2007), whereas negative or partial results were obtained with 0.1 µM NVP-AAM077 or less; concentrations at which inhibition of NR2B-containing NMDARs is low (Berberich et al., 2005; Toyoda et al., 2005; Weitlauf et al., 2005; Bartlett et al., 2007; Le Roux et al., 2007; Tran et al., 2007; von Engelhardt et al., 2007).

Concurrently, ifenprodil is highly selective for NR2B-containing NMDARs. However, it has two caveats: its maximal potency is around 80% and it can potentiate NMDAR currents at low agonist concentration since it increases the agonist affinity of NMDARs (Williams, 1993). Thus, ifenprodil may affect NMDARs in an unexpected manner in cases where low, ambient extracellular concentrations of glutamate are present. Therefore both the use of NVP-AAM077 and ifenprodil may explain some of the contradictory results between studies investigating the signalling roles of NR2 subunits.

In conclusion, the present results show that in DIV 7-11, dissociated hippocampal neurons mainly express NR2B-containing NMDARs. In these neurons at this developmental stage, NR2B-NMDARs are able to mediate death, survival, synaptic potentiation and depression. These results support the hypothesis that stimulation (length, intensity and subcellular localization of NMDARs), rather than subunit composition, dictates the type of signalling triggered by NMDAR activation at this time in development.

The results presented in this Chapter were published in Martel et al. (2009a). Figure 3.7 was published in Papadia et al. (2008). Both research papers are included at the end of this thesis.
Chapter 4
Pro-death p38 signalling requires a neuronal context and does not contribute to NMDAR-induced non-neuronal cell death
1. Chapter introduction

It is well documented that calcium entry through NMDARs can mediate cell death (Olney, 1969; Arundine and Tymianski, 2004). Moreover, calcium flux through this subtype of glutamate receptor is more efficient at triggering neuronal death than through other routes (Sattler et al., 1998). This “source specificity” indicates the presence of a pro-death signalling cassette/complex, formed by NMDAR coupling to other proteins such as PSD-95 and neuronal NOS (Sattler et al., 1999).

However, acute expression of NMDARs in various non-neuronal cell types also proves to be cytotoxic (Cik et al., 1993; Anegawa et al., 2000). When cell lines such as HEK 293, COS, Hela or CHO cells are transfected with pcDNAs coding for the NR1 and NR2A or NR2B subunits of the NMDAR, these cells die in a manner that mimics excitotoxicity in neurons. In contrast, transfection with the less calcium-permeable NR2C and NR2D subunits has been suggested to be less excitotoxic (Boeckman and Aizenman, 1996; Raymond et al., 1996). Expression of NMDARs in a heterologous system offers the opportunity to study excitotoxicity in a simpler cellular model, compared to the complexity and heterogeneity of dissociated neuronal cultures. Others who have investigated this issue by using recombinant expression of NMDAR subunits have focussed on the receptor itself. For instance, the influence of subunit phosphorylation by different kinases on NMDAR activity and trafficking was elucidated by studying NMDAR in cell lines (Vissel et al., 2001; Woodward, 2002; Hawkins et al., 2004; Wu et al., 2007). Moreover, the interaction of MAGUKs with the different NR2 subunits and their effect on the receptor surface expression and properties have also been elucidated in non-neuronal cellular models (Dong et al., 2004; Cousins et al., 2008a; Cousins et al., 2008b; Sornarajah et al., 2008).

However, the reconstitution of NMDAR-mediated cell death in non neuronal cells raises some intriguing questions. From the neuronal studies the “source-specificity” hypothesis postulates that signalling complexes are tethered at the mouth of the NMDAR channel, and that these complexes then recruit pro-death signals during excessive NMDAR activation. Thus, whilst NMDAR expression also induces non-
neuronal cell death, it is unclear if this phenomenon also shares the "source-specificity" of neuronal excitotoxicity.

The JNK and p38 SAPKs are two key mediators of neuronal excitotoxicity (Borsello et al., 2003a; Cao et al., 2005; Segura Torres et al., 2006; Soriano et al., 2008). Inhibiting either of these pathways using two chemical compounds, SP600125 and SB203530 respectively, protects cortical neurons from a moderate agonist-induced insult, as seen in Figure 4.1 (experiment done by Dr Francesc X. Soriano). It is however unknown if these pro-death cascades are contributing to recombinant NMDAR-mediated toxicity. Indeed, elucidating the non-neuronal excitotoxicity pathways could help identify the pro-death signals that rely on NMDAR-bound signalling complexes in neurons.

Thus, in the present study we sought to recapitulate NMDAR-mediated cell death in a non-neuronal cell line to investigate its reliance on the JNK and p38 SAPKs. The following experimental findings are presented in this Chapter:

- Expression of NR1/NR2B subunits in the mouse pituitary cell line AtT20 led to the production of functional, surface-expressed NMDARs;
- By measuring the amount of co-expressed luciferase enzyme activity in these cells, the levels of excitotoxic death could be measured following agonist application;
- Using this viability assay, recombinant NMDAR-induced toxicity was found to depend on the JNK pathway and not on the p38 SAPKs;
- The NMDAR-mediated Ca\(^{2+}\) load needed to induce toxicity was compared between neurons and non-neuronal cells, and was found to be more than 4 times higher in AtT20 cells, consistent with the absence of p38 signalling in the non-neuronal excitotoxicity.

In conclusion, this deviation between non-neuronal cell and neuronal pro-death signals suggests that NMDARs are more effective at recruiting excitotoxic cascades in neurons. Moreover, the results indicate that the two SAPK cascades have different induction requirements, as the p38 signals could not be activated by NMDARs outside a
neuronal context. The results presented in this Chapter were published in Soriano et al. (2008) and Martel et al. (2009b).
Figure 4.1: In cortical neurons, both p38 and JNK SAPKs participate in excitotoxic signalling downstream of NMDARs. Experiment done by Dr Francesc X. Soriano (data not shown in Soriano et al., 2008). Neurons were pre-treated for 1 hour with SB203580 (p38 inhibitor 5 µM; p38i) or SP600125 (JNK inhibitor 1 µM; JNKi) before 1 hour treatment with glutamate (n = 3). Cells were then returned to fresh medium and cell death was assessed 16-24 hours later. Both p38 and JNK pathways inhibition were neuroprotective at moderate agonist stimulation. However, only when both SAPKs were inhibited were neurons protected against a strong excitotoxic insult (100 µM NMDA), indicating the synergistic action of both pathways in neurons. * p < 0.05 compared to control.
2. Results

2.1 Expression of NMDARs in AtT20 cells
To study the toxicity of NMDAR in non-neuronal context, we used the mouse LAF1 pituitary gland tumour cell line AtT20 (European Collection of Cell Cultures number 87021902). These cells were chosen because they do not express any glutamate receptors and grow at a moderate rate in DMEM medium containing 10% FBS (about 1 cell cycle per 2-3 days in these conditions). Also, their transfection efficiency using Lipofectamine is low enough to control the basal amount of cell death when expressing NMDAR but sufficiently high for use in the various assays described here. Indeed in a high transfection efficiency paradigm, the spontaneous cell death would release glutamate in the medium and create a positive feedback of toxicity for other transfected cells.

Because cell lines do not possess a highly negative membrane potential like neurons, the potency of NMDAR blockade by Mg\(^{2+}\) is low in cell lines and causes spontaneous cell death (Cik et al., 1993). Therefore, at the end of the transfection period, medium was replaced with fresh medium containing NMDAR antagonist APV.

Transient expression of NMDARs in AtT20 cells was achieved by transfecting pCis vectors containing the full length mouse cDNAs encoding for the full-length NR1-1a and NR2A or NR2B sequences, as described in Material and Methods. The NR1-1a splice variant of NR1 was used as it is the most abundantly expressed form (Stephenson, 2006).

Transfection efficacy was measured by immunostaining of the NR1 subunit. To do so, AtT20 cells were fixed 24 hours after the end of the transfection with NR1/NR2B and luciferase plasmids. Immunohistochemistry was then performed using an antibody against the NR1 epitope and the fluorescence visualized as seen in Figure 4.2. The efficiency of transfection was established to be around 5-10% of cells.
Figure 4.2: Transfection of plasmids encoding for the NR1 and NR2B subunit leads to expression of functional, surface-expressed NMDARs in AtT20 cell line. A. AtT20 cells were transfected with pDNA encoding for full-length NMDAR subunits along with luciferase. Cells were fixed 24 hours post-transfection and probed for NR1 immunoreactivity. B. Example of glutamate-induced NMDAR currents in transfected AtT20 cells. Coverslips with AtT20 cells were transfected with NR1, NR2B and enhanced green fluorescent protein (eGFP). 18-72h later, a fluorescent cell was patch-clamped at -60 mV in Mg$^{2+}$-free medium and fast applications of glutamate (100 µM) and glycine (100 µM) were applied near the transfected cell (around 10 s) to evoke currents.
To ensure that subunits formed functional, surface-expressed receptors, as well as to verify that cells expressing the reporter plasmid (luciferase of eGFP) were also transfected with the other plasmids, electrophysiological recordings to assess NMDAR-mediated currents were performed on AtT20 cells 18-72 hours after transfection. Coverslips covered with AtT20 cells were transferred into the recording chamber filled with external solution without Mg$^{2+}$. GFP-positive cells were patch-clamped and voltage-clamped to -60 mV. Glutamate (100 µM) and glycine (100 µM) were applied with an independent line positioned near the patched cell for quick stimulation and washout. Transfected AtT20 cells exhibited an inward current following agonist application, confirming the presence of functional NMDARs (see example trace in Figure 4.2B showing an evoked current in a NR1/NR2B-expressing cell).

2.2 Measurement of NMDAR-mediated toxicity with a luciferase reporter
To compare the NMDAR-mediated excitotoxicity observed in our neuronal culture model to the one reconstituted in a heterologous system, we focussed on the NR2B-NMDAR subtype. Indeed, neurons at DIV 7-11 -the time at which we study the neuronal pro-death signals- predominantly express NR2B-containing NMDARs (Chapter 3 and Martel et al., 2009a). As mentioned earlier, transient expression of NR2B-NMDARs in cell lines (much like NR2A-NMDARs) causes spontaneous toxicity because of the chronic activation of the receptors (Boeckman and Aizenman, 1996; Raymond et al., 1996). Therefore, the competitive NMDAR antagonist D-APV was added to the medium at the end of the transfection period to prevent the spontaneous toxicity. Thus, addition of exogenous NMDA/glutamate in these assays competes with APV in a dose-dependent manner.

Using this principle, we assayed viability in cells transfected with pNR1/pNR2B along with a constitutively active luciferase vector. 16-24 hours post-transfection with NMDAR subunits and luciferase plasmids, cells were stimulated with glutamate and the luciferase activity in the cell lysates was subsequently measured. Only living transfected cells contribute to the chemi-luminescence readings, as AtT20 cells undergoing excitotoxicity lose their membrane integrity and release their content in the medium.
NMDAR-expressing AtT20 cells died in a dose-dependent manner in response to glutamate, as the amount of luciferase activity decreased with increasing agonist concentration (Figure 4.3; n = 10 experiments). Residual luciferase signal at 1 mM Glutamate, normalized to 0%, accounted for 45 ± 7%. Presumably, this residual signal accounted for cells expressing only the reporter plasmid, or else missing one of the NMDAR subunit plasmid -therefore lacking in functional receptors.

The decrease in luciferase signal was due to NMDAR activation since it was completely blocked when open-channel blocker MK-801 (10 µM; n = 4 experiments) was added 30 minutes prior to glutamate. As a further control to assess the need for functional NMDARs, the luciferase assay was done on AtT20 cells transfected only with NR2B and luciferase pDNA (at a 4:1 ratio; n = 4 experiments). Without concomitant NR1 expression NR2B cannot assemble in a functional, surface-express channel/receptor. Indeed, NR2B-only-expressing cells did not show any variation of viability following the agonist application, confirming the toxicity dependence on functional NMDAR activation.
Figure 4.3: NMDAR-dependent cell death is reconstituted in non-neuronal cell line AtT20 expressing NR1 and NR2B subunits. A. Excitotoxicity in AtT20 cells expressing NR2B-containing NMDARs and measured by loss of luciferase signal in lysates. 16-24 hours after transfection, AtT20 cells (in medium containing 50 µM D-APV) were subjected to various concentrations of glutamate for 5-7 hours after which a luciferase assay was performed. As opposed to cells expressing only the NR2B subunit (n = 4) or when MK-801 (10 µM, n = 4) was added during stimulation, NR1/NR2B-expressing AtT20 cells died in a glutamate dose-dependent manner. B. Glutamate-induced cell death followed by GFP signal. Cells transfected with NR1/NR2B/eGFP were visualized 16-24 hours after transfection and treated to 400 µM glutamate overnight, which caused excitotoxic loss of GFP-positive cells (scale bar 20 µM).
To confirm that loss of luciferase signal corresponded to glutamate-induced cell death of NMDAR-expressing AtT20 cells, GFP/NMDAR-expressing cells were monitored visually. 16-24 hours after transfection, GFP-positive AtT20 cells were observed under fluorescence microscopy. Cells were then subjected to 400 µM glutamate overnight. The following day, a loss in the GFP-positive cells was seen, as some –but not all- AtT20 cells succumbed to excitotoxicity (Figure 4.3B). Thus, cells expressing functional NMDARs died in presence of agonist. This confirmed that the decreased measurements of luciferase luminescence were due to the loss of cellular integrity and cell death, as opposed to an indirect effect on the reporter enzyme activity.

Altogether these results show that, much like in HEK 293 and CHO cells, expression of NMDAR subunits in mouse pituitary cells AtT20 reconstitutes NMDAR-dependent cell death in a non-neuronal system. Moreover, this excitotoxicity can be reliably monitored by the loss of luciferase signal.

2.3 Excitotoxicity in AtT20 cells expressing NMDARs involves JNK SAPK

Having developed a reliable method to assess NMDAR-mediated toxicity in transfected AtT20 cells, we then studied the reliance of this phenomenon on two mediators of neuronal excitotoxicity. The p38 and the JNK SAPKs are two key death pathways that contribute to neuronal death following pathological levels of NMDAR activation (Kawasaki et al., 1997; Borsello et al., 2003a; Rivera-Cervantes et al., 2004; Molz et al., 2008; Soriano et al., 2008).

AtT20 cells were transfected with NR1/NR2B and luciferase pDNAs and stimulation with various concentrations of glutamate in presence of SB203580 (p38 SAPK inhibitor, 5 µM) or SP600125 (JNK inhibitor, 1 µM).

Inhibition of the JNK pathway by SP600125 protected the AtT20 cells from glutamate-induced toxicity, as even at 1 mM glutamate the viability signal remained at 84 ± 20% (all 3 concentrations significantly different from no drug controls; n = 3 experiments; Figure 4.4). However, inhibition of the p38 pathway did not alter the viability pattern of glutamate-induced cell death (n = 3 experiments). This suggests that, unlike in neurons, the p38 SAPK pathway does not appear to play an excitotoxic role in
NMDAR-expressing AtT20 cells. On the other hand, the JNK pathway contributes to the agonist-induced toxicity both in neurons and non-neuronal cells.

A possible explanation for the lack of reliance on the p38 pathway in non-neuronal excitotoxicity could simply be that this type of SAPK is absent or non-functional in this particular cell line. Thus, we verified the presence and functionality of p38 using Western blotting of AtT20 cells homogenates. Anisomycin, a protein synthesis inhibitor (1 µM), and hydrogen peroxide (H$_2$O$_2$, 200 and 500 µM) are two cellular stresses activators that are known to lead to p38 phosphorylation –the activated state of p38.

As seen in Figure 4.5, both anisomycin and H$_2$O$_2$ induced phosphorylation of the p38 SAPK, which shows that this stress pathway is indeed present and functional in AtT20 cells. Therefore, the absence of role of p38 in the NMDAR-induced toxicity is not due to a specific defect on this pathway in AtT20 cells, but comes from a context-sensitive difference in the NMDAR downstream events.
Figure 4.4: Excitotoxicity in AtT20 cells expressing NR2B-containing NMDARs is dependent on JNK SAPK but not on p38 SAPK. AtT20 cells were transfected with plasmids encoding for NR1, NR2B and luciferase. 16-24 hours post-transfection, p38 inhibitor SB203590 (5 µM) or JNK inhibitor SP600125 (1 µM) was added for 30 min. Cells were stimulated with glutamate for 5-7 hours then a luciferase assay was done to assess the amount of AtT20 cell viability (n = 3). Inhibition of p38 did not alter the dose-dependent toxicity of glutamate whereas inhibition of JNK abolished NMDAR-mediated non-neuronal cell death. * p < 0.05 compared to vehicle.
Figure 4.5: The p38 SAPK pathway is present and inducible in the AtT20 cell line. AtT20 cells were treated with anisomycin (Aniso; 1 µM) or hydrogen peroxide (H$_2$O$_2$; 50, 200 and 500 µM) for 2 hours. Equal amount of proteins were loaded on an electrophoresis gel then transferred to a PVDC membrane. Activation of p38 was detected with a phospho-specific p38 antibody and p38 immunoreactivity was used as a loading control. Both anisomycin and hydrogen peroxide induced activation of the p38 SAPK, indicating that this molecular pathway is present and can be induced in this particular cell type.
2.4 Estimation of the calcium load shows lower Ca$^{2+}$ requirement for neuronal death

The absence of NMDAR-dependent pro-death p38 signal in non-neuronal cells suggests that toxicity may be “facilitated” in neurons, in which NMDAR activation induces both p38 and JNK SAPK pathways. Therefore, we hypothesized that the effectiveness of Ca$^{2+}$ influx through NMDAR to induce death might be greater in neurons, due to the multitude of death-signals.

To estimate the respective calcium load in both cellular models of excitotoxicity, we measured the amount of $^{45}$Ca$^{2+}$ influx needed to trigger a 50% decrease in viability. $^{45}$Ca$^{2+}$ load following 10 minutes glutamate applications was therefore quantified in both neurons and AtT20 cells. This data was then correlated with cell death at the same agonist concentration, to find the amount of calcium entry at the half-maximal toxic dose of glutamate (Figure 4.6; n = 3 experiments each). This “EC$_{50}$” value of calcium (in CPM) was then divided by the number of neurons per well, or the number of transfected AtT20 cells based on NR1 immunoreactivity. At that point, the estimate represented the amount of calcium (in CPM) entering each cell with 10 minutes of agonist, at a concentration that triggers 50% cell death. However, this amount did not properly represent the relative concentration of intracellular calcium in both cell types, since it did not account for the difference in cell volume between neurons and AtT20 cells. To normalize the Ca$^{2+}$ influx to cell volume, cells were transfected with eGFP. 3D-deconvolved models of the eGFP fluorescence were subsequently made by Dr Colin Rickman and Dr Rory Duncan (Figure 5.7A; n = 6). Average cell volumes of AtT20 cells and of cortical neurons were obtained from these deconvolutions.

For equal volumes, the amount of calcium entry needed to trigger 50% death was four times higher in AtT20 cells expressing NR2B-NMDARs, relative to the amount found for neurons (Figure 4.7B). Thus, Ca$^{2+}$ influx through NMDARs appeared more potent at killing neurons than non-neuronal cells, which reflects the absence of pro-death p38 signalling in AtT20 cells.
Figure 4.6: Determination of the amount of Ca\(^{2+}\) influx needed for half-maximal toxicity in cortical neurons and AtT20 cells. A. Glutamate-induced toxicity in cortical neurons, assessed by counting the percentage of pyknotic nuclei. The concentration of glutamate needed to induce 50% cell death was determined (n = 3). B. Amount of \(^{45}\text{Ca}^{2+}\) intake following 10 min stimulation with glutamate (n = 3). C. Glutamate-induced toxicity in AtT20 cells expressing NR2B-containing NMDARs, as measured by a luciferase assay (n = 7). D. Same as in B; determination of the \(^{45}\text{Ca}^{2+}\) influx EC\(_{50}\) required to kill transfected AtT20 cells (n = 3).
Figure 4.7: Increased NMDAR-mediated Ca\textsuperscript{2+} influx is required to kill NMDAR-expressing AtT20 cells than to kill cortical neurons. A. 3D-deconvolved models of the eGFP-expressing cells made by Dr Colin Rickman and Dr Rory Duncan (scale bar 5 µm). B. The \textsuperscript{45}Ca\textsuperscript{2+} influx required to kill 50% of neurons and NMDAR-expressing AtT20 cells (NR-AtT20) was divided by the number of neurons or transfected AtT20 cells, then divided by the estimated volumes of each type of cell – measured using data shown in A by Dr Colin Rickman and Dr Rory Duncan. The estimated calcium load required to kill 50% of non-neuronal AtT20 cells was more than 4 times higher than what was found for cortical neurons, consistent with the absence of p38 pro-death signalling. * p < 0.05 compared to NR-AtT20 cells.
3. Chapter discussion

To study the pro-death signalling downstream of NMDAR-mediated calcium influx, we sought to recreate this toxicity by expressing NMDAR in a heterologous system. We used AtT20 cells, a mouse pituitary cell line that offer the advantages of a moderate cell cycle time, a 5-10% rate of transfection with Lipofectamine and not expressing any endogenous glutamate receptors. Along with an eGFP or luciferase plasmid, plasmids containing the full-length mouse sequences for NR1-1a, NR2A and NR2B, all under control of a mammalian CMV promoter were transiently transfected in AtT20 cells. NR1 immunoreactivity could be detected 16-24 hours after transfection, as well as glutamate-induced whole-cell currents, confirming the expression of fully functional NMDARs.

The recombinant expression of NMDAR in a mammalian cell line is an approach that has been used previously in HEK 293, COS and CHO cell lines, but not in AtT20 cells. Indeed, in cells that do not express any endogenous NMDAR subunits, the exogenous subunits form a pure NMDAR population. This principle has been extensively used in publications from other groups to investigate the NMDAR biophysical properties, assembly and interaction with MAGUKs (Cik et al., 1993; Anegawa et al., 2000; Rutter and Stephenson, 2000; Lynch and Guttmann, 2002; Rutter et al., 2002; Hawkins et al., 2004).

This simplified model has been used by Boeckman and Aizenman (1996) to demonstrate a correlation between NMDAR calcium permeability and the amount of cytotoxicity induced by the receptors. In CHO cells, they found that expression of receptors composed of NR2C, which has low Ca$^{2+}$ permeability, fails to induce death. Similarly, expression of NR2A/B in conjunction with a mutated NR1 subunit (N616R) with reduced calcium permeability induces lower levels of toxicity in contrast to wild-type NR1 (Boeckman and Aizenman, 1996). A similar approach was used to show the effects of NMDAR subunits interaction with MAGUKs on the receptor surface expression, clustering and channel properties (Rutter et al., 2002; Dong et al., 2004; Cousins et al., 2008b; Sornarajah et al., 2008); or to study the NR2 subunit...
phosphorylation or degradation by calpains (Woodward, 2002; Dong et al., 2004; Zheng et al., 2006; Wu et al., 2007).

On the other hand, the cellular signals involved in recombinant NMDAR-induced toxicity have never been investigated. Thus, we focussed here on the death signals recruited in the NR2B-NMDAR-mediated AtT20 cell toxicity, as a difference in signalling could be informative about the reliance on a neuronal signalling complex.

Indeed, expression of NMDARs in AtT20 cells recapitulated neuronal-like agonist-induced toxicity. Our stimulation approach differed from what others have used; whilst other studies also tried to reduce the chronic NMDAR activity with antagonists between the transfection and the subsequent experimentation (Wu et al., 2007; Sornarajah et al., 2008), our cells were in constant presence of D-APV from the end of transfection. This allowed us to induce agonist-dependent toxicity as glutamate overcame the antagonist in a dose-dependent manner. This was quantified by measuring the luminescence of the luciferase reporter plasmid from the surviving cells. This viability measurement was specific to NMDAR-induced cell death since both the blockade of NMDARs with MK-801 and the absence of functional receptors (NR2B subunit alone) showed no decrease of the luciferase signal with glutamate.

Using the luciferase viability assay, we tested the role of two SAPKs in the non-neuronal excitotoxicity. The p38 and JNK pathways are two key mediators of NMDAR-induced pro-death signalling in neurons (Kawasaki et al., 1997; Borsello et al., 2003a; Rivera-Cervantes et al., 2004; Molz et al., 2008). Inhibiting either of these pathways protects cortical neurons from a moderate agonist-induced insult (see Figure 4.1 and Soriano et al., 2008). Moreover, despite the inefficiency of inhibiting one SAPK alone to protect neurons at high agonist concentrations, disruption of both cascades synergistically decreases excitotoxicity; indicating that these two death signals act independently in neurons.

Interestingly, it did not seem to be the case for excitotoxicity in AtT20 cells. Whereas inhibition of JNK was effective at preventing the decrease of the luciferase signal of viability, inhibition of p38 did not prevent cell death at any glutamate concentration. Thus, unlike in neurons the p38 pathway did not participate in NMDAR
pro-death signalling, even though the p38 cascade appeared present and inducible in the AtT20 cell line. The lack of p38 pro-death signal was reflected in the relative NMDAR-mediated Ca$^{2+}$ load that was required to induce cell death in neurons compared to AtT20 cells: the Ca$^{2+}$ load was estimated to be more than 4 times more potent in neurons. However, it is important to note that this estimation of calcium entry is not quantitative and do not account for local, subcellular concentration changes in free calcium. Indeed, Ca$^{2+}$ is widely used as a second messenger in cells and Ca$^{2+}$ signalling can be highly localized. As an example, nuclear calcium activates CaMKIV and CREB-dependent gene transcription whereas cytoplasmic Ca$^{2+}$ elevation recruits PI3K-Akt (Hardingham, 2006a; Soriano et al., 2008). Thus, our estimate does not account for Ca$^{2+}$ microdomains but agrees with the difference in pro-death signals induced by NMDARs in and out of a neuronal context.

The SAPK and Ca$^{2+}$ load discrepancies also supports the notion of “source specificity” of NMDAR-mediated excitotoxicity (Sattler et al., 1998). The presence of intracellular binding partners (among them PSD-95/nNOS) forming signalling complexes at the C-terminus tail of NMDAR subunits affects the downstream signals following Ca$^{2+}$ influx through the receptor in neurons (Sattler et al., 1999). On the other hand, expression of NMDAR in a different cellular context did not recruit the p38 pathway, which suggests that the induction of this SAPK pathway necessitates NMDAR-bound molecular machinery that is absent from AtT20 cells. This will be further explored in the next chapter.

On the contrary, our observation that the JNK SAPK is playing a role in non-neuronal NMDAR toxicity, much like in neurons, suggests that this particular cascade is not source-specific and is recruited as a general stress-induced death signal. Therefore, a NMDAR-coupled signalling cassette may not be required for JNK activation; as the high intracellular calcium load following receptor activation is sufficient for its induction and subsequent toxicity (Raymond et al., 1996; Anegawa et al., 2000).

In conclusion, our investigation of NMDAR-mediated toxicity in non-neuronal cells AtT20, by comparison to neuronal excitotoxicity, demonstrates that two independent pro-death signals may have different requirement for NMDAR signalling.
complexes. This finding could prove important in the design of an effective anti-
excitotoxic strategy. The results appearing in this Chapter were published in Soriano et al. (2008) and Martel et al. (2009b), included at the end of this thesis.
Chapter 5
The PDZ-ligand pro-death signalling is neuron-specific and can be disrupted without affecting other NMDAR-mediated signals
1. Chapter introduction

The coupling of the NMDAR-mediated calcium influx to different signalling cascades could be a potential avenue for the design of anti-excitotoxic strategies (Ikonomidou and Turski, 2002). Since NR2B-containing NMDAR activity can mediate to neuronal survival or death (Hardingham, 2006b; von Engelhardt et al., 2007; Martel et al., 2009a), as well as playing a central role in synaptic plasticity (Berberich et al., 2005; Le Roux et al., 2007; Martel et al., 2009a), understanding how these receptors are coupled to these signalling routes is crucial for a selective therapeutic design against excitotoxicity.

The C-terminus tail of the NR2B subunit contains about 650 amino acids (Salter and Kalia, 2004). Analysis of the C-terminus structure suggests that its state alternates between folded and unfolded, depending on its interactions with other proteins (Ryan et al., 2008). The NR2B subunit has been shown to interact with the autophosphorylated form of CaMKII (Barria and Malinow, 2005; Park et al., 2008), the p85 subunit of PI3K (Waxman and Lynch, 2005a) and phospholipase C-gamma (Gurd and Bissoon, 1997). Also, the NR2B C-terminus can be phosphorylated by the Src-family of protein-tyrosine kinases (Salter and Kalia, 2004), ubiquitinated by Mind Bomb-2 (Jurd et al., 2008) and cleaved by calpains (Wu et al., 2007). The role of these modifications and interactions is not completely understood, but implies that the cytoplasmic C-terminus of the NR2 subunit is an important regulatory domain for NMDAR function, assembly, localization and signalling properties. This is further supported by the fact that transgenic animals lacking the C-terminus portion of a NR2 subunit exhibit the same phenotype as if they were deficient in the entire subunit –which is lethal in the case of NR2B (Sprengel et al., 1998).

The final four amino acids (ESDV) at the very end of the NR2 C-terminus sequence form a PDZ-binding motif, a type of protein-protein interaction domain (Kim and Sheng, 2004). The PSD-95-like MAGUK family of proteins is enriched in the postsynaptic density of glutamatergic synapses and can interact with glutamate receptors on their PDZ domains (Gardoni, 2008). They are thought to play a role in receptor trafficking and the stabilization of the postsynaptic density because of their capacity to associate with the cytoskeleton (Kim and Sheng, 2004; Elias and Nicoll, 2007; Gardoni,
Moreover, since they bind to several proteins in addition to associate with NMDARs, PSD-95-MAGUKs can form signalling complexes at the mouth of the NMDAR channel (Kim and Sheng, 2004).

PSD-95 has been found to form such a signalling complex and to trigger pro-death signals following NMDAR overactivation (Sattler et al., 1999; Aarts et al., 2002; Cui et al., 2007). PSD-95 interacts strongly with the NR2B subunit and with nNOS, a Ca\(^{2+}\)-activated NO-producing enzyme (Christopherson et al., 1999; Cui et al., 2007; Cousins et al., 2008b). Thus, it can link NR2B-NMDAR-mediated Ca\(^{2+}\) influx with NO production, which can trigger excitotoxic cascades in an ischemic context (Cui et al., 2007).

In order to disrupt this signalling complex, Aarts et al. (2002) constructed an oligopeptide comprised of the last nine amino acids of the NR2B sequence (KLSSIESDV), fused with a cell-membrane transduction domain of the human immunodeficiency virus-type 1 Tat protein to render the construct cell-permeable. Addition of this peptide, named TAT-NR2B9c, to cell culture medium disrupts the interaction of NR2B-containing NMDARs and PSD-95 in neurons, as well as NMDAR-induced NO production (Sattler et al., 1999; Aarts et al., 2002). Strikingly, abolition of this signalling cassette results in neuroprotection from NMDAR-dependent toxicity both in vitro and in vivo (Sattler et al., 1999; Aarts et al., 2002; Cui et al., 2007; Sun et al., 2008). A similar conclusion implicating the NR2B-PSD-95-nNOS signalling cascade in CGCs has been published by Cao et al. (2005), using a peptide disrupting the binding between PSD-95 and nNOS.

Thus, the PDZ-binding domain of NR2B-containing NMDARs has a role in the induction of excitotoxic signals via PSD-95. However, it is unclear which death pathways are recruited downstream of the PDZ ligand in neurons. Moreover, the possible side effects of disrupting the PDZ interactions from the NR2B subunit have not been fully studied. The TAT-NR2B9c peptide does not have any obvious effect on NMDAR surface expression and does not change NMDAR-mediated currents and calcium influx (Sattler et al., 1999; Aarts et al., 2002). However, considering the diversity of proteins that can be associated with NR2B via the PDZ ligand, disrupting
the NR2B/PSD-95 association can have undesired consequences on neuronal physiology and needs investigation.

The ability of the NR2B-PDZ ligand to promote excitotoxicity involves the neuronal proteins PSD-95 and nNOS (Cui et al., 2007). This NMDAR-associated signalling complex supports the “source-specificity” hypothesis, which postulates that pro-death signals are specifically recruited by the NMDAR-mediated Ca\(^{2+}\) influx as opposed to other sources. We showed in Chapter 4 that the acquired excitotoxicity in AtT20 cells expressing NMDARs did not recruit the same SAPK pathways as in neurons. This difference may result from the absence of the PDZ-bound pro-death signalling in non-neuronal cells; this hypothesis will be verified in this Chapter.

Thus, in the study presented here we sought to investigate if the neuroprotective disruption of the association between NR2B and PSD-95 causes unwanted alterations in neuronal function. Specifically, we aimed to verify if the disruption of the PDZ ligand by TAT-NR2B9c affects NMDAR-dependent plasticity, or has any obvious physiological effect on neuronal excitability and synaptic transmission. In addition, to further characterize the role of the NR2B PDZ ligand in neuronal excitotoxicity, we revisited the toxicity in non-neuronal cells to assess if the PDZ binding domain also has a pro-death-inducing capacity in this context; as a difference could explain the discrepancy between pro-death pathways recruited in AtT20 cells and neurons during excitotoxicity.

In conjunction with results obtained by Dr Francesc Soriano (Soriano et al., 2008), the following experimental findings are presented in this Chapter:

- The disruption of the NR2B/PSD-95 association did not affect neuronal survival, excitability or the ability of NMDARs to signal to potentiation at the concentration and incubation time used for neuroprotection;

- In AtT20 cells expressing NMDARs, TAT-NR2B9c did not alter excitotoxicity. Also, toxicity remained unchanged with truncated NR2B subunits –missing the PDZ binding domain, confirming that excitotoxicity did not rely on this domain in non-neuronal cells;
In cortical neurons disruption of the PDZ ligand did not affect the JNK death signal but prevented the p38 death signal recruitment (data from Dr Francesc Soriano), which correlates with the absence of induction of this SAPK in AtT20 cells.

In conclusion, we found that the NR2B/PSD-95 interaction signalling to p38 is a neuronal pro-death signal that can disrupted by TAT-NR2B9c, a cell-permeable peptide mimicking the last 9 amino acids of the NR2B C-terminus. Whilst neuroprotective, the disruption of the PDZ-ligand did not affect pro-survival signals, plasticity signals, neuronal transmission or excitability. Thus, TAT-NR2B9c appeared to selectively disrupt pro-death signalling while sparing normal NMDAR function. The results presented in this Chapter have been published in Soriano et al. (2008) and in Martel et al. (2009b).
2. Results

2.1 NR2B-NMDARs mediate death of cortical neurons
We have previously shown that NR2B-containing NMDARs were able to signal to death in hippocampal cells (Chapter 3 and Martel et al., 2009a). We first confirmed that this observation was still valid for cortical neurons, and concurrently assessed the relative sensitivity of the luciferase viability assay (on transfected neurons) compared to the usual quantification of pyknotic nuclei.

The 100 µM glutamate stimulation triggered an almost complete loss of luciferase signal and increase in nuclear pyknosis (Figure 5.1; n = 3). In both assays, inhibition of NR2B-NMDARs by ifenprodil protected neurons from excitotoxicity. Thus, as in hippocampal neurons, NR2B-containing NMDARs were the principal mediators of neuronal death. Moreover, the luciferase luminescence quantification as a viability indicator followed reliably the values from nuclei counting, giving an additional validation for the technique. It also ruled out the possibility that the transfection step modifies the susceptibility of neurons to death.
Figure 5.1: NR2B-containing NMDARs mediate excitotoxicity in cortical neurons (DIV 8-10). For the luciferase assay neurons were transfected with a plasmid encoding for luciferase. Twenty-four hours later, transfected and untransfected neurons were treated as indicated with ifenprodil (3 µM) for 30 min then glutamate (100 µM) was applied to the wells for 1 hour. Stimulation was stopped by replacing the well content to fresh, drug free-medium and death was assayed 16-24 hours after. Both cell death counted by nuclear pyknosis and by luciferase measurement showed that cell death was prevented by inhibiting NR2B-containing NMDAR
2.2 TAT-NR2B9c is neuroprotective against agonist-induced neuronal death

The efficacy of the TAT-NR2B9c peptide in protecting neurons against NMDAR-mediated toxicity has been proven previously (Aarts et al., 2002). To ensure that the TAT-NR2B9c peptide was neuroprotective in our hands, we pre-incubated cortical cells DIV 8-9 with either TAT-NR2B9c or a control peptide comprised of the Tat sequence alone (both 2 µM) and stimulated with NMDA.

In agreement with previous studies (Aarts et al., 2002; Cui et al., 2007), TAT-NR2B9c protected neurons against NMDA-induced toxicity (Figure 5.2), whereas the Tat sequence peptide alone did not affect cell death. At 40 µM NMDA, a dose that triggered 63 ± 4% cell death in control cells, the TAT-NR2B9c peptide reduced the amount of pyknotic nuclei to 12 ± 2% (n = 3 experiments). Thus, disruption of the NR2B/PSD-95 interaction, and the consequent uncoupling of NMDAR-to-NO-production, effectively interfered with the pro-death signalling downstream of NR2B-NMDAR activity. Moreover, the displacement of PSD-95 from the PDZ binding domain of the NR2B subunit did not seem to affect the pro-survival NMDAR signals since in the absence of agonist, the amount of basal death remained unchanged with the TAT-NR2B9c peptide. This result suggests that the NR2B PDZ-ligand mediates pro-death, but not pro-survival signalling within cortical neurons.
Figure 5.2: Disruption of the PDZ domain interactions is neuroprotective against NMDAR-mediated toxicity. Cortical neurons were pre-treated for 1 hour with TAT-NR2B9c, a cell-permeable peptide mimicking the PDZ binding domain of the NR2B subunit (1 µM). Cell death was then induced by 1 hour NMDA stimulation, after which medium was changed. Neuronal death was assayed 16-24 hours after by counting the number of pyknotic nuclei as a percentage of total. Disrupting the NR2B-PDZ interactions protected neurons against agonist-induced cell death (n = 3). * p < 0.05 compared to control.
2.3 Disruption of the PDZ-signalling cassette does not affect excitability or potentiation signalling

The neuroprotective effects of disrupting PSD-95 from binding to NR2B-NMDARs can induce undesirable side-effects that would undermine the usefulness of this approach. Also, the functionality of cells after surviving an excitotoxic insult in presence of TAT-NR2B9c remained unclear. Thus, we performed electrophysiological recordings to investigate the firing properties of the neuronal network 24 hours after stimulation with NMDA in presence of 2 µM TAT-NR2B9c.

To do so, neurons (treated with 20 or 40 µM NMDA) were transiently depolarized to evoke action potentials. Similar to control cells, (agonist + TAT-NR2B9c)-treated cells (Figure 5.3A; n = 5 cells for each condition) were able to fire characteristically-shaped action-potentials when depolarized; indicating that these neurons were functional.

To assess the network functionality of the TAT-NR2B9c-protected neurons after a toxic dose of NMDA, sEPSCs were recorded from neurons stimulated with 40 µM NMDA after being incubated with TAT-NR2B9c or with NMDAR antagonist APV.

As expected, cells submitted to 40 µM NMDA appeared highly damaged, and recordings from surviving cells showed very little, if any sEPSCs (0.023 ± 0.009 Hz compared to control at 0.12 ± 3 Hz; Figure 5.3B-C; n = 7 cells). This low frequency of spontaneous activity reflects the high amount of cell death in the network, which results in a much lower connectivity of the cell to other neurons. On the other hand, cultures pre-treated with NMDAR antagonist APV or the peptide TAT-NR2B9c both showed levels of activity comparable to the average control frequency, which is consistent with the highly reduced toxicity. Additionally, the normal frequency of sEPSCs in the peptide-treated-surviving cells shows that the TAT-NR2B9c peptide not only prevents cell death, but preserves the overall connectivity and excitability of the neuronal network.

It is important to note that, without any agonist stimulation, incubation with TAT-NR2B9c did not alter the frequency of sEPSCs, indicating that it has no effect by itself on synaptic transmission and excitability (Figure 5.4; n = 5-8 cells). We also
verified if incubation with TAT-NR2B9c was affecting the basal mEPSC frequency, which would indicate an alteration of basal synaptic neurotransmitter release or postsynaptic properties. Cells incubated with 2 µM TAT-NR2B9c for 1 hour displayed a frequency of mEPSCs similar to untreated cells (1.2 ± 0.3 Hz; Figure 5.4; n = 4 cells). Together with the observation that the peptide did not alter the sEPSC frequency in the network, the unchanged basal mEPSC frequency confirms that disruption of the PDZ-signalling cassette by TAT-NR2B9c did not modify the properties of the synaptic transmission.
Figure 5.3: Cortical neurons protected from excitotoxicity by the TAT-NR2B9c peptide show normal excitability and network activity. Cells on coverslips were treated with 2 µM TAT-NR2B9c for 1 hour then NMDA as indicated for another hour. Medium was changed to fresh and recordings were made 16-24 hours later. A. Surviving neurons fire action potential as normal. In current-clamp, cells were depolarized beyond -40 mV (by 300 ms pulses at 1 Hz) to elicit action potentials (n = 5). Example traces of the firing responses triggered in surviving cells. B. Neurons protected from NMDA toxicity by TAT-NR2B9c, or D-APV (50 µM) experienced normal levels of synaptic activity, as measured by the sEPSC frequency. Spontaneous currents were recorded at -70 mV for 3 min; only events bigger than 200 pA and spaced by more than 1s were considered for analysis (n = 7). C. Example traces of the sEPSC recordings used in B. * p < 0.05 compared to control.
Figure 5.4: Incubation with the TAT-NR2B9c peptide does not alter basal mEPSC frequency or sEPSC frequency. Cortical neurons were treated with 2 µM TAT-NR2B9c for at least 1 hour, after which electrophysiological recordings were made. A. mEPSCs were recorded for 5-10 minutes (at least 300 events) and their frequency analysed. Disruption of the PDZ domain interactions did not alter the mEPSC frequency (n = 8 and 5, respectively). B. Spontaneous activity at -70 mV was monitored for 3-6 minutes and frequency of events larger than 150 pA and spaced more than 1s apart was calculated. TAT-NR2B9c-treated cells exhibited similar amount of endogenous activity than untreated ones (n =8 and 5).
We then sought to investigate if displacement of the PDZ-signalling complex by TAT-NR2B9c was disrupting the ability of NR2B-NMDARs to mediate plasticity signalling. We previously shown that in hippocampal neurons at DIV 8-11, a period of bicuculline-induced bursting was triggering a potentiation of synaptic transmission dependent on NR2B-containing NMDARs (Abegg et al., 2004; Arnold et al., 2005; Martel et al., 2009a and Chapter 3).

This protocol induced a strong increase in mEPSC frequency in control cells (2.9 ± 0.4 Hz compared to untreated at 1.3 ± 0.2 Hz; Figure 5.5; n = 9 cells each). As reported in Chapter 3, this potentiation required NMDAR activation, since antagonism with MK-801 completely blocked its induction (1.4 ± 0.4 Hz; n = 10 cells). However, disruption of the PSD-95 association to the NR2B subunit by TAT-NR2B9c did not influence the plasticity signals emanating from the NMDARs, as bicuculline induced an increase of mEPSC frequency (2.9 ± 0.5 Hz; n = 8 cells).

Altogether, these results show that disruption of the NR2B/PSD-95/nNOS complex by TAT-NR2B9c effectively protects neurons against excitotoxicity without affecting other types of signals or neuronal function. The cells surviving a toxic dose of NMDA with the help of the TAT-NR2B9c peptide were able to fire action-potentials and exhibited normal connectivity and excitability. TAT-NR2B9c did not affect the basal synaptic transmission, as measured with sEPSC and mEPSC frequency, and the survival of neurons. Also, the displacement of PSD-95 from the PDZ ligand of NR2B leaved the ability of the receptor to mediate plasticity signals. Thus, these results confirm a role of the PDZ ligand of NR2B in pro-death signalling, but not in survival or potentiation signalling.
Figure 5.5: Disruption of the NR2B/PSD-95 interaction by TAT-NR2B9c does not block synaptic plasticity signalling unlike conventional NMDAR antagonists. Hippocampal neurons were treated with MK-801 (10 µM, for 30 minutes) or TAT-NR2B9c (2 µM, for 1 hour) and in the presence of these compounds were subjected to bicuculline (50 µM) application. After 15 minutes of bicuculline-induced bursting, coverslips were put in fresh, drug-free medium and allowed to settle for 30 minutes. mEPSCs were recorded at -70 mV for 5-10 minutes (minimum of 300 events) and their frequency calculated (n = 8-10 for each condition). A. Example traces of mEPSC recordings with the different compounds. B. Treatment with TAT-NR2B9c did not alter the induction of this synaptic potentiation model, unlike NMDAR open-channel blocker MK-801. * p < 0.05.
2.4 Specific signalling to p38 by the PDZ-signalling complex

This section covers complementary results obtained by co-author Dr Francesc Soriano, published in Soriano et al. (Soriano et al., 2008).

Several pro-death pathways have been shown to participate in NMDAR-mediated excitotoxicity. We and others have shown that the SAPKs p38 and JNK are key mediators in agonist-induced neuronal death (see Figure 4.1 in Chapter 4; Kawasaki et al., 1997; Borsello et al., 2003a; Rivera-Cervantes et al., 2004; Molz et al., 2008; Soriano et al., 2008). These cascades are recruited independently following NMDAR activation since their inhibition appears to synergistically protect cells when submitted to a strong insult. Indeed, disruption of the NR2B/PDS-95 interaction by TAT-NR2B9c prevents p38 activation following NMDA. Treatment with TAT-NR2B9c also impairs the activation/phosphorylation of an upstream activator of p38 in neurons, ASK1 (Takeda et al., 2004). On the other hand, displacement of the PDZ pro-death signalling cassette does not inhibit the JNK cascade activation by NMDA, as assessed by the phosphorylation of serine 73 on c-Jun. These results indicate that the two SAPK death pathways have a different requirement on the PDZ ligand of NR2B. Thus, the PSD-95/nNOS signalling complex attached to the PDZ domain of NR2B-NMDARs is able to signal to p38, but not to JNK (Soriano et al., 2008).

Furthermore, these two excitotoxic pathways rely on two spatially distinct pools of Ca\(^{2+}\). Concomitant with its requirement on the NMDAR-signalling complex, submembranous calcium in the immediate vicinity of calcium-permeable receptors is sufficient to trigger the p38 phosphorylation (Soriano et al., 2008). On the contrary, the JNK pro-death cascade is initiated by calcium uptake in mitochondria. Indeed, NMDA application causes an increase in the vesicular Ca\(^{2+}\) load which is prevented when the mitochondrial membrane potential, the driving force for calcium uptake in the organelle, is depolarized by carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; and by adding oligomycin to prevent cytoplasmic ATP depletion). Consequently, disrupting this mitochondrial calcium uptake interferes with the activation of the JNK pathway, but not of p38 or ERK1/2 (Soriano et al., 2008). Moreover, recruitment of JNK following mitochondrial calcium uptake is stopped when in presence of a mitochondrially targeted
antioxidant, showing that reactive oxygen species (ROS) production triggers the induction of this pro-death signal in the mitochondrion (Soriano et al., 2008).

Thus, the two SAPK death signals have very different locus of induction downstream of NMDAR channel opening in neurons. The SAPK JNK, which also mediated excitotoxicity in non-neuronal cells expressing NMDARs (see Figure 4.4), does not rely on the NR2B-bound signalling cassette. Its induction follows Ca\(^{2+}\) uptake and ROS production in the mitochondria. However the SAPK p38, which did not participate in the acquired NMDAR-induced toxicity in AtT20 cells, requires the NR2B PDZ-bound signalling complex.

2.5 mEPSC potentiation signalling is independent of the NR2B PDZ-ligand but not of the p38 cascade

The displacement of PSD-95 from the C-terminus of the NR2B subunit by the TAT-NR2B9c peptide specifically inhibits the p38 pro-death signal, without affecting NMDARs-mediated potentiation of mEPSCs. To support the notion that the PDZ binding domain did not have any role in our plasticity model, we tested the mEPSC potentiation dependence on the SAPK pathways. Coverslips were treated with SB203580 (5 \(\mu\)M) or SP600125 (1 \(\mu\)M), inhibitors of p38 SAPK and JNK respectively, before the bicuculline stimulation.

JNK inhibition did not affect significantly the mEPSC frequency potentiation (5 ± 1 Hz, Figure 5.6, n = 7 cells), but showed a tendency to increase mEPSC frequency potentiation. However, we did not pursue the investigation of JNK further, as other results indicate that the PDZ-ligand is not involved in NMDAR-dependent signalling to JNK SAPKs (Soriano et al., 2008).

On the other hand, inhibition of p38 by SB203580 completely blocked the induction of the potentiation (1.5 ± 0.3 Hz; n = 5 cells). This was not due to the drug triggering a decrease in mEPSC frequency, as the drug without bicuculline stimulation did not alter the basal mEPSC frequency in neurons (1.5 ± 0.4 Hz; n = 6 cells). This result suggests that this model of NMDAR-dependent synaptic potentiation required the presence of a functional p38 pathway. On the other hand, the uncoupling of the p38
cascade from the NMDAR-signalling complex by TAT-NR2B9c did not impair the plasticity signals in this model (Figure 5.5). Thus, selective disruption of the signalling cascades to p38 offer a clear advantage over the global inhibition of the p38 pathway, which not only disrupt death signals but also potentiation.
Figure 5.6: mEPSC frequency potentiation is impaired when p38 is globally inhibited. Hippocampal neurons were treated with JNK inhibitor SP600125 (1 µM) or p38 inhibitor SB203580 (5 µM) for 30 min before cells were stimulated –where indicated– with bicuculline (50 µM) for 15 min. 30 min after stimulation, mEPSCs were recorded for 5-10 min (minimum of 300 events) and their frequency determined. The p38 inhibition disrupted the synaptic potentiation model; this was not due to an effect of SB203580 on basal mEPSC frequency since it did not alter mEPSC frequency in non-stimulated neurons (n = 5-7 cells each). * p < 0.05.
2.6 NR2B-PDZ binding domain does not signal to death in non-neuronal cells

Whilst p38 and JNK SAPK pathways are both key mediators of excitotoxicity in cortical neurons, in non-neuronal cells AtT20 expressing NR2B-containing NMDARs the toxicity only involved JNK. Indeed, p38 inhibition by SB203530 did not protect the mouse pituitary cell line from the acquired agonist-induced toxicity (Figure 4.3 from Chapter 4). In neurons, p38 phosphorylation/activation occurs via the signalling cassette formed of PSD-95/nNOS on the PDZ binding domain of the NR2B C-terminus. Incubation with the TAT-NR2B9c peptide disrupts interactions at the PDZ domain and protects neurons against excitotoxicity. The apparent lack p38 signalling in AtT20 cells implies that, contrary to neurons, non-neuronal cells would not be protected by the neuroprotective TAT-NR2B9c peptide. To verify this, AtT20 cells were transfected with NR1/NR2B and luciferase vectors, then subjected to glutamate in presence or absence of TAT-NR2B9c and a luciferase viability assay was performed.

AtT20 cells transiently expressing NR2B-NMDARs died in an agonist concentration-dependent manner, as previously shown in Chapter 4. However, glutamate-induced toxicity did not vary in cells pre-incubated with TAT-NR2B9c (Figure 5.7; n = 3 experiments). Thus, unlike neurons submitted to a similar toxic insult, non-neuronal cells were not protected by disruption of the PDZ ligand. The lack of cytoprotection with TAT-NR2B9c suggests that the PDZ binding domain of the NR2B subunit is not able to signal to death in AtT20 cells.
Figure 5.7: In NMDAR-expressing AtT20 cells, disruption of PDZ ligand does not protect cells against agonist-induced toxicity. AtT20 cells were transfected with plasmids encoding for NR1/NR2B and luciferase then put in medium containing D-APV (50 µM). 16-24 hours later cells were treated with TAT-NR2B9c (2 µM) for 1 hour prior to stimulation and subjected to various glutamate doses for 5-7 hours. Luciferase activity from the cell lysates was then measured. Unlike in neurons, the TAT-NR2B9c peptide did not diminish the amount of glutamate-induced cell death (n = 3).
To further support this observation, we used a truncated variant of the NR2B subunit. The PDZ binding motif consists of the last 4 amino acids (ESDV) of the C-terminus tail of the protein. To study the NR2 PDZ motif and characterize its interaction with PSD-95, Rutter et al. (2000) created a truncated version of the subunits. This truncation was achieved by mutating the last glutamate (E) codon (GAG), of the ESDV sequence, to a Stop codon (TAG). This point mutation effectively removes the last 4 amino acids from the C-terminus tail, which form the PDZ consensus motif E(T/S)XV.

Thus, we transfected AtT20 cells with this truncated NR2B subunit (NR2B<sub>trunc</sub>; gift from the Prof Stephenson), together with the NR1 and luciferase reporter plasmids. We first needed to verify that the truncated subunit was expressing, assembling and functioning in an equivalent manner to the wild-type version. Using radioactive $^{45}$Ca$^{2+}$ as a quantifiable input, cells were stimulated by glutamate in medium enriched with $^{45}$Ca$^{2+}$.

The radioactive calcium load measured following NMDAR activation in transfected AtT20 cells increased with glutamate in a dose-dependent manner—and in an inversely proportional relation to viability (Figure 5.8; n = 3 experiments). Importantly, both wild-type and truncated NR2B-NMDAR showed a similar calcium influx. Therefore, NR2B<sub>trunc</sub>-NMDAR appeared to express and function normally, and the truncation of the PDZ ligand did not modify the calcium load mediated by the receptors.
Figure 5.8: Truncation of the PDZ domain of the NR2B subunit does not alter NMDAR-mediated Ca\(^{2+}\) influx or toxicity in non-neuronal cells AtT20. Cells were transfected with plasmids encoding for NR1, luciferase and wild-type (WT) or truncated NR2B (lacking the last 4 amino acids). Experiments were done 16-24 hours later. A. Cells were stimulated with glutamate as indicated for 5-7 hours then luciferase assay was done on cell lysates. Truncation of the NR2B subunit did not modify the agonist-induced toxicity profile (n = 3). B. AtT20 cells were stimulated with glutamate for 10 min in medium enriched with \(^{45}\)Ca\(^{2+}\). Intracellular \(^{45}\)Ca\(^{2+}\) from the lysates was then quantified (in count per minute; cpm). NMDARs composed of truncated NR2B allowed the same amount of Ca\(^{2+}\) influx into AtT20 cells than wild-type (n = 3).
Having demonstrated that the NR2B subunit lacking a PDZ motif forms NMDARs functionally undistinguishable from their wild-type equivalents, we investigated the toxicity of NR2B<sub>trunc</sub>-NMDARs using the luciferase viability assay.

The agonist-induced toxicity gave similar profiles for NR2B<sub>trunc</sub>-NMDAR- and NR2B-NMDAR-expressing AtT20 cells (Figure 5.8A; n = 3 experiments). Thus, the absence of NR2B PDZ binding domain did not influence toxicity in the heterologous system, confirming our result with TAT-NR2B<sub>9c</sub> and demonstrating that the PDZ ligand does not signal to death in this cellular model.

In neurons, PSD-95 and nNOS associate to the PDZ binding domain of NR2B to form the pro-death signalling complex to p38, and disruption of this complex by TAT-NR2B<sub>9c</sub> is neuroprotective. In non-neuronal AtT20 cells, the absence of a signalling complex coupling NMDARs to p38 activation is implicitly due to their lack of neuronal proteins PSD-95 and nNOS. Thus, we probed for the presence of these proteins in AtT20 cells using Western blotting of cell homogenates.

As expected, the AtT20 cell homogenates did not exhibit significant immunoreactivity to either PSD-95 or nNOS (Figure 5.9); whereas neuronal protein extracts showed high levels of both molecules. This confirmed that the inability of NR2B-NMDARs to signal to the pro-death p38 pathway was due to the absence of PSD-95 and nNOS in non neuronal cells, unlike in neurons where they form a signalling cassette on the PDZ ligand of NR2B.
Figure 5.9: Significant protein levels of PSD-95 and neuronal nitric oxide synthase (nNOS) are absent from AtT20 cell homogenates. Protein levels in neuronal culture (Neu) and AtT20 cell (AtT) homogenates were measured with a BCA protein assay and equal quantities were loaded on an electrophoresis gel for western blotting. Membranes were probed for PSD-95 and nNOS, stripped of antibodies and re-probed for calmodulin as a loading control. Whereas cortical neurons extract showed evident immunoreactivity to both proteins, AtT20 cell homogenates exhibited extremely low levels of PSD-95 and nNOS.
3. Chapter discussion

NR2B-NMDARs have the ability to signal to synaptic potentiation and depression, but also to survival and death of neurons, depending on the stimulus (Chapter 3 and Martel et al., 2009a). In the quest to find an effective anti-excitotoxic strategy, one must consider that the intrinsic physiological roles of the NMDAR in neuronal survival and in learning and memory have to be spared (Ikonomidou and Turski, 2002). Thus, it is of great importance to elucidate the molecular pathways signalling to these different outcomes. A better understanding of the events downstream of NMDAR activation could allow a selective disruption of the pro-death cascades whilst leaving other, beneficial routes unscathed.

The NR2B subunit has an extensive cytoplasmic C-terminus tail that interacts with other proteins which then assemble into a signalling complex. One particular domain, the ESDV sequence at the very end of the C-terminus, is a consensus PDZ binding motif and has been found to associate with PSD-95. PSD-95 also binds to nNOS (Christopherson et al., 1999) and effectively couples NMDAR-mediated calcium entry to NO production and p38 phosphorylation (Soriano et al., 2008). Aarts et al. (2002) created an oligopeptide composed of the last 9 amino acids of the NR2B subunit fused with a cell-permeant Tat-sequence, TAT-NR2B9c. This peptide specifically dissociates PSD-95 and NR2B -despite the ubiquity of proteins with PDZ motifs, and disrupts the NMDAR-to-NO-production signalling cassette (Cui et al., 2007). Incubation of neurons with the TAT-NR2B9c peptide protects neurons from excitotoxic conditions both in vitro and in vivo, even when applied 1 hour after the insult (Aarts et al., 2002).

We showed in Soriano et al. (2008) that disruption of PSD-95 binding to the PDZ domain of NR2B-NMDARs is neuroprotective by uncoupling NMDAR activation from p38 induction in cortical neurons. Another key mediator of neuronal excitotoxicity, the JNK SAPK, appears unaffected by this disruption, indicating its independence from the NR2B PDZ ligand. Instead, the JNK cascade requires Ca\(^{2+}\) uptake to the mitochondria and production of ROS. The lack of “source-specificity” of the JNK pathway agrees with our previous observations concerning acquired excitotoxicity in a non-neuronal context. In AtT20 cells expressing NR2B-containing NMDARs, the sole reliance of
NMDAR-mediated toxicity was on JNK pathway, since the recombinant receptors do not interact with their normal neuronal partners. Indeed, PSD-95 and nNOS were not present in detectable quantities in AtT20 cells. Consequentially, NMDAR-mediated toxicity in non-neuronal cells was not rescued from ablation of the PDZ motif from the NR2B subunit, nor by the TAT-NR2B9c peptide. Therefore, unlike in cortical neurons, the PDZ motif did not exhibit a pro-death, p38-inducing pathway in this cellular model.

As shown in Chapter 4, this was reflected in the relative NMDAR-mediated Ca\(^{2+}\) load that was required to induce cell death in both models. However, additional results in Soriano et al. (2008) highlight the importance of considering micro-domains of calcium, as JNK and p38 have different spatial requirements for Ca\(^{2+}\). The PDZ-bound signalling cassette only requires a submembranous calcium elevation: chelating cytoplasmic Ca\(^{2+}\) with EGTA-AM abolishes the activation of Akt but spares the p38 pathway (Soriano et al., 2008). Calcium in the immediate vicinity of NMDARs triggers NO production by nNOS, which in turn recruits the p38 pro-death cascade. Adding to neurons a NO donor (SNAP) or NOS inhibitors (L-NAME and 7-NI) respectively activates or abolishes p38 signalling, confirming that p38 is downstream of NMDAR-mediated NO production in cortical neurons (Soriano et al., 2008). This is also true in cerebellar granule cells, as Cao et al. (2005) demonstrated using a peptide disrupting the PSD-95 and nNOS interaction. In their study, uncoupling of PSD-95 and nNOS reduces glutamate-induced p38 activation and cell death, much like the TAT-NR2B9c peptide used here and by Aarts et al. (2002) in cortical neurons.

The way NO regulates p38 is not clear, as well as the significance of this relation for neuronal physiology. NO is a second messenger that modulates synaptic transmission (Rameau et al., 2007). However deregulated nNOS activation, as when NMDARs are overactivated, triggers excitotoxicity likely through the excessive production of ROS (Keynes and Garthwaite, 2004). Similarly, NMDAR-mediated p38 activation occurs in non-toxic conditions: low glutamate concentrations and bicuculline-induced bursting both trigger p38 phosphorylation at levels that appear similar to that observed following a toxic stimulus (Waxman and Lynch, 2005a; Soriano et al., 2008). Indeed, a functional p38 pathway was required for the induction of the bicuculline-
induced mEPSC potentiation. Thus, spatially distinct p38 pathways or isoforms may play important physiological roles in neurons and general, unselective inhibition could compromise such roles.

However, unlike with general p38 SAPK inhibition, the selective uncoupling of p38 from the NR2B PDZ ligand by TAT-NR2B9c did not abolish our NMDAR-dependent model of potentiation or affect the basal survival of neurons. The absence of alteration of these signals shows the advantages of targeting a specific signalling route such as the PDZ pro-death cassette, over global chemical inhibition of p38 which may cause undesirable side effects on physiological neuronal signalling for survival or plasticity (Waxman and Lynch, 2005a).

Disruption of the PDZ ligand by the TAT-NR2B9c peptide is also protective in vivo against middle cerebral artery occlusion (MCAO) and three pial vessel occlusion (3PVO), two models inducing ischemic brain damage in rats (Aarts et al., 2002; Soriano et al., 2008). In both cases, treatment with the peptide reduces the infarct size substantially. Concurrently, TAT-NR2B9c is neuroprotective in a p38-dependent manner since the damage caused by 3PVO is reduced similarly with the p38 inhibitor SB202190. Others have targeted with similar success the JNK pathway, using a peptide disrupting the access of JNK to its targets (D-JNKI1; Borsello et al., 2003b).

In vitro, when a high concentration of NMDA is applied (100 µM), inhibition of both p38 and JNK SAPKs is necessary for neuroprotection (see Figure 4.1 in Chapter 4 and Soriano et al., 2008). This is also true in vivo: with a stronger ischemic insult, created by MCAO with 90 min closure of the ipsilateral common carotid artery (MCAO+), TAT-NR2B9c or D-JNKI1 alone do not reduce infarct size significantly but do so when combined (Soriano et al., 2008). This multi-faceted approach is a promising anti-excitotoxic strategy as it shows the effectiveness of disrupting two separate pro-death signals, one depending on the NR2B PDZ ligand and the other not.

Importantly, disruption of the PSD-95 association with NR2B-NMDARs was protective without modifying neuronal excitability or firing properties. Presence of the peptide for 1 hour did not alter the network activity and synaptic transmission, measured by sEPSC and mEPSC frequency. Neurons surviving a toxic concentration of NMDA
still exhibited normal firing and sEPSC frequency, an indication that glutamatergic neurons still formed a functional and excitable network. Displacement of PDS-95 was also not detrimental to our model of synaptic potentiation. Investigations of any potential side-effects on other pro-survival cascades do not show any defects with the TAT-NR2B9c peptide (Soriano et al., 2008): disruption of the PDZ ligand in neurons does not alter bicuculline-induced activation of Akt and CRE-dependent gene expression, two key synaptic NMDARs pro-survival signals (Soriano et al., 2008).

PSD-95 is thought to be important for the surface delivery, stabilization, synaptic targeting and clustering of the molecules it interacts with (Kim and Sheng, 2004). Thus, one can expect that disrupting the interaction of PSD-95 with NR2B would have strong repercussions on the synaptic structure. It has been shown that modulating the amount of PSD-95 expression in neurons has a direct effect on the amount of surface-expressed AMPARs, likely via TARPs like stargazin (Elias and Nicoll, 2007). Knockout mice lacking PSD-95 show impairments in their AMPAR-mediated synaptic transmission and in plasticity; more precisely a deficit in LTD and a facilitated LTP (Carlisle et al., 2008). Defects in AMPAR-mediated transmission were not observed when we disrupted PSD-95 binding to NMDARs, suggesting that the TAT-NR2B9c peptide was not affecting the role of PSD-95 in AMPAR trafficking.

The role of PSD-95 in NMDAR surface expression, clustering and function is still unclear. In non-neuronal cells co-expression of NMDARs (NR1/NR2A-B) with PSD-95 enhances their surface expression and decrease their sensitivity to glutamate (Rutter and Stephenson, 2000; Rutter et al., 2002). In neurons, mutation of the PDZ motif of NR2B appears to decrease NR2B-NMDAR surface expression and synaptic localization (Prybylowski et al., 2005). It has also been found that the binding of PSD-95-like MAGUKs to the very C-terminal PDZ motif of NR2B prevents the receptor internalization by hiding the consensus internalization motif YEKL in close proximity (last 11 to 7 amino acids; Roche et al., 2001). It is important to note that these observations do not discriminate between the PSD-95-like MAGUKs, which may share this role in NMDAR trafficking. Thus, disrupting the PSD-95/NR2B association might be compensated for by another member of the PSD-95-like MAGUK family. Indeed, in
the PSD-95 knockout mice neurons, the surface-expression and clustering of NMDARs do not seem to be altered (Migaud et al., 1998). Additionally, others using the TAT-NR2B9c peptide to disrupt the NR2 PDZ ligand have not measured any changes in NMDAR expression and Ca\(^{2+}\)-mediated influx (Aarts et al., 2002), which indicates that the disruption does not alter significantly NMDAR trafficking.

Cui et al. (2007) demonstrated that among all other PDZ domain-containing proteins, PSD-95 and nNOS were key effectors of NMDAR-mediated excitotoxicity. Acute disruption of the NR2B/PSD-95/nNOS interaction (around an hour timescale in our experiments) did not show any significant consequences on glutamatergic transmission and plasticity. However, this displacement of PSD-95 from the PDZ binding motif of NR2B-NMDARs was neuroprotective, by uncoupling NMDARs to the p38 SAPK activation. In non-neuronal cells, excitotoxicity was independent of the PDZ ligand; the PDZ motif of NR2B was not promoting pro-death signalling due to lack of neuron-specific proteins PSD-95 and nNOS.

Thus, targeting of the NR2B PDZ ligand appeared to specifically disrupt the p38 pro-death signal without affecting the normal NMDAR functions. In conjunction with the use of a peptide like D-JNKI1 to disrupt the JNK cascade (Borsello et al., 2003b), this may prove to be an effective therapeutic strategy against excitotoxicity. The results presented in this Chapter have been published in Soriano et al. (2008) and Martel et al. (2009b). Both research papers are included at the end of this thesis.
Chapter 6
The C-terminus tail of the NR2 subunit influences NMDAR-mediated neuronal death
1. Chapter introduction

The majority of NMDAR channels are composed of two NR1 and two NR2 subunits; the latter conferring distinct biophysical properties to the channel. Receptors composed of NR2A, as opposed to NR2B (the two predominantly expressed NR2 subunits in forebrain neurons), possess higher open probability, peak currents and faster activation/deactivation kinetics (Chen et al., 1999; Erreger et al., 2005). On the other hand, NR2B-NMDARs have longer kinetics but desensitize more (Erreger et al., 2005). What it ultimately signifies at the synapse in terms of Ca$^{2+}$ influx intensity and duration is still relatively speculative, since most of the studies on the subject have been done in non-neuronal models (Yashiro and Philpot, 2008). Indeed, at the synapse, the biophysics of NMDARs can be modulated by binding to cytoskeletal and signalling proteins, as well as by covalent modifications (Sobczyk et al., 2005). In addition, the properties of tri-heteromeric receptors -carrying both NR2A and NR2B subunits- are unclear; albeit it has been reported that the majority of NMDARs in the hippocampus exists as di-heteromeric complexes (NR1/NR2A or NR1/NR2B; Al-Hallaq et al., 2007).

During a single pulse of neurotransmitter release, it is thought that NR2B-containing NMDARs can carry more Ca$^{2+}$ per unit of current in individual dendritic spines (Sobczyk et al., 2005). However, the amount of Ca$^{2+}$ influx mediated by NMDARs is predicted to depend on the stimulation frequency, in addition to the NR2A/B subunit composition (Erreger et al., 2005). Thus, these observations suggest that the NR2A/B subunit composition may affect NMDAR-mediated toxicity due to the difference in channel kinetics.

On the other hand, the NR2A and NR2B C-termini share only 30% sequence homology. This difference implies that the NR2 composition of the NMDAR in neurons can also affect the intracellular signalling interactions, which are regulated by their C-termini.

A link between the NR2 subunit composition of NMDARs and excitotoxicity could prove useful in the quest for an effective neuroprotective compound, in that selective inhibition of “toxic receptors” could minimise the undesired side-effects seen with complete NMDAR blockade (Ikonomidou and Turski, 2002). Whilst the evidence
is still controversial, it has been reported that NR2B-containing NMDARs are coupled to pro-death signalling whereas NR2A-ones are mediating pro-survival signals, both \textit{in vitro} and \textit{in vivo} (Zhou and Baudry, 2006; Liu et al., 2007; Chen et al., 2008). However, we showed previously that NR2B could induce both pro-death and pro-survival signals (Chapter 3 and Martel et al., 2009a), and others have demonstrated that both NR2 subunits are able to induce excitotoxicity (von Engelhardt et al., 2007).

It is also possible that the two NR2 isoforms exert different pro-death and pro-survival signalling properties because of their subcellular localization. NR2A-NMDARs are preferentially, but not exclusively, inserted at synaptic sites (see Chapter 3; Tovar and Westbrook, 1999; Thomas et al., 2006; Martel et al., 2009a). Because of this partitioning, it is difficult to distinguish if the signalling properties of the receptors are due to their localization or their subunit composition itself. However, both the localization and the identity of the associated signalling complexes are likely dependent on the NR2A and NR2B C-terminus. Therefore, if the NR2 subunit composition influences the ability of NMDAR to induce toxicity other than by modifying the magnitude of Ca$^{2+}$ influx, this influence would involve the C-terminus.

Thus, we devised an approach to evaluate the contribution of both channel properties and C-terminus interactions to NMDAR-mediated excitotoxicity. Overexpression of NR2 subunits has been shown to increase the total number of functional NMDARs in cerebellar granule cells (Prybylowski et al., 2002). Therefore, we sought to use this approach to modify the NR2 subunit composition of NMDARs in hippocampal neurons and investigate the ability of NR2A/B to induce excitotoxicity. To distinguish between the influence of the receptor/channel properties and C-terminal interactions, we then hypothesized that swapping the C-terminus of NR2A/B would indicate which of these two factors has a critical role in determining the death-inducing ability of each subunit. The experiments presented in this Chapter have led to the following findings:

- Overexpression of NR2A or NR2B increased the amount of NMDARs in hippocampal neurons. The overexpressed subunit accounted for a large proportion of the
whole population of NMDARs, as assessed by measuring the current sensitivity to ifenprodil in NR2A-overexpressing neurons;

- NR2B-overexpression led to increased levels of NMDA-induced neuronal toxicity compared to NR2A-overexpressing neurons;

- To evaluate if the NR2 C-terminus had an influence on the difference in excitotoxicity, chimeric NR2 subunits with swapped C-terminus were designed;

- Overexpression of chimeric subunits showed that the NR2B C-terminus was more potent than the NR2A C-terminus in inducing excitotoxicity, but did not completely invert the difference in toxicity between NR2A and NR2B overexpression.

In conclusion, these findings suggest that both the receptor/channel portion and the C-terminus of the NMDAR determine its ability to induce excitotoxicity. This study enables further investigation of the properties of the NR2 subunit intracellular tail and is the first step in the identification of the subunit-specific interactions that affects viability signals in neurons.
2. Results

2.1 Overexpression of NR2 affects the pool of functional NMDARs in hippocampal neurons

We showed previously that hippocampal neurons at DIV 8-11 possess predominantly NR2B-containing NMDARs (Chapter 3 and Martel et al., 2009a). At this stage, their NMDAR-mediated currents are inhibited by almost to the same extend by ifenprodil as a population of recombinant NR2B-NMDARs. Thus, we hypothesized that overexpression of exogenous NR2 subunits would have a clear, characterizable effect on the NMDAR-mediated currents in these neurons. Perhaps surprisingly, overexpression of NR2 subunits in wild-type neurons is a strategy that has not been extensively used, presumably because of the confounding issue of the presence of endogenous subunits. It has been shown, however, that in cerebellar granule cells overexpressing NR2 increases the number of functional NMDARs, indicating that it is this subunit –and not the NR1- that limits the export of functional NMDARs from the endoplasmic reticulum to the cytoplasmic membrane (Prybylowski et al., 2002).

Thus, we transfected neurons at DIV 7-9 with NR2A- or NR2B-encoding plasmids. The NR2 subunits were of the mouse wild-type sequence, apart from a small mutation in their M4 transmembrane domain inserting a Not1 restriction enzyme site (GCGGCCGC). In the NR2A sequence, the sequence mutations were inserted without changing the codons (Ala825 and Ala826). In NR2B the creation of a Not1 site only changed the amino acid sequence at Gly826Ala (but not Ala827). The M4 domain is conserved between NR2A and NR2B, apart from the Gly826 of NR2B and the very last M4 amino acid. This change from glycine to alanine in the NR2B M4 domain is unlikely to affect the receptor properties, as both amino acids bear small, non-polar and neutral side chains. Forty-eight hours after transfection, currents evoked by 100 μM NMDA were recorded from these neurons (DIV 9-11).

Current densities of transfected hippocampal neurons were reliably increased by overexpression of either NR2A or NR2B vectors. Globin-transfected cells had a current density of 60 ± 5 pA/pF, whereas NR2A and NR2B overexpressing neuron currents
reached 102 ± 6 pA/pF and 101 ± 7 pA/pF respectively (Figure 6.1; n = 30, 20, 16 cells). Therefore overexpression of either NR2A or NR2B subunits increased the number of functional NMDAR in hippocampal neurons to an equal extent and in agreement with that reported in cerebellar granule cells (Prybylowski et al., 2002).

The overexpression of NR2 subunits raised the amount of surface-expressed NMDAR by more than 60%, but it remained unclear how much of this receptor population actually resulted from expression of the introduced receptors. Ifenprodil is a highly specific NR2B-NMDAR antagonist and DIV 9-11 neurons mostly exhibit endogenous NR2B-NMDARs. Thus, we studied the change in ifenprodil sensitivity of the NMDA-induced currents in transfected cells, as an indicator of overexpressed subunit incorporation.

Similar to untransfected hippocampal neurons (see Chapter 3; Martel et al., 2009a), globin-overexpressing cells currents were inhibited by ifenprodil by 77 ± 3% (23 ± 3% of initial current remained; Figure 6.2; n = 12 cells). Neurons transfected with the NR2B plasmid exhibited equally high inhibition (24 ± 4% of ifenprodil-insensitive current fraction; n = 3 cells). Thus, as expected the vast majority of NMDARs in globin and NR2B over-expressing cells are di-heteromeric NR1/NR2B. On the other hand, the ifenprodil sensitivity of currents in NR2A overexpressing neurons was decreased to 23 ± 3% (77 ± 3% of the initial currents were unaffected by ifenprodil; n = 9 cells). This sharp change in ifenprodil sensitivity shows that a large proportion of the endogenous receptors has been replaced by di-heteromeric NR1/NR2A receptors, or has incorporated a NR2A subunit. Indeed, the potency of ifenprodil on tri-heteromeric NMDARs is still undefined. However, evidence obtained with chimeras in oocytes suggests that ifenprodil has a high affinity for NR1/NR2A/NR2B receptors, but inhibits their current by as little as 20% (Hatton and Paoletti, 2005). Thus, our observation that NR2A-overexpressing neurons showed only 23 ± 3% inhibition by ifenprodil indicates that the exogenously-provided subunits account for an important fraction of the pool of functional NMDARs in these cells.
Figure 6.1: Overexpression of NR2A or NR2B increases NMDA-induced currents in hippocampal neurons. Neurons (DIV 7-8) were transfected with NR2A, NR2B or globin and eGFP plasmids. 48-72 hours later, currents evoked by 100 µM NMDA were recorded from transfected neurons and current values (pA) were normalized by the measured cell capacitance (pF). Compared to control (globin), NR2A- and NR2B-overexpressing neurons (DIV 9-11) exhibited similarly increased current densities (n = 16-30 cells each). * p < 0.001.
Figure 6.2: A large proportion of NMDARs incorporate exogenously provided subunits in hippocampal neurons overexpressing NR2 subunits. Neurons (DIV 7-8) were transfected with globin, NR2A or NR2B plasmids. 48-72 hours after, ifenprodil (3 µM) sensitivity of NMDAR-mediated currents were measured (100 µM NMDA). In globin (n = 12) and NR2B-overexpressing neurons (n = 3), the ifenprodil sensitivity reflected a near-pure NR2B-containing NMDAR population. However, in NR2A-transfected cells the ifenprodil-insensitive fraction of current rose to 77 ±3% (n = 9), indicating that a large quantity of NMDARs contained at least one NR2A subunit. * p < 0.001.
2.2 Overexpression of NR2 subunits in hippocampal neurons influences excitotoxicity

The overexpression increased currents by more than 60% with both NR2A and NR2B. To compare the death-inducing properties of each subunit type, we followed visually transfected cells (with Globin or NR2 and eGFP) before and after a moderately toxic agonist stimulation. We aimed for a concentration of NMDA that would place the toxicity in a dynamic range where it could be influenced by the subtype of NMDARs present. The toxicity threshold in our assays is around 20 µM NMDA (Soriano et al., 2006), however since the neurons were transfected with NR2 subunits and possessed higher current density, we used 20 µM NMDA as the maximal stimulus.

As seen in Figure 6.3, around 60% of GFP-positive cells in the three overexpressing conditions survived in the absence of any NMDA stimulation. Since the cells were first visualized 48 hours after transfection, the spontaneous “death-inducing” effect of NR2 overexpression on cell survival should already be accounted for at that time point. At 10 µM NMDA, NR2B-overexpressing neurons showed significantly more vulnerability than control cells (46 ± 3% surviving cells as opposed to 68 ± 7% of globin control; n = 5 experiments). Expectedly, 20 µM NMDA induced a higher amount of cell death. However, NR2A overexpression did not modify the level of excitotoxicity compared to neurons with no additional NR2 subunit (37 ± 2% and 40 ± 1%, respectively). Strikingly, only 9 ± 2% of cells transfected with the NR2B plasmid survived the stimulation at this concentration of NMDA, a level distinct from both globin- and NR2A-overexpressing neurons. These results clearly show that increasing the number of NR2B-containing NMDARs renders neurons more vulnerable to excitotoxicity, whereas an equal increase of NR2A-NMDARs does not affect the cells survival. This suggests that each of the two NR2 subunits influence differently excitotoxicity in hippocampal neurons; albeit there is no indication whether it is due to the subtype of channel or to different signalling properties of the C-termini.
Figure 6.3: Overexpression of NR2A and NR2B subunits influences agonist-induced neuronal death differentially. 48 hours after transfection with globin/NR2 and eGFP, GFP-positive neurons’ position were marked, then neurons were treated as indicated with NMDA for 1 hour. Stimulation was ended by addition of NMDAR antagonist MK-801 (10 μM). 16-24 hours later, marked GFP-expressing hippocampal neurons were visualized (under both GFP and DAPI fluorescence) and percentage of cell death was assessed. Whereas overexpression of the NR2A subunit did not alter excitotoxicity, the toxicity was increase at both 10 and 20 μM NMDA stimulations in neurons overexpressing NR2B (n = 5). * p < 0.01; ** p < 0.001.
2.3 Creation of NR2 chimeras with swapped C-terminus domains

To further investigate the difference in cell death between NR2A- and NR2B-overexpressing neurons, we sought to exchange the C-terminus tails of the NR2 subunits to see if that would modify their ability to induce excitotoxicity. As described in Material and Methods, we used the inserted NotI site in the transmembrane domain M4 to cleave the sequence of both NR2 plasmids. The NR2A N-terminal fragment was obtained by digestion with EcoRI and NotI. The fragment was then ligated with the pCis-NR2B plasmid (with the NR2B N-terminal fragment excised), to obtain the construct NR2A(2BCTD) (NR2A with the NR2B C-terminus; Figure 6.4).

The NR2B(2ACTD) (NR2B with the NR2A C-terminus; Figure 6.4) was obtained by ligating the NR2B N-terminal portion (isolated using ClaI and NotI), the NR2A C-terminal domain (NotI and EcoRI) and the empty pCis vector (ClaI and EcoRI).

Apart from the very last amino acid (W on NR2A, C on NR2B), the sequence of the M4 domain is conserved between NR2A and NR2B. Therefore, this swap created NR2 isoforms having their wild-type mouse sequence until the very last amino acid of the M4 domain, but possessing the other subtype cytoplasmic sequence from thereon.
Figure 6.4: Mutant NR2 subunits with swapped C-terminal domains. Up: linear representation of the NR2 subunit domains. Down: schematic illustrations of the NR2 subunits. The NR2A sequence appears in grey whereas the NR2B depiction is white. Using the NotI restriction site in the M4 transmembrane domain of each subunit, chimeric constructs were assembled. NR2A(2B\textsuperscript{CTD}) consists in the N-terminal fragment of NR2A(1-824) and the cytoplasmic C-terminal tail of NR2B(826-1482). On the contrary, NR2B(2A\textsuperscript{CTD}) is composed of the receptor portion of NR2B(1-825) and the C-terminal domain of NR2A(825-1464). Amino-terminal domain (ATD); carboxy-terminal domain (CTD).
Using these NR2 subunits with differing C-terminus, we then verified that these chimeras formed functional receptors that retained the channel properties of their parent receptors. Thus, we assessed the ifenprodil sensitivity of the NMDAR-mediated current in non-neuronal cells transfected with the relevant plasmids. HEK 293 cells were used because of their rapid proliferation and high rate of transfection with Lipofectamine. HEK 293 cells were transfected with plasmids encoding for NR1 subunit and eGFP along with a NR2 plasmid.

HEK 293 cells expressing NR1 and any of the four NR2 variants exhibited NMDA-induced currents, indicating that the NR2A(2B<sup>CTD</sup>) and NR2B(2A<sup>CTD</sup>) chimeras formed functional NMDARs. Moreover, the C-terminal chimera gave currents that exhibited an ifenprodil sensitivity of similar magnitude to what would be expected for the N-terminal region contained within the receptor. Like NR1/NR2A-NMDARs, receptors composed of NR1/NR2A(2B<sup>CTD</sup>) were largely unaffected by ifenprodil (92 ± 6% and 89 ± 9% of currents remained; Figure 6.5; n = 2 cells each). On the other hand, both NR1/NR2B- and NR1/NR2B(2A<sup>CTD</sup>)-NMDARs were highly sensitive to inhibition by ifenprodil (11 ± 1% and 8 ± 4%; n = 2 cells). These inhibition values suggest that the receptors made of C-terminal NR2 chimeras share the same channel properties than the wild-type receptors of the same subtype; this is to be expected since the original and mutant NR2s retain the same transmembrane (channel-forming sequence) and extracellular N-terminal domains (the site of ifenprodil binding; Hatton and Paoletti, 2005).
Figure 6.5: NMDARs composed of NR2 subunits with their C-terminal domain swapped retain the ifenprodil sensitivity of their wild-type counterparts. A. HEK293 cells were transfected with NR1, eGFP and the indicated NR2 constructs. NMDAR-mediated currents sensitivity to NR2B-specific antagonist ifenprodil (3 µM) was measured 48-72 hours later and expressed as percentage of NMDA-induced current (100 µM) remaining after drug application (n = 2). Receptors containing NR2B(2A<sup>CTD</sup>) retained the same level of ifenprodil sensitivity as NMDAR composed of NR2B(WT), whereas NR2A(2B<sup>CTD</sup>)-containing receptors were barely antagonised by the compound as with NR2A(WT). B. Example traces of currents evoked in transfected HEK293 cells.
2.4 Overexpression of NR2A(2B<sub>CTD</sub>) and NR2B(2A<sub>CTD</sub>) in neurons affects current in the same manner as NR2A and NR2B overexpression

Having determined that the NR2 subunits with interchanged C-terminal tails were able to produce functional receptor that retained the same ifenprodil sensitivity as their native counterparts in non-neuronal cells, we then studied the chimeric expression in hippocampal neurons to assess the effect of overexpressing NR2A(2B<sub>CTD</sub>) or NR2B(2A<sub>CTD</sub>) on NMDA-induced currents. Again, neurons were patch-clamped at -60 mV 48 hours after transfection with NR2 and eGFP plasmids (2:1) and currents evoked by 100 µM NMDA were recorded.

As shown if Figure 6.6, neurons overexpressing the constructs NR2A(2B<sub>CTD</sub>) and NR2B(2A<sub>CTD</sub>) exhibited higher currents than globin-expressing control cells (NR2A(2B<sub>CTD</sub>) = 102 ± 6 pA/pF; NR2B(2A<sub>CTD</sub>) = 94 ± 6 pA/pF; globin = 61 ± 3 pA/pF; n = 16, 13 and 30 cells respectively). These elevated levels of NMDAR-mediated currents were of similar magnitude to currents in NR2A- and NR2B-overexpressing cells, indicating that overexpression of either NR2 subunit increased equally the number of functional receptors.

Next, we assessed the ifenprodil sensitivity of the currents; not only to evaluate the proportion of receptors having incorporated an exogenously provided subunit (in the case of NR2A and NR2A(2B<sub>CTD</sub>)), but also to ensure that the C-terminal mutants were behaving like their native counterparts in a neuronal expression system. As it was the case for NR2B overexpression, NR2B(2A<sub>CTD</sub>) overexpression resulted in an ifenprodil inhibition of 81 ± 4% by 3 µM ifenprodil (unblocked current 19 ± 4% as illustrated in Figure 6.7; n = 3 cells). Moreover, transfection with the NR2A(2B<sub>CTD</sub>) plasmid rendered the NMDAR-mediated currents largely insensitive to ifenprodil (76 ± 5% of initial current remained with 3 µM ifenprodil). This low level of inhibition by ifenprodil in both NR2A and NR2A(2B<sub>CTD</sub>) (23 ± 3% and 24 ± 5%) suggests that a large proportion of the functional NMDAR pool had incorporated at least one of these exogenous subunits.
Figure 6.6: Chimeric subunit overexpression increases NMDAR-mediated current in transfected hippocampal neurons. Neurons were transfected with a NR2 subunit and eGFP plasmids as indicated and currents were recorded 48 hours after. NMDA-induced currents (100 µM) were normalized with the cell capacitance and compared to globin-transfected neuron currents. All four NR2 subunits increased NMDAR currents by more than 50% of control-transfected neurons, showing that C-terminal chimeric subunit overexpression augment the number of functional receptors to a similar level than wild-type NR2A and NR2B (n = 13-30 cells each). * p < 0.001 compared to globin. Figure courtesy of Dr Giles Hardingham.
Figure 6.7: Ifenprodil sensitivity of NMDA-induced currents shows that an equally large proportion of NMDARs incorporates C-terminal mutant NR2 subunits compared to wild-type when overexpressed in neurons. Hippocampal neurons were transfected with plasmids as indicated (along with eGFP) and patched-clamped 48 hours later. Ifenprodil (3 µM) sensitivity of the NMDA-induced currents (100 µM) reflected a near-pure NR2B-containing pool of NMDARs in globin (n = 12), NR2B(WT) (n = 3) and NR2B(2A\text{CTD}) (n = 3). However, the fraction of current insensitive to ifenprodil increased to 76 ± 5% in neurons overexpressing NR2A(2B\text{CTD}) (n = 9), a level similar to NR2A(WT) overexpression (n = 9), indicating that a large proportion of the functional NMDARs possess a NR2A subunit subtype. * p < 0.001.
2.5 The NR2-C-terminus influences the subunit’s potency for excitotoxicity

As described above, NR2B-overexpression caused the cells to be more vulnerable to an excitotoxic insult whereas NR2A overexpression did not alter the agonist-induced toxicity, despite similar current increases. To verify if the intracellular C-terminal tail of the NR2 subunits have a role in their different ability to promote death, we transfected hippocampal neurons with the NR2A(2B\textsuperscript{CTD}) and NR2B(2A\textsuperscript{CTD}) chimeras along with eGFP and subjected such cultures to an excitotoxic NMDA stimulation (20 µM). Cell were visualized before and 24 hours after stimulation to assess viability (Figure 6.8A).

Strikingly, swapping the NR2A C-terminus for the NR2B sequence equivalent caused an increase in cell death (from 36 ± 4% to 63 ± 9% cell death; Figure 6.8B; n =5 experiments). Inversely, changing the C-terminus of NR2B for the NR2A tail reduced the toxicity following the NMDA application (from 84 ± 5% to 56 ± 7% neuronal death). Thus the NR2B C-terminal domain appeared to promote death better than the NR2A C-terminus. However, the C-terminal exchange was not sufficient to completely invert the toxicity to the levels of the wild-type subunits; suggesting that the intracellular tail alone was not the only determinant for excitotoxicity, but that the rest of the subunit was also important.
Figure 6.8: Changing the C-terminal domain of the NR2 subunit affects the amount of agonist-induced cell death in overexpressing hippocampal neurons. Neurons were transfected with eGFP and NR2 or globin (control vector) plasmids as indicated. 48 hours later, pictures of GFP-positive neurons were taken then cells were stimulated with NMDA (20 µM) for 1 hour, then MK-801 (10 µM) was added to cultures. 16-24 hours after stimulation, GFP and DAPI fluorescence pictures of previously-marked neurons were obtained to assess the neuronal viability. A. Example pictures of a neuron without any agonist treatment and of a neuron dying from excitotoxicity as seen with GFP and DAPI fluorescence (scale bar 20 µM). B. Swapping the C-terminus of the NR2A subunit for the 2B<sup>CTD</sup> enhanced the amount of neuronal death. On the contrary, changing the cytoplasmic tail of the NR2B subunit for the NR2A variant decreased excitotoxicity (n = 5). * p < 0.05.
2.6 Excitotoxicity also depends on the NR2 subunit regardless of their C-terminal domain

Whilst the C-terminus of the NR2B subunit augmented the toxicity of NMDARs and the NR2A one decreased it, the swapping was insufficient to reproduce the levels of cell death observed with each wild-type subunit overexpression. The NR2A and NR2B subunits have a moderately different \( EC_{50} \) values for NMDA, with NR2A having less affinity for the agonist than NR2B (64 µM and 42 µM, respectively; Frizelle et al., 2006). Thus, the concentration of NMDA used to induce neuronal death (20 µM) would not be expected to be equipotent at NR2A and NR2B-containing NMDARs; it is to be expected that less of the NR2A population is activated compared to the NR2B population. However, subunits where the only difference was in the C-terminal domain would give a similar level of activation. To verify this, we measured currents evoked by 20 µM NMDA in neurons overexpressing NR2A, NR2A(2B\(^{CTD}\)), NR2B or NR2B(2A\(^{CTD}\)) subunits.

Expressed as a ratio against globin-transfected cells, currents from both NR2A and NR2A(2B\(^{CTD}\)) overexpressing neurons were only 1.3 ± 0.1 and 1.4 ± 0.2 times greater (Figure 6.9; \( n = 11 \) and 10 cells). On the other hand, currents from cells overexpressing NR2B or NR2B(2A\(^{CTD}\)) were more than 2 times the currents of control neurons (2.18 ± 0.2 and 2.18 ± 0.3; \( n = 14, 13 \) cells). Thus, at the concentration of NMDA used to induce toxicity in the hippocampal culture, cell overexpressing the NR2B subunit allow a larger ion influx in the neurons, regardless of its C-terminus tail (globin 25 ± 3 pA/pF; NR2A 34 ± 3 pA/pF; NR2A(2B\(^{CTD}\)) 36 ± 4 pA/pF; NR2B 56 ± 6 pA/pF; NR2B(2A\(^{CTD}\)) 56 ± 7 pA/pF). Therefore, the larger cation entry in cells expressing the NR2B isoforms contributed to the higher amount of cell death observed in Figure 6.3 and 6.8.
Figure 6.9: The C-terminal domain does not alter the NMDAR-mediated current, although the rest of the subunit does. 48 hours after transfection with globin/eGFP or NR2/eGFP as indicated, currents induced by the same concentration of NMDA used to trigger excitotoxicity (20 µM) were measured in hippocampal neurons. A. Current values (in pA/pF) were normalised to the amount of current in control-transfected neurons. All NR2-overexpressing neurons exhibited higher current densities than globin-transfected cells (#). However, both NR2A(WT) and NR2A(2B^{CTD}) showed lower current values than cells overexpressing the NR2B subtypes at this concentration of agonist (n = 13-14 cells each). B. Example traces of currents recorded from GFP-positive neurons. * and # (against globin) p < 0.05.
We combined the toxicity data with the amount of current each wild-type and chimeric subunit exhibited to visualize each NR2 subunit contribution to excitotoxicity. As shown in the scatter plot in Figure 6.10, both the subunit type (NR2A or NR2B) and the C-terminus (-2A\text{CTD} or -2B\text{CTD}) defined the magnitude of cell death in NR2-overexpressing neurons. The NR2B-NMDAR channel induced higher levels of cell death due to the higher amount of current it passed; whereas the NR2B-C-terminal domain also increased the NMDAR-mediated toxicity, but without increasing the current. This suggests that the ability of the NR2 C-terminus to trigger excitotoxicity depends on the intracellular interactions taking place on the C-terminal domain, such as the coupling NR2B/PSD-95/nNOS discussed in previous chapters. The exact identity of the pro-death signals involved in the present experiments has not been investigated, but taken together, these results show that the NR2B-C-terminal tail preferentially signals to death compared to the NR2A C-terminus.
Figure 6.10: The C-terminal domain of the NR2B subunit is more effective at promoting death than the C-terminal domain of NR2A. A scatter plot (left) comparing the effect of each NR2 construct with regard to the current increase (see figure 6.9) and NMDA-induced neuronal death (see figure 6.8). “Con” denotes globin-transfected control cultures. At 20 µM NMDA, the subtype of NR2 affected the amount of NMDAR-mediated currents, which in turn augmented the amount of cell death in overexpressing neurons (arrows 3 and 4). However swapping the cytoplasmic tail between NR2A and NR2B did not alter the currents, but influenced excitotoxicity of transfected hippocampal neurons (arrows 1 and 2). Thus, the NR2B and NR2A C-terminal domains demonstrate a differential ability to trigger pro-death signals.
3. Chapter discussion

NMDARs can induce both pro-survival and pro-death signals, as well as being key players in synaptic plasticity. Pro-death signalling from the NMDAR is occurring during glutamate deregulation and this excitotoxicity has been implicated in pathologies such as hypoxia, ischemia, epilepsy, Parkinson’s disease and Huntington’s disease (Waxman and Lynch, 2005b). NMDAR subunit composition is possibly a determinant in the type of signalling downstream of the receptor activation: understanding each subunit role in neuronal viability could be therapeutically exploited to interfere more selectively with NMDAR toxicity with minimal disruption of its roles in synaptic transmission (Ikonomidou and Turski, 2002; Hardingham and Bading, 2003). Indeed, the C-terminal portion of the NR2A and NR2B subunits interact with various scaffolding, anchoring and signalling molecules; these associations form signalling cassettes coupling the NMDAR to downstream neuronal events.

We have previously shown that NR2B-containing NMDAR were able to signal to synaptic potentiation and depression, as well as to pro-death and pro-survival signals (Chapter 3; Martel et al., 2009a). Here, we demonstrated that NR2B-NMDARs were better than NR2A-NMDAR at inducing cell death. We found that, in hippocampal neurons overexpressing NR2 subunits, the amount of current the NR2B-NMDARs pass at a low but toxic concentration of agonist was higher than for NR2A-NMDARs. This can be explained by NR2B-containing receptors having lower deactivation and desensitization constants, in addition to their higher potency for NMDA (Frizelle et al., 2006; Erreger et al., 2007).

However, we discovered that the C-terminal intracellular tail of the NR2 subunits also determined the receptor ability to induce cell death, regardless of the rest of the subunit. By creating chimeras of NR2A and NR2B containing the C-terminal domain of the other subunit, we measured a change of toxicity in neurons overexpressing the chimeras (NR2A(2B<sup>CTD</sup>) or NR2B(2A<sup>CTD</sup>)) compared to the wild-type subunits (NR2A or NR2B). This difference in toxicity occurred without any concurrent changes in NMDAR currents and agonist potency. Thus, this indicates that the C-terminal domain of the NR2B subunit was better at triggering cell death than the NR2A C-terminal
sequence. This was not due to a differential modulation of the channel properties by an interaction with intracellular molecules because each C-terminal chimera passed the same amount of current as their wild-type homolog at 20 µM NMDA. It is therefore unlikely that the difference in cell death observed by changing the C-terminus of a subunit is due to a modulation of the receptor biophysical properties (desensitization, probability of opening, etc).

As discussed in the other Chapters, it has been previously suggested by Liu et al. (2007) that NR2A-containing NMDARs promote survival whereas NR2B-NMDARs trigger death. Their report suggests that the NR2A-mediated neuroprotection occurs both at synaptic and extrasynaptic locations; and inversely, that NR2B-NMDARs promote neuronal death, both at synaptic and extrasynaptic sites. Thus, they conclude that subunit composition, rather their localization determines the direction of the viability signals; the apparent ability of synaptic NMDARs to induce pro-survival signals would therefore be explained by the NR2A enrichment at the synapse.

Our present results support partially the conclusions of Liu et al. (2007). Whilst we used overexpression/enhancement of functional NMDARs, instead of their antagonism approach, our results also suggest that subunit composition has an influence on excitotoxicity, with NR2B being preferentially pro-death compared to NR2A. We did not, however, verify the precise localisation of our overexpressed constructs, nor did we examine the putatively differential effect of synaptic/extrasynaptic NMDARs stimulation on excitotoxicity. Further work is required to answer this question.

In vivo results supporting the notion that NR2A-NMDARs are neuroprotective whereas NR2B-NMDARs are pro-apoptotic have been recently published (Chen et al., 2008). On the other hand, the conclusions raised by Liu et al. (2007) and Chen et al. (2008) are still not accepted unequivocally, as different results were obtained in older neurons by von Engelhardt et al. (2007). In their study, von Engelhardt et al. (2007) observed that blockade of either NR2A or NR2B-NMDARs is inefficient at protecting neurons against excitotoxicity. Only when both subtypes are inhibited did they measure a reduction in cell death, implying that when a particular type is blocked the other carries the toxicity signalling. However, they noticed that at a concentration of NMDA
that induces around 50% cell death, adding NVP-AAM077 (50 nM, a concentration where it inhibits 75% of NR2A- but only 25% of NR2B-NMDARs) exacerbates the neuronal death. Interestingly, this suggests that co-recruitment of NR2A-NMDARs has an ameliorating, protecting effect which is lost in presence of NVP-AAM077.

From our results, we cannot specify whether the difference in cell death between the NR2A and NR2B C-terminal domain is due to a NR2A-mediated pro-survival or a NR2B-associated pro-death signal. This will be determined in future studies by creating mutations at different domains of the C-terminus, as well as by studying the downstream pathways preferentially activated by each NR2 subunit. This will allow identification of the sequences implicated in the C-terminus pro-survival/death signalling, and of the intracellular proteins interacting with the NMDAR in creating signalling complexes. Posttranslational modifications like phosphorylation or cleavage by calpains can also offer an explanation for the differential ability of the C-terminal domains to induce toxicity (Waxman and Lynch, 2005b). For example CaMKII, PKC and Src family kinases can phosphorylate serine or tyrosine residues on the NR2 tail, which can modify the receptor activity or change their localization (Waxman and Lynch, 2005b).

The accessibility of those phosphorylation sites by kinase enzymes, or cleavage sites by calpains can be determined by other proteins binding to a domain nearby. For example, the NR2B association with PSD-95 induces phosphorylation on Tyrosine 1336 by Src family kinase Fyn, and also protects against calpain truncation (Wu et al., 2007). Such a finely tuned autoregulatory mechanism for NR2B-NMDAR activity is only one of many possibilities by which each subunit can signal differently. As mentioned, this will be investigated further by swapping and mutating specific domains of the intracellular tails, which will allow identification of the events contributing to the death signals evoked by each subunit.

Concurrently, a recent publication observed a inverse correlation between the NR2B subunit expression and the number of synaptic AMPARs (Hall et al., 2007). When NR2B was genetically removed from neurons in development, an increase in synaptic AMPAR-mediated currents could be measured from these neurons. Conversely, overexpressing NR2B led to a decrease in surface and synaptic AMPARs, through a
mechanism involving TARP (transmembrane AMPAR regulatory protein) expression (Hall et al., 2007). Therefore, it remains a possibility that our experiments using NR2 construct overexpression affects the neuron’s excitability through changes in AMPAR numbers, which could then influence their vulnerability to a toxic agonist insult—since synaptic activity induces pro-survival processes (Hardingham, 2006a; Papadia et al., 2008). This issue will be addressed in future experiments by assessing the AMPAR-mediated currents in neurons overexpressing NR2 subunits.

We did not assess the proportion of synaptic and extrasynaptic NMDARs in our cultures following overexpression of NR2 subunits. In cerebellar granule cells, Prybylowski et al. (2002) observed an increase in extrasynaptic NMDAR punctas when the neurons were transfected with NR2-encoding plasmids. Since activation of synaptic or extrasynaptic NMDARs trigger different types of viability signalling this could be a crucial issue for neuronal survival. It has also been suggested that the subunit composition of the receptors dictates their surface mobility, as NR2B-containing NMDARs are more mobile and more likely to be localized at extrasynaptic sites (Groc et al., 2006). This subunit-dependent trafficking may be governed by C-terminal domain processes, which raises the possibility that our NR2 C-terminus chimeras display an altered surface mobility compared to the un-mutated subunits. In turn, this could then influence the synaptic/extrasynaptic signalling balance in the transfected neurons and modify the toxicity signals accordingly.
Summary of experimental findings

Chapter 3. The NR2 subunit composition has been reported to govern the type of signalling downstream of NMDAR, but the supporting evidence is still controversial. Thus, to assess if the NR2A/NR2B subunit composition could explain the dichotomy of NMDAR signals, the ability of a single subtype of receptor to signal to different outcomes was investigated. Developing hippocampal neurons were used at time when the vast majority of NMDAR was found to be composed of NR2B subunits, both at synaptic and extrasynaptic sites. In these neurons, NR2B-containing NMDARs were able to trigger agonist-induced cell death and mediate activity-induced neuroprotection. In addition, both synaptic potentiation and depression could be induced and required NR2B-containing NMDAR activation. Thus pro-survival, pro-death and plasticity signals were evoked depending on the type of stimulation, but not on the NR2 subunit composition: in these developing hippocampal neurons, all the types of signalling necessitated NR2B-NMDAR activity.

At a later developmental stage, NR2A subunit expression increased and was found to preferentially partition at synaptic sites. Further experiments will address if a developmentally-regulated difference in NMDAR signalling appears at this later stage. However, in immature hippocampal neurons the NR2 subunit composition cannot account for the diversity of NMDAR-dependent signals.

Chapter 4. Recombinant NMDAR expression in mammalian cell lines has been previously shown to recreate excitotoxicity in a non-neuronal context. However, this paradigm has mainly been used to study the NMDAR and its regulation by other proteins, but the pro-death signals involved in the acquired excitotoxicity have never been investigated. Whilst many pro-death signals have been identified in neurons, the molecular link between their activation and NMDAR channel opening are often unclear. The p38 and JNK SAPKs are two of such pathways contributing to neuronal excitotoxicity. Thus, to elucidate the requirements for the induction of these two pro-
death cascades, we determined if they participated in non-neuronal excitotoxicity. Inhibition of the p38 pathway did not prevent agonist-induced death in non-neuronal cells expressing NMDARs, whereas JNK inhibition abolished it completely. In agreement with the lack of p38 activation, excitotoxicity in non-neuronal cells was estimated to require a greater Ca$^{2+}$ influx that neurons. This deviation from neuronal excitotoxicity suggests that the p38 cascade requires a neuronal context for its induction as a pro-death signal, and is informative for further studies of this pro-death pathway in neurons. This method can also be used in further experiments to investigate other NMDAR-dependent signals and their requirement for neuronal signalling complexes.

Chapter 5. The PDZ binding domain at the very end of the NR2 C-terminus can associate with PSD-95, and the latter protein can assemble signalling complexes at the mouth of the NMDAR channel. Disruption of the PDZ-ligand by the cell-permeable peptide TAT-NR2B9c protects neurons against ischemic and excitotoxic damages. Results obtained by co-author Dr Francesc Soriano indicate that the PDZ-bound signalling complex triggers pro-death p38 activation, whereas JNK induction is independent of the PDZ ligand (Soriano et al., 2008). Considering that p38 was not activated during NMDAR-dependent toxicity in non-neuronal cells, the ability of the PDZ-ligand to mediate cell death may be absent in such context.

Thus, the role of the PDZ motif in recombinant NMDAR-mediated toxicity was investigated. Contrary to neurons, non-neuronal cells expressing NMDARs were not protected by TAT-NR2B9c. Moreover, truncation of the NR2B PDZ motif did not modify the acquired excitotoxicity.

Whilst these results confirmed the crucial role of the PDZ-ligand in neuronal excitotoxicity, it remained uncertain if disruption of NR2B/PSD-95 by TAT-NR2B9c altered other types of NMDAR-dependent signals. At the concentration at which it exerts neuroprotection, TAT-NR2B9c did not alter neuronal survival, excitability or normal synaptic transmission. It also did not interfere with a model of NMDAR-dependent synaptic potentiation, suggesting that the uncoupling of NMDARs and pro-death signalling did not affect the physiological functions of NMDARs in neurons.
Chapter 6. The ability of NMDARs to signal to both survival and death is not strictly dependent on the NR2 subunit composition, as it was presented in Chapter 3. However, the presence of different signalling complexes may still influence the ability of NR2A/B-containing NMDAR to induce excitotoxicity.

To investigate such a possibility, NR2A or NR2B subunits were overexpressed in hippocampal neurons. In both cases NR2 overexpression augmented the amount of functional NMDAR, however only NR2B overexpression increased excitotoxicity levels. To distinguish if the differential abilities to induce excitotoxicity were due to the NR2 subunit channel properties/agonist affinity or to differing intracellular interactions, chimeric NR2A/B with swapped C-termini were created. Swapping the C-terminal domains altered the toxicity in overexpressing neurons, such that the NR2B C-terminus augmented the toxicity compared to the NR2A C-terminus. However, changing the intracellular tail of the subunit did not completely invert the amount of cell death. The remaining influence may involve the rest of the subunit/channel, as the currents passing through the NR2B subtypes (mutated or not) were higher than for NR2A subtypes at the NMDA concentration used to induce excitotoxicity.

These results suggest that both the NR2 channel properties and the NR2 C-terminus contribute to the excitotoxicity. Since the NR2 C-terminus may bind to specific signalling complexes but also determine to localization of the NMDARs, further experiments are needed to investigate the influence of NR2 overexpression on synaptic and extrasynaptic NMDAR pools. Additionally, NR2 chimeras will be created with partial C-terminal domains swapped between NR2A and NR2B, in an attempt to identify the domains and signalling complexes involved in the differential viability signals. Determination of the cascades recruited by both subunits in excitotoxic conditions could allow for a more specific targeting of the pro-death signalling pathways downstream of NMDARs; a critical milestone in the search for an effective antiexcitotoxic strategy.


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