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Exzitotoxizität im ZNS bei Mausmutanten mit veränderten Glutamatrezeptoren

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1.Introduction

1.1 Preface

The great challenge of neural science is to elucidate the mechanism by which humans act, perceive, learn and remember. Nerve cells and their connectivity in the brain accomplish all of these processes. In order to understand how the brain integrates and stores perceptions, it is fundamental to study the mechanisms of neuronal signaling and also how connections between neurons are formed and modified by experience. Furthermore, a big challenge is to elucidate the mechanisms that might contribute to the pathogenesis of human central neuronal cell loss induced by acute insults such as hypoxia-ischemia, hypoglycemia, sustained epilepsy and brain trauma.

1.2 Signal transmission in the central nervous system

The central nervous system (CNS) consists of two major classes of cells: nerve cells (neurons) and glial cells (glia). The human brain contains 10^{11} neurons and 10x more glia. The traditional view is that glia provide structure to the brain, sometimes insulate neuronal groups and synaptic connections from each other. Certain classes of glia cells guide the migration of the neurons and direct the outgrowth of the axons. Furthermore, glia help to form the blood-brain barrier, remove cellular debris and secrete trophic factors. However, this view of glia acting only as support cells has recently been challenged. Recent studies suggest that glia respond to action potentials with a rise in intracellular Ca^{2+} (Dani et al., 1992), that they have the ability to modulate synaptic strength (Araque et al., 1998; Parpura and Haydon, 2000) and even that they are able to release neurotransmitter (Innocenti et al., 2000).

Nevertheless, the majority of fast signaling is mediated by neurons, which are classically divided into two functional classes: principal (or projection) neurons and interneurons. Neurons have a highly polarized structure consisting of the cell body (soma), dendrites for receiving signals from other neurons, the axon, which projects to target cells, and presynaptic terminals for neurotransmitter release at synapses with targets. Nerve cells transmit signals either electrically by directly exchanging ions via gap junctions (electrical synapses) or chemically by neurotransmitter release at

the specialized sites of contact with other neurons (chemical synapses). These chemical synapses consist of the presynaptic axon terminal and the postsynaptic dendrite or soma.

Nerve cells are able to transmit signals because of their ability to generate an action potential, a regenerative electrical signal, which spreads along the axon actively without attenuation. Generation of the action potential takes place in the axon hillock, which is the initial segment of the axon; the axon thus plays the role of the output element of the neuron, whereas the dendrites are input elements. However, the finding that following initiation, action potentials actively backpropagate into the dendrites, providing a retrograde signal of neuronal output to the dendritic tree, has extended the notion of dendrites as being input units (Stuart and Sakmann, 1994; Yuste and Tank, 1996; Johnston et al., 1996). Briefly, action potentials are mediated by sequential opening of voltage-activated Na^+ and K^+ channels. Voltage gated Na^+ channels have the highest open probability when the membrane potential is depolarized to a threshold level. This results in further depolarization and the membrane potential is driven toward the equilibrium potential for Na^+ (around +50 mV). The surrounding membrane, which also contains voltage-activated Na^+ channels, is subsequently equally depolarized resulting in a spread of the excitation. The inactivation of Na^+ channels and the opening of voltage-gated K^+ channels terminate the action potential. The subsequent outflow of K^+ repolarizes the membrane by restoring the initial charge distribution. When an action potential arrives at the presynaptic terminal, it opens the voltage-dependent Ca^{2+} channels and the resulting Ca^{2+} influx triggers neurotransmitter release from the presynaptic vesicles into the synaptic cleft. The neurotransmitter diffuses across the synaptic cleft and binds to its postsynaptic receptors leading to an opening or closing of the ion channels, thereby altering the membrane conductance and the potential of the postsynaptic cell.

Receptors are divided into two classes: ionotropic and metabotropic. Ionotropic receptors are membrane proteins that contain an ion channel. In contrast, metabotropic receptors act indirectly by activating a G-protein-coupled second messenger cascade that modulates channel activity. Whereas ionotropic receptors mediate fast synaptic activity in the millisecond range, metabotropic receptors mediate synaptic actions in the second-to-minute range, often associated with changes in neuronal excitability and synaptic strength.

1.3 Excitatory and inhibitory synaptic transmission

In the CNS, synaptic transmission can be either excitatory or inhibitory. Glutamate is the main excitatory neurotransmitter in the vertebrate brain. After release from the nerve terminals, glutamate crosses the synaptic cleft and activates three different types of ionotropic receptors: L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors.

AMPA and kainate receptors are responsible for fast ionic inward current, thereby contributing to the early peak of the excitatory postsynaptic potential (EPSP). NMDA receptor channels are blocked by Mg^{2+} at resting membrane potential and are relieved from Mg^{2+} block when the membrane is depolarized (Mayer et al., 1984; Nowak et al., 1984). Thus, under physiological ionic conditions, both glutamate and depolarization of the membrane are necessary to open the NMDA receptor channel. Since NMDAR channels activate and deactivate relatively slowly compared to non-NMDAR channels, NMDA receptors contribute only to the late component of the EPSP.

Another class of glutamate receptors is represented by metabotropic receptors, which act on ion channels via a cascade of second messengers. Whereas glutamate always has an excitatory effect via ionotropic glutamate receptors (iGluRs), via metabotropic glutamate receptors (mGluRs) it can produce either excitation or inhibition.

The main inhibitory transmitters in the CNS are γ-Aminobutyric acid (GABA) and glycine. GABA receptors are divided into ionotropic $GABA_A$ receptors and metabotropic G-protein coupled $GABA_B$ receptors. $GABA_A$ receptors form Cl^- permeable channels, which open upon binding of GABA, thereby hyperpolarizing the membrane. Excitatory synapses are typically located on the dendrites (often on protrusions called spines and more rarely directly on the shaft), whereas inhibitory synapses can often be found on the soma and on the dendritic shafts. Thus, spatially and temporally distinct excitatory and inhibitory signals (EPSPs and respectively IPSPs) will sum within a neuron and be either sub- or suprathreshold for the generation of an action potential at the axon hillock.

1.4 Ionotropic glutamate receptors

1.4.1 Classification of ionotropic glutamate receptors

The majority of excitatory neurotransmission in the brain is mediated by ionotropic glutamate receptors. Ionotropic glutamate receptors (GluRs) are ligand-gated ion channels, which are selectively permeable for cations, principally Na^+, K^+ and sometimes Ca^{2+} ions. Although the majority of ionotropic GluRs is expressed in the central nervous system, there is evidence that subpopulations exist in the pancreas (Inagaki et al., 1995), osteoclasts and osteoblasts (Chenu et al., 1998), skin (Ault and Hildebrand, 1993) and cardiac ganglia (Gill et al., 1998). Numerous glutamate receptor-like (GLR) genes have been identified in plant genomes, and plant GLRs are predicted, on the basis of sequence homology, to retain ligand-binding and ion channel activity (Davenport, 2002).
On the basis of their responsiveness to certain glutamate derivatives, ionotropic GluRs are classified as AMPA, NMDA and kainate receptors.
AMPA receptor (AMPAR) channels are heteromers of the GluR-A to GluR-D subunits, kainate receptors are subdivided into GluR-5 to GluR-7, KA-1 and KA-2 (Hollmann and Heinemann, 1994; Wisden and Seeburg, 1993), and the subfamilies of NMDA receptors (NMDARs) will be explained in detail below. A fourth class of ionotropic GluR is represented by the δ1 and δ2 receptors which share between 18 to 25 % sequence identity with the other GluR subunits. When expressed in a heterologous system, they do not bind glutamate and do not form functional channels, neither alone nor with other GluRs (Lomeli et al., 1993; Araki et al., 1993). However, they seem to play an important role, since a point mutation in δ2 receptors leads to the phenotype of “lurcher mice”, with spontaneous degeneration of Purkinje cells and cerebellar ataxia (Zou et al., 1997).

1.4.2 Sructure of ionotropic glutamate receptors

Ionotropic GluRs share the same transmembrane topology, but differ in their pharmacological and kinetic profile, ion permeability, expression patterns and trafficking. They typically consist of three transmembrane domains (M1, M3, M4) and a re-entrant membrane loop (M2) (Fig.1). The N-terminus is located on the extracellular side and controls proper assembly of receptor complexes (Ayalon and Stern, 2001).

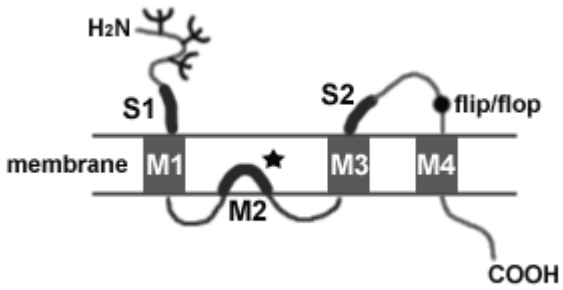

Fig. 1: *Schematic structure of an ionotropic glutamate receptor subunit*
The three transmembrane domains M1, M3 and M4 are shown as gray boxes, the membrane loop M2 forms the channel pore. The star indicates the position of key amino acids regulating the ion selectivity, e.g. the Q/R for AMPA, N for NMDA receptors. S1 and S2 designate the two ligand-binding domains. The alternatively spliced flip/flop exon occurs in AMPA receptor and is located extracellulary. Potential glycosilation sites are shown as trees in the N-terminal region (Ayalon and Stern, 2001).

The carboxy-terminal (C-terminal) domain is located on the intracellular side. It influences receptor function (Sprengel et al., 1998) and interacts with trafficking, motor, signaling and scaffolding proteins, assuring proper receptor localization and regulation (Barry and Ziff, 2002). The agonist-binding domain consists of two segments (S1 and S2) that are formed by the region preceding the M1 domain and the loop between the M3 and M4 domain. The S1 and S2 lobes form a clamshell structure that undergoes a conformational change to enclose the ligand upon binding (Stern-Bach et al., 1994; Armstrong et al., 1998).

1.4.3 The Q/R/N site of ionotropic glutamate receptors

The Q/R/N site of the M2 segment is occupied by a glutamine (Q) or an arginine (R) residue in AMPA and kainate receptors and by asparagine (N) in NMDA receptors. Site-selective RNA editing (Sommer et al., 1991; Higuchi et al., 1993) of the GluR-B pre-mRNA results in an arginine residue in 99 % of the GluR-B population, whereas the other three AMPAR subunits feature glutamine at this site. Heteromeric AMPAR channels containing one GluR-B(R) subunit are impermeable to Ca^{2+} (Burnashev et al., 1992; Hume et al., 1991), demonstrating the dominance of GluR-B(R) in terms of Ca^{2+} permeability. In principal neurons most AMPAR channels are Ca^{2+}-impermeable, whereas

interneurons express Ca^{2+}-permeable AMPAR channels. The Q/R/N site furthermore affects single channel conductance, blockade by intracellular polyamines and permeation of anions (Hume et al., 1991; Swanson et al., 1996; Ruppersberg et al., 1993). In the NMDAR, this critical channel site does not only control Ca^{2+} permeability but also voltage-dependent block by Mg^{2+} and inhibition by protons, Zn^{2+} and MK-801 (Burnashev et al., 1992; Kashiwagi et al., 1997; Sakurada et al., 1993).

1.4.4 Subunit stoichiometry

Functional and crystallographic studies show that GluR channels most probably consist of four subunits assembled as a dimer-of-dimers (Premkumar and Auerbach, 1997; Rosenmund et al., 1998; Laube et al., 1998; Sobolevsky et al., 2004). In the case of the NMDA receptors, each channel contains two NR1 and two NR2 subunits (Behe et al., 1995; Sheng et al., 1994). The specific subunit composition not only determines the biophysical characteristics of the GluR channel but also its trafficking during basal transmission and synaptic plasticity (Barry and Ziff, 2002; Barria and Malinow, 2002). For example, AMPA receptors assembled from GluR-B and GluR-C subunits are constitutively delivered to the postsynaptic membrane, whereas delivery and insertion of GluR-A and GluR-B heteromers is regulated by synaptic activity (Hayashi et al., 2000; Shi et al., 2001).

1.5 The NMDA receptor

1.5.1 Role of the NMDA receptor during synaptic transmission

During synaptic transmission, one of the most noticeable features of the NMDAR-mediated component of the synaptic current is its slow rise and decay, compared with the much faster AMPAR-mediated component. NMDAR channels are Ca^{2+}-permeable and exhibit voltage-dependent block by Mg^{2+}. These properties make them key players in synaptic development, synaptic plasticity and excitotoxic cell death. It is generally agreed that the glutamate concentration rises rapidly to millimolar levels in the synaptic cleft with a time constant of about 1 ms. However, NMDAR channels open roughly 10 ms after glutamate has been released and continue to open and close repeatedly for several hundred milliseconds (Dzubay and Jahr, 1996; Behe et al., 1999). The

slow decay time therefore reflects, in part, the high affinity of NMDARs, with glutamate generating exceptionally prolonged channel activity until the transmitter molecules finally dissociate.

1.5.2 Modulation and pharmacology of NMDAR channel function

The NMDAR family appears unique among ligand gated ion channels and requires the binding of both the transmitter (glutamate) and the coagonist (glycine) for its activation (Dingledine et al., 1999). In addition, a variety of molecules present in the extracellular milieu bind to, or interact with these receptors, influencing their behavior (Fig. 2).

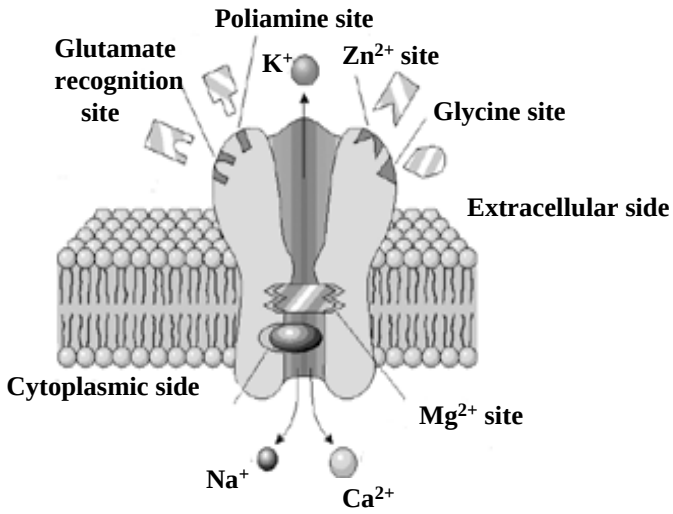

Fig. 2: *Schematic representation of the NMDA receptor complex*

The NMDA receptor complex possess a glutamate recognition site to which receptor agonists and competitive antagonists bind, as well as other binding sites for glycine, polyamines, Mg^{2+} and Zn^{2+}. Channel opening permits an influx of Na^{+} and Ca^{2+} ions, and an efflux of K^{+} ions (After Scatton, 1993).

Furthermore, at the cell's resting membrane potential, Mg^{2+} ions bind to a site located deep within the ion channel to occlude the flow of cations (Johnson and Ascher, 1990; Li-Smerin and Johnson, 1996). Activation of the channel requires depolarization in order to relieve the blockage by Mg^{2+} ions. NMDAR channels can be blocked pharmacologically by the competitive antagonist D-5-amino phosphonate pentanoic acid or by the open channel blocker MK-801. The non-competitive

antagonist ifenprodil acts through the proton sensor of the NR1 subunit and has an IC_{50} (concentration producing half-maximal inhibition) that is 400-fold lower for NR2A- than for NR2B-containing receptors (Cull-Candy et al., 2001). Many of the important NMDAR properties are influenced by the subunit composition of the receptor.

1.5.3 NMDAR subunits and splice variants

NMDARs are divided into three classes: the ubiquitously expressed NR1 subunit, a family of four distinct NR2 subunits (A, B, C and D), and two NR3 subunits (A, B) (Hollmann, 1999; Moriyoshi et al., 1991; Monyer et al., 1992,1994; Sugihara et al., 1992; Das et al., 1998). NR1 exists as eight distinct isoforms owing to the presence of three independent sites of alternative splicing (Dingledine et al., 1999). Similarly, each of the NR2 and NR3 subunits (apart from NR2A) has several splice variants, although the functional relevance of the different splice variants remains unclear. To form a functional NMDAR channel, NR1 and NR2 subunits are required (Dingledine et al., 1999; Meguro et al., 1992; Monyer et al., 1992), with NR1 containing the binding site for the coagonist glycine and NR2 containing the glutamate-binding site (Anson et al., 1998; Laube et al., 1998; Kuryatov et al., 1994). The NR3 subunit can co-assemble with NR1/NR2 and seems to confer a modulatory function on channel properties (Perez-Otano et al., 2001; Matsuda et al., 2002). NR3B-containing receptors are not permeable to Ca^{2+} and show little of the Mg^{2+}-dependent blockade that is characteristic of NMDA receptors (Chatterton et al., 2002). Neurons have a large excess of the NR1 subunits relative to NR2 subunits; this pool of NR1, which is unassembled with NR2, does not reach the cell surface and is rapidly degraded (Chazot and Stephenson, 1997a; Huh and Wenthold, 1999). It has been hypothesized that unassembled NR1 and NR2 subunits are being retained in the endoplasmic reticulum until assembly, which then allows exit and expression on the cell surface (Wenthold et al., 2003).

1.5.4 Developmental profile of NMDAR subunits and effect on kinetics

Whereas NR1 is continuously and ubiquitously expressed in the CNS, expression of the distinct NR2 subunits depends on the developmental stage and brain region (Monyer et al., 1994; Akazawa et al., 1994). NR2B and NR2D subunits predominate in the neonatal brain, whereas NR2A and NR2C are

expressed during postnatal development. The NR2A and NR2B subunits are the most widespread NR2 subunits; the NR2C subunit is largely restricted to the cerebellum. In CA1 and CA3 pyramidal neurons the NR2B subunit is expressed prenatally, whereas the NR2A subunit progressively increases its expression (Monyer et al., 1994; Sheng et al., 1994) and synaptic incorporation during postnatal development (Stocca and Vicini, 1998; Tovar and Westbrook, 1999) (Fig.3).

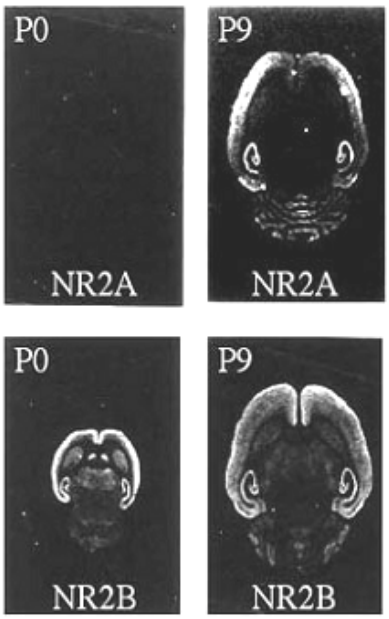

Fig. 3: *Developmental NMDA subunit gene expression in the rat brain (X-ray film autoradiographs)*

NR2A mRNA is widely expressed in the adult brain; NR2B mRNA is mainly forebrain specific: hippocampus and neocortex. NR2A gene expression increases strongly during postnatal development in neocortex, hippocampus and cerebellar granule cells. In contrast, during same postnatal periods, NR2B expression stay either constant, increases but less so than that of NR2A, or decreases (e.g. in cerebellar granule cells) (Monyer et al., 1992).

The gradual replacement or supplementation of NR2B by NR2A increases the decay time of synaptic NMDA-mediated currents (Cull-Candy et al., 2001). Although NR2A subunits are incorporated synaptically late in development, NR2B subunits are still highly persistent at extrasynaptic sites (Stocca and Vicini, 1998; Rumbaugh and Vicini, 1999; Tovar and Westbrook, 1999).

Immunoblot analysis, using antibodies against distinct NMDA receptor subunits, indicates that the NR2A and NR2B subunit proteins have developmental profiles in cultured cortical neurons similar to those seen *in vivo*. NR1 and NR2B subunits display high levels of expression within the first week. In contrast, the NR2A subunit is barely detectable at 7 days *in vitro* (DIV) and then gradually increased to mature levels at DIV21 (Li et al., 1998) (Fig 4).

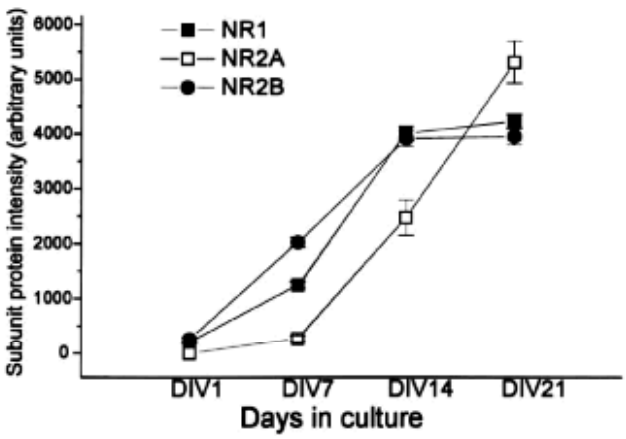

Fig. 4: *Developmental expression pattern of NR2A and NR2B subunits in vitro*
Protein expression for all NMDA receptor subunits increases with development. While the NR1 and NR2B subunits are expressed as early as DIV1, the NR2A subunit is barely detectable at DIV7. Furthermore, while the expression of NR1 and NR2B subunits reach a plateau at DIV14, the expression of NR2A subunit increases further from DIV14 to DIV21 (Li et al., 1998).

When expressed recombinantly, the different NR2 subunits confer different properties to the NMDAR channel, with the NR2A subunit increasing overall macroscopic current amplitude, accelerating deactivation and increasing desensitization (defined as inactivation in the presence of the agonist) and the NR2B subunit having the opposite effects (Monyer et al., 1992; Vicini et al., 1998). However, some native NMDAR channels contain more then one type of the NR2 subunit in the same assembly (Dunah et al., 1998; Sheng et al., 1994; Chazot and Stephenson, 1997b; Luo et al., 1997). Although studies in a heterologous system suggest the formation of functional

triheteromeric assemblies of NR1/NR2A/NR2B (Vicini et al., 1998; Tovar and Westbrook, 1999), it is still unknown to what extent these assemblies form *in vivo*.

NR3B is expressed postnatally in ventral horn motor neurons where it might participate in transmission in the spinal cord, a region in which glycine has long been known to have synaptic actions (Chatterton et al., 2002).

1.5.5 The C-terminal tail of the NMDAR

The critical role of the C-terminal domain in receptor trafficking and stabilization at synaptic sites has been shown not only for $GABA_B$ and AMPA receptors (Calver et al., 2001; Osten et al., 2000; Pagano et al., 2001; Passafaro et al., 2001; Shi et al., 2001a), but also for NMDARs (Mori et al., 1998; Sprengel et al., 1998; Steigerwald et al., 2000; Sheng and Pak, 2000). NR2A and NR2B both contain very long C-terminal tails (627 and 644 amino acids), including a PDZ binding domain as a site for protein-protein interactions (Kornau et al., 1997). The NR1 C-terminal tail is much shorter (105 amino acids) and the predominant splice variant in the hippocampus (NR1-1a) has no PDZ binding motif (Laurie and Seeburg, 1994; Nakanishi et al., 1992).

The C-termini of NR2 subunits of NMDA receptor interact with the PDZ domains of membrane-associated guanylate kinases (MAGUKs), which include postsynaptic density-95 (PSD-95), PSD-93 and synapse-associated protein (SAP102) (Garner et al., 2000; Sheng, 2001; Tomita et al., 2001) (Fig. 5). MAGUKs, which are linked to a number of other postsynaptic proteins, mediate interactions with signal transduction molecules and may function to retain NMDA receptors at synapses (Kennedy, 1998).

The C-terminus of the NR2 subunits has been also implicated in controlling the endocytosis rate (Roche et al., 2001). Truncation of large regions of the NR2A C-terminus in gene-manipulated mice causes a loss of synaptic enrichment of the NR2A subunit and the appearance of NMDA EPSCs with smaller amplitudes and slower kinetics (Steigerwald et al., 2000), indicating that the C-terminus of NR2 subunits might be critical for normal synaptic localization.

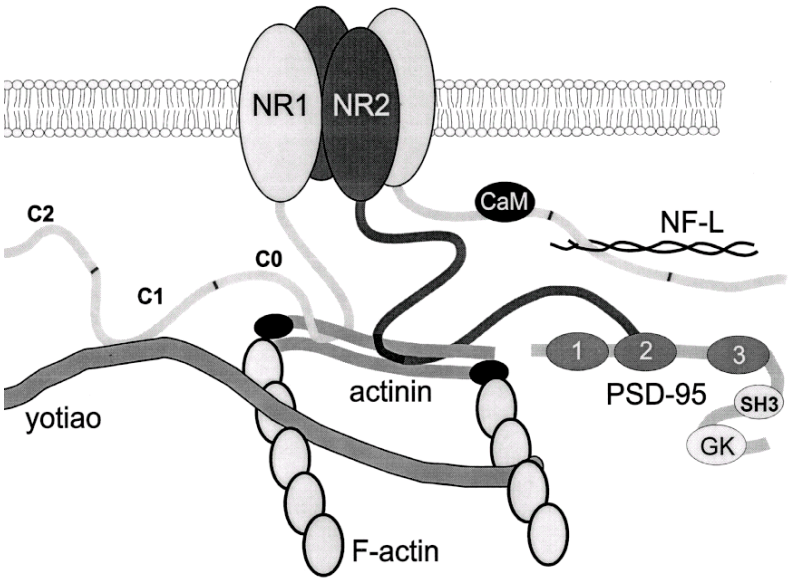

Fig. 5: *Multiple mechanisms for anchoring NMDA receptors*
The NMDA receptor, here depicted as a heteromultimer of NR1 and NR2 subunits within the cell membrane, is anchored to various structural elements via its C-terminal tails. NR1 interacts through its invariant C0 exon to the actin-binding protein • -actinin, which is competitively inhibited by calmodulin (CaM) binding to the same C0 region. The alternatively spliced C1 exon of NR1 binds both to the putative cytoskeletal-associated protein yotiao and to the neurofilament subunit NF-L. NR2 subunits interact with PSD-95 and the NR2B subtype can also bind to • -actinin. PDZ domains of PSD-95 are labeled 1, 2, and 3 (adapted from Sheng & Pak).

The C-terminal tails of NR2A and NR2B are potential targets for tyrosine kinases, CaM kinase II (CaMK II) and protein kinases A and C (Moon et al., 1994; Wang and Salter, 1994; Köhr and Seeburg, 1996; Omkumar et al., 1996; Leonard and Hell, 1997; Tingley et al., 1997).
Studies in mice lacking the NR2A C-terminal domain ($NR2A^{\Delta C/\Delta C}$) suggest that C-terminal signaling events are also critical for LTP induction (Kohr et al., 2003).

1.5.6 Mice expressing C-terminally truncated NR2 subunits

Mice that express NMDAR subunits lacking a large intracellular C-terminal receptor domain were constructed by replacing in embryonic stem cells the C-teminal exons of the NR2A and NR2B

subunit genes with the neomycin phosphotransferase (neo) gene preceded by in-frame translational stop codons (Sprengel et al., 1998) (Fig. 6).

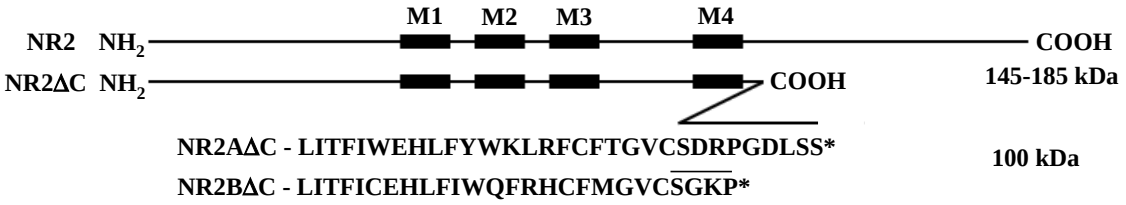

Fig. 6: *NR2 subunits, full-length and truncated forms*

The black boxes indicate the hydrophobic segments M1-M4 and the C-terminal aminoacids are listed below. The overlying refer to the synthetically introduced residues (Sprengel et al., 1998).

Phenotypically these NR2$^{\bullet C/\bullet C}$ mice resemble those mice that are deficient in that particular subunit. Thus, mice expressing the NR2B subunit in a C-terminally truncated form (NR2B$^{\bullet C/\bullet C}$ mice) die perinatally, and NR2A$^{\bullet C/\bullet C}$ mice are viable but exhibit impaired synaptic plasticity and contextual memory, indicating that the deletion of the C- terminal domain induced a functional knock-out of the receptor. This functional NMDA receptor knock-out was not due to a lack of functional NMDA receptor channels but appeared to reflect defective intracellular signalling of activated NMDA receptors. The study with these mice clearly demonstrated that the intracellulary located C-termini of the NR2 subunits are indispensable for the physiological functionality of the respective NMDA receptor subtypes.

1.5.6 NMDAR subunits and synaptic plasticity

It has been hypothesized that the developmental switch from NR2B to NR2A at synaptic sites may be responsible for the decreased plasticity observed in older animals (Carmignoto and Vicini, 1992; Crair and Malenka, 1995). Interestingly, mice overexpressing NR2B show enhanced long-term potentiation (LTP) and improved performance in behavioural tests for learning and memory (Tang et al., 1999), which might be useful for developing strategies to combat cognitive disabilities. Mice with a targeted disruption of the NR2A subunit have reduced LTP and deficiencies in certain learning tests (Sakimura et al., 1995).

1.5.7 NMDARs and disease

Ca^{2+} influx via NMDAR channels is believed to be essential in triggering synaptic plasticity, but excessive Ca^{2+} influx can cause neuronal death. Consequently, blocking NMDARs is neuroprotective in animal models of stroke and seizure (Lee et al., 1999), making NMDAR antagonists a promising tool for treating these diseases. Interestingly, studies which used this strategy found that some non-competitive antagonists elicited dopaminergic hyperactivity and schizophrenic behavior (Rowley et al., 2001). In addition, mice with reduced NR1 expression (~5 % of normal level, Mohn et al., 1999) or NR2A knock-out mice (Miyamoto et al., 2001) show symptoms associated with schizophrenic behavior. NR2B antagonists were also suggested for pain treatment, since overexpression of NR2B leads to increased sensitivity to inflammatory pain (Wei et al., 2001). The NMDAR blocker amantadine is used in Parkinson therapy, although it is unclear whether its therapeutic effect is caused by the NMDAR blockade (Blanpied et al., 1997).

Stroke is perhaps the condition with the stronger evidence for the role of excitotoxic mechanism, therefore high expectations were directed towards therapy based on blocking excitatory amino acid receptors. Unlike other organs, the brain can neither synthesize nor store energy reserves, and relies exclusively on blood-borne glucose for its energy source. Any interruption in cerebral blood flow will lead rapidly and irrevocably to energy depletion and a dramatic fall in the intracellular levels of ATP. The immediately consequences is an increase in the concentrations of extracellular glutamic acid followed by cerebral ischaemia. In a rat model of focal ischaemia, the extent of the rise in extracellular glutamic acid has been shown to be correlated with the size of the lesions that subsequently develop (Benveniste et al., 1984).

1.6 Excitotoxic cell death

1.6.1 Basic characteristics

Excitotoxicity is a term originally used by John Olney to refer to the ability of glutamate and structurally related amino acids to destroy neurons (Olney, 1968). The basic nature of excitotoxicity has been well established by *in vivo* studies beginning more then 20 years ago (Coyle et al., 1981; McGeer and McGeer, 1982). High systemic doses of glutamate produce characteristic pathological changes in the circumventricular regions of young rodent or monkey brains, for example, the arcuate nucleus of the hypothalamus (Olney, 1969; Olney et al., 1972). Within 30 minutes of glutamate

administration, electron microscopy reveals massive acute swelling of neuronal cell bodies and dendrites, whereas axons and terminal buttons originating from cell bodies outside of the affected region remain largely intact. These *in vitro* studies support the notion that injury may result from the glutamate-induced overexcitation, and suggest also that a significant injury component may be specifically triggered by excess of Ca^{2+} influx.

1.6.2 Mechanism of excitotoxicity

In vitro studies of excitotoxicity using cell culture models have implicated the NMDA receptor subtype as being the principal vehicle of excitotoxic damage. In most cell culture models, it appears that the excitotoxic effect of glutamic acid can be blocked by NMDA receptor antagonists (Choi et al., 1988). In classical excitotoxicity experiments, elevated levels of extracellular glutamic acid cause persistent depolarization of the neuron. This triggers a cascade of intracellular events that eventually lead to cell death: sodium influx (A), calcium influx (B) and the glutamatergic loop (C). These three elements are not independent and occur in parallel. Sodium entry is responsible for early necrotic events, calcium entry for delayed neurodegenerative events, and exocytosis of glutamic acid for spread and amplification of the degenerative process.

A. Depolarization is initiated primarily by activation of AMPA receptors and subsequently by activation of voltage-dependent sodium channels. This leads to sodium entry and further depolarization. The entry of sodium ions is followed by a passive entry of chloride ions in order to maintain ionic equilibrium (Rothman, 1985). This is followed by entry of water, due to the osmotic gradient, that causes an increase in cell volume (osmotic swelling) and a dilution of the cytoplasmic contents, leading to disruption of organelles. The final consequence of osmotic swelling is cell lysis and the release of the cell content into the extracellular milieu. This osmotic component of excitotoxicity is potentially reversible if the depolarizing stimulus is removed (Choi, 1987).

B. Intracellular calcium levels are usually very low (~10^{-7} M) but when the cell is excessively depolarized the Ca^{2+} levels rise persistently. The principal source of elevated intracellular free Ca^{2+}, in the presence of extracellular glutamic acid, is through the opening of NMDA receptor channels, whose Mg^{2+} block will have been relieved upon depolarization. Using cell culture models, it has been demonstrated that, at least *in vitro*, removal of extracellular calcium from the extracellular milieu prevents the neurotoxicity of glutamic acid (Choi, 1985). However, the ability of neurons to withstand excitotoxic damage may well be related to their ability to buffer intracellular calcium

(Iacopino et al., 1992; Lukas and Jones, 1994). The rise in intracellular free Ca^{2+} concentration stimulates the activity of numerous enzymes and triggers other Ca^{2+} dependent protein-protein interactions that are ultimately deleterious to cell homeostasis, leading to neuronal death. Nucleases disrupt the organization of chromatin in the nucleus and fragment DNA (Orrenius et al., 1989), cytosolic proteases such as calpain attack the cytoskeleton (Siman et al., 1989; Mills and Kater, 1990), Ca^{2+} dependent cytosolic kinases modify the phosphorylation state on cytoplasmic proteins and thus disrupt cell function (Favaron et al., 1990). A final consequence of the elevation of the intracellular Ca^{2+} concentration is the activation of the exocytosis of neurotransmitter-containing vesicles from nerve endings.

C. During the course of excitotoxicity, there is a strong increase in the extracellular concentrations of glutamic acid. The principal cause is the release of intracellular cytosolic glutamic acid due to cell lysis, slowing of glutamic acid transport subsequent to depolarization and the Ca^{2+}-dependent exocytosis of synaptic vesicles. The large concentrations of glutamic acid can then diffuse towards other neurons and depolarize them in their turn. The extracellular glutamic acid thus plays a facilitator role, further depolarizing already weakened neurons and spreading the depolarization to healthy neurons in the vicinity. Such elevations of extracellular glutamate concentrations have been well documented in cerebral ischaemia (Benveniste et al., 1984).

1.6.3 Clinical trials

Over the last 5 years, several clinical trials have been undertaken in stroke with NMDA receptor antagonists. The results of these studies have been disappointing. Although results of pilot studies did suggest some efficacy, larger controlled trials have failed to demonstrate a clear therapeutic benefit. In most cases, the side effects of the drug used and, in some cases, increased mortality have preempted continued clinical research. These side effects include CNS depression and phychodysleptic effects. Three low-affinity NMDA receptor antagonists, sinnabidol (Belayev et al., 1995), ARL 15896 (Cregan et al., 1997) and MS-153 (Umemura et al., 1996) remain in clinical development for stroke and it remains to be seen whether any clinical benefit will overcome potential safety concerns from associated side effects.

1.6.4 Role of the NR1 subunit in excitotoxicity

Cultured neurons obtained from NR1 knock-out mice are resistant to NMDA- and glutamate-induced excitotoxicity (Tokita et al., 1996). Similar results were obtained in cultures by suppressing NR1 expression with antisense oligonucleotides (Wahlestedt et al., 1993), a procedure that also reduces focal ischaemic infarction *in vivo* (Wahlestedt et al., 1993*).* Investigations using mice expressing NR1 mutations in the site critical for Ca^{2+} permeation (N598Q and N598R) showed that NMDA receptor-mediated signaling was perturbed, and the mice failed to develop autonomic functions, such as feeding and breathing (Single et al., 2000). All these observations can only suggest that functional NMDA receptors are necessary for triggering receptor-dependent excitotoxicity, but cannot indicate whether the NR1 subunit plays a distinct role in mediating neuronal death or survival.

1.6.5 Role of the NR2 subunits in excitotoxicity

It is well documented that immature cultured neurons are less vulnerable to glutamate neurotoxicity (Choi et al., 1987; Barish et al., 1991; Marks et al., 1996; Wahl et al., 1989). This vulnerability parallels the temporal expression pattern of different subunits, as the expression of NR2B and NR2D begins at least as early as E14, whereas NR2A and NR2C are first detected postnatally (Monyer et al., 1994; Sheng et al., 1994; Wenzel et al., 1997). Thus, a developmental switch in NR2 subtype expression may underlie the increase in susceptibility to excitotoxicity over time. This developmental switch in NR2 subtype expression is dependent on neuronal activity (Audinat et al., 1994; Lindlbauer et al., 1998). It was demonstrated (Mizuta et al., 1998) that cortical neurons exhibited increased glutamate sensitivity on day 11 in culture, whereas they were not affected by glutamate on culture days 7-9. The authors concluded that glutamate neurotoxicity in these neurons was mainly mediated by a heteromeric NR1-NR2B receptor, as they detected levels of NR2B and NR1 on both days (8 and 11), whereas NR2A protein levels were hardly detectable at these ages *in vitro*.

The development of mutant mice provides further insights into the role of distinct NR2 subunits in neurotoxicity. Morikawa et al. (Morikawa et al., 1998) investigated the role of NR2A and NR2B subunits in brain ischaemia using mutant mice deficient in NR2A (Sakimura et al., 1995; Kiyama et al., 1998) and double mutants deficient in NR2A and NR2B. NR2A deficiency resulted in a pronounced reduction in infarct volume. Infarct volume in mice lacking both NR2A and NR2B was

not different than in NR2A deficiency alone. The authors suggested that NR2A subunits play an important role in glutamate neurotoxicity.

Alternatives to knock-out mice are mutant mice expressing truncated NR2 subunits. Most express gateable receptors that are synaptically activated, but are defective in intracellular signaling and synaptic localization (Sprengel et al., 1998; Mori et al., 1998; Steigerwald et al., 2000). These data suggest that the different NR2 subunits link NMDARs to downstream molecules that mediate cellular responses to ions entering through the NMDAR channel.

1.7 The aim of this study

Since excitotoxicity has been implicated in a variety of neuropathological conditions, understanding the pathways involved in this type of cell death is of critical importance to the future clinical treatment of many diseases. The N-methyl-D-aspartate (NMDA) receptor has become a primary focus of excitotoxic research because early studies demonstrated that antagonism of this receptor subtype was neuroprotective.

Due to the high permeability to Ca^{2+}, NMDA receptors are playing a critical role in neuronal cell death. Extensive studies have documented that excitotoxicity is mediated by NMDA receptors due to an excessive Ca^{2+}-influx triggered by prolonged NMDA channel activation. However, the contribution to excitotoxicity by the two major NR2 NMDA receptor subtypes, NR2A and NR2B, has remained enigmatic.

This question is investigated in primary cortical cell cultures prepared from wild-type and gene-manipulated mice expressing NR2A or NR2B subunits with altered signaling characteristics using excitotoxicity experiments, Ca^{2+} influx analysis, subtype-specific pharmacological tools and electrophysiological measurements.

Due to the lack of effective treatment in stroke therapy, research in the field of the NMDA receptor antagonists has been extremely active in the last decade. This study indicates that both NR2A and NR2B subunits are playing a critical role in excitotoxicity, and any NMDA receptor subtype specific therapeutic intervention in stroke would lead to a failure.

2. Materials and Methods

2.1 Cell culture

In order to prepare primary neuronal cell cultures from gene-manipulated mice, cortical cells from each individual embryo were dissociated and plated separately. After culturing the cells, the body of each embryo was used to genotype by PCR analysis the heterozygous, homozygous and wild-type sister cultures.

2.1.1 Plates preparation

Glass coverslips placed into culture wells (24 wells plate, Greiner) were coated each with 300 µl neuron-friendly substrate poly-D-lysine (5 mg/ml). The plates were kept in the 5 % CO_2 incubator for 24 hours. The poly-D-lysine substrate was washed two times with 1xPBS and the plates were allowed to dry about two hours before plating the dissociated cortical neurons.

2.1.2 Dissection

The pregnant mouse was euthanized with halothane. Before making any incision, the abdomen was wiped with 70 % ethanol. In order to minimize possible contamination from the fur or skin, the first cut was done through the skin; the instruments were then rinsed with 70 % ethanol and the cut through the abdominal wall was performed. The two horns of the uterus were gently removed and placed in a sterile Petri dish containing cold PBS/ HEPES/ Glucose solution (4ºC). The remaining steps were carried out in a laminar flow hood. The fetuses were removed from the uterus, decapitated and the heads were transferred into a 50 ml Flacon tube containing cold PBS/ HEPES/ Glucose solution (4ºC). The heads of the embryos were grasped firmly with a pair of Durmont-style forceps by inserting the tips of the forceps deeply into the orbits. Using fine scissors, a midline incision through the skin and skull was performed, beginning at the level of the decapitation and continuing forward to the orbits. Then, with a second pair of forceps, this tissue was reflected away to each side

so that the entire cortex was revealed. The brain was removed and transferred to a dissecting dish by inserting the flat face of the forceps beneath the olfactory bulbs.

The hippocampi are C-shaped structures on the medial aspect of the cerebral hemispheres and were removed in the next steps under a dissecting microscope at 10 to 15x magnification. The procedure started by separating the cerebral hemispheres from the diencephalon and brainstem. With the basal aspect of the brain facing up, the next cut was done along the boundary between the diencephalon and the cerebral hemispheres. One blade of the forceps was placed into the space between the hemisphere and diencephalon at the posterior pole, and then the cut was done forward and medially around the diencephalon. The hemispheres were then separated so that the position of the hippocampus on the medial aspect it could be seen. The brainstem and the diencephalon were discarded.

In the next steps, the meninges and the choroid plexus were removed. The meninges form a thin layer covering the surface of the brain and they can be easily recognized by the blood vessels, being a highly vascularized structure. The choroid plexus lies within the ventricle, attaching to the meninges at the fimbrial edge of the hippocampus. The hemispheres were stabilized with a pair of forceps and with the help of another pair of forceps the meninges was grasped and tugged gently. By beginning at one end of the hippocampus, it is usually possible to pull away the meninges overlying the hippocampus as a single sheet. This procedure was done very gently, otherwise the traction on the meninges could tear the underlying hippocampus and its removal became more difficult.

Once the meninges had been removed, the hippocampus was cut out. The two cerebral hemispheres were lifted with the tip of the closed forceps and transferred into a 15 ml Falcon tube containing cold PBS/ HEPES/ Glucose solution (4ºC).

2.1.3 Enzymatic digestion of the cortex

In the next step, the PBS/ HEPES/ Glucose solution was replaced with PBS cold solution (4ºC). Using a 5 ml sterile pipette the cerebral hemispheres were transferred into a Petri dish and with a sharp scalpel the tissue was chopped into very small pieces. Using the same pipette, the chopped tissue was placed back into the 15 ml Falcon tube containing cold PBS solution (4ºC). This solution was then fast replaced with 1.5 ml 2.5 % Trypsin and incubated at 37ºC for 7 minutes. During this time the tube was shacken gently to prevent the setting of the tissue at the bottom of the tube and to

allow a better contact between the tissue and the enzyme. After the incubation, the trypsin was washed out two times with 5 ml warm PBS solution (37ºC) and then replaced with 1.5 ml Neurobasal Media. Using a regular Pasteur pipette with a fire-polished tip (to avoid mechanical tissue disruption) the tissue was dissociated by pipetting 10 to 12 times up and down. This step was followed by a second dissociation, in which the fire-polished Pasteur pipette had a smaller diameter, about half size than the original one. This step allowed a better dissociation of the tissue up to individual cells.

2.1.4 Plating the cells

The density of the cells was determined using a hemacytometer. Only the viable cells were counted; these cells were easily recognized using a drop of 0.8 % solution of Trypan blue to a small volume of cell suspension. After 4 minutes the viable cells (the dye-excluding cells) were counted. Under normal conditions, 85 % to 95 % of the cells should exclude Trypan blue. Plating of cells was done rapidly to prevent the pH of the medium to become basic. The plating density used for all the experiments was $3x10^5$ cells/ ml.

2.1.5 Feeding and maintenance of neuronal cultures

Cultures were kept at 37ºC in a 5 % CO_2 incubator. The cultures were fed 24 hours after plating the cells and the medium was changed every four days. During feeding, the culture medium was never changed completely; the neurons depend on the conditioning of the medium by glial cells for their long-term survival. At each feeding only half of the medium was replaced. Cells were used for experiments after 14 and 21 DIV.

2.1.6 Reagents and solutions

1xPBS without Calcium and Magnesium, sterile filtered (PAA Laboratories GmbH, H15-002)

PBS/ HEPES/ Glucose solution 7.38 pH:

100 ml 10xPBS (Gibco, 14200-067)

6 g D-(+)-Glucose (Sigma, G-7021)

7.38 g Hepes (Roth, 9105-2)
adjust pH to 7.38 with 2N NaOH

Neurobasal Medium without L-glutamine (Gibco, 21103-049)
B 27 Supplement 50x (Gibco, 17504-044)
L-glutamine 0.2M (Gibco, 25030-024)
Penicillin-Streptomycin (Gibco, 15140-114)
Culture medium:

500 ml Neurobasal Medium
10 ml B27 Supplement (50x)
1.25 ml L-glutamine (0.2M)
5 ml Penicillin-Streptomycin

0.25 % Trypsin in PBS w/o Calcium & Magnesium sterile filtered (PAA, L11-002)
Poly-D-Lysine Hydrobromide 1mg/ ml (Sigma, P-1149)

2.2 Neuronal toxicity experiments

Brief exposure to different concentrations of NMDA (0 to 100 µM NMDA) in the presence of 10 µM glycine was carried out at 37ºC in a 5 % CO_2 incubator for 5-10 minutes, using an exposure solution of serum-free media (Neurobasal Media without B27 Supplement). The exposure solution was then replaced with culture medium and the plates were returned to the incubator for LDH measurements the following day.

2.3 Assessment of neuronal injury

2.3.1 LDH assay

Overall neuronal injury was determined quantitatively by the measurement of lactate dehydrogenase (LDH) in the bathing medium, an index proportional to the total number of damaged neurons (Koh

and Choi, 1987). The LDH assay was performed 24 hours after NMDA exposure, at which point the cell death process was largely complete.

Lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. With the use of the Cytotoxicity Detection Kit (Roche), LDH activity could be easily measured in culture supernatants by a single measurement at one time point. The use of spectrophotometric microtiter plate reader (ELISA reader) allowed the simultaneous measurement of multiple probes and thereby guaranteed the easy processing of a large number of samples.

Background LDH levels were determined in sister cultures subjected to sham wash and subtracted from experimental values to yield the signal specific to experimentally induced injury. LDH values were scaled to the near-maximal mean value found in sister culture exposed to 100 μM NMDA for 24 hours (equal to 100 % cell loss).

Cytotoxicity (%) = [(Experimental Value – Low Control)/ (High Control – Low Control)] x 100

2.3.2 Trypan blue dye exclusion

Overall neuronal cell injury was estimated by examination of cultures with phase-contrast microscopy at 100x magnification. The examination was performed 24 hours later after NMDA exposure. In some experiments, this examination was verified by subsequent bright-field examination of trypan blue staining.

Cells are able to exclude most large hydrophilic molecules from their cytoplasm until membrane integrity has been severely compromised. In this assay, cell viability is assessed by the ability of the healthy neurons to exclude trypan blue, a very hydrophilic dye. Cells were incubated in 0.4 % trypan blue solution for 10 minutes. Nonviable cells were distinguished by their dark blue staining.

2.4 Mouse genotyping

The tips of mouse tails were digested by proteinase K (1 mg/ml) in TENS buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1 % SDS) at 55ºC. After precipitation in 1 volume of isopropanol and washing with 70 % ethanol, genomic DNA was diluted in 300 μM sterile H_2O

(millipore) at 55ºC. For PCR analysis 1 µl of this solution was used in an overall volume of 25 µl containing PCR buffer (Gibco BRL), 2 mM $MgCl_2$, dNTPs (0.2 mM per nucleotide), specific oligonucleotides (0.4 µM each), 0.5 U Taq polymerase and ddH_2O. An initial denaturating step (20 sec, 95ºC) was followed by 35 cycles each with 20 s at 94ºC (denaturing), 30 s at 55ºC (priming) and 50 s at 72ºC (elongation). After the last cycle, a final elongation was conducted for 10 min at 72ºC. The size of PCR products was determined by agarose gel electrophoresis. The following oligonucleotides were used for genotyping the respective mouse lines (expected bands in parentheses):

NR1 $^{-/-}$: N1IN18up1, N1EX18do1 (wt: 1140 bp, -/-: 480 bp)

NR2A$^{\Delta C/\Delta C}$: rspneo10, rspneo26 (wt: 700 bp, ΔC/ΔC: 1700 bp)

rspneo6, rspneo26 (wt: 700 bp, ΔC/ΔC: 1100 bp)

NR2B$^{\Delta C/\Delta C}$: 2B1, 2B3, rspneo6 (wt: 400 bp, ΔC/ΔC: 200 bp)

Sequences of oligonucleotides (5'-3')

N1IN18up1	AGGGGAGGCAACACTGTGGAC
N1EX18do1	CTGGGACTCAGCTGTGCTGG
rspneo6	GCAATCCATCTTGTTCAATGGC
rspneo10	CCTGCTGATGGAGAAGAGCC
rspneo26	AGAAGCTAATGTACCTGAGG
2B1	CGACATTGCTTCATGGGTGTCTGTTCTGGCA
2B3	GGAACAAGCACCTTCTTGTCTC

The genotyping was performed after cortical cell preparation from each individual embryo.

2.5 Immunocytochemistry

Cultured neurons were fixed with 4 % paraformaldehyde, 4 % sucrose in PBS for 10 minutes at 4ºC and permeabilized with 0.25 % Triton X-100 for 5 minutes. Cells were preincubated in 10 % goat serum for 30 minutes at room temperature and then incubated in primary antibody in phosphate-buffered saline (PBS) containing 5 % goat serum overnight at 4ºC. The concentrations of primary antibodies were:

rabbit N-terminal anti-NR2A subunit: 1 μg/ml (Belle)

mouse anti-MAP2: 1.5 μg/ml (Calbiochem)

mouse anti-NeuN: 1.5 μg/ml (Chemicon)

mouse anti-synapsin I: 1 μg/ml (Chemicon)

For double labelling, appropriate combinations of primary antibodies were incubated together. After washing three times in PBS, cells were incubated with goat anti-rabbit and/or anti-mouse (Dianova FITC-conjugated 1:50 or Dianova Cy3-conjugated 1:200) secondary antibodies for 1 hour at room temperature. Coverslips were mounted on slides using Mowiol as mounting medium and visualized using a microscope equipped for the visualization of fluorescence. Neurons were visualized through 10x, 40x and 100x objectives, and images were captured using a digital camera and transferred to a computer workstation.

2.6 Colocalization analysis

Control and experimental groups of cells were always run in parallel within the same immunohistochemical procedure. MethaMorph 6.1 software was used to interactively define the NR2A and synapsin I clusters at two-fold cytoplasmic background for evaluation of cluster density and size in single-channel analysis. Generating a dilated binary mask of synapsin clusters from the same multichannel image determined cluster localization. All NR2A cluster regions with an average binary pixel value that was greater than zero were defined as colocalized with synapsin I. Data collected with MetaMorph were exported to a Microsoft Excel spreadsheet.

2.7 ^{45}Ca analysis

Cultures were washed with HEPES-buffered control salt solution (HCSS) that contains: 120mM NaCl, 5mM KCl, 1.6mM MgCl2, 2.3mM CaCl2, 15mM glucose, 20mM HEPES, and 10mM NaOH. In the next step, cells were incubated in the presence of different concentrations of NMDA (0 to 100 μM) in HCSS solution containing $^{45}CaCl_2$. After 5 minutes, the exposure solution was quickly washed out with three rinses of HCSS and the cells were lysed by addition of 0.2 % sodium dodecyl sulfate (SDS) solution at 37ºC. Radioactivity was determined in the cell lysate. Calcium influx through other channels was prevented by applying NMDA together with 10 μM 6-cyano-7-

nitroquinoxaline-2-3-dione (CNQX, an AMPA/ kainate antagonist) and 2 μM nimodipine (a Ca^{2+} channel blocker).

2.8 Immunoblot analysis

Primary cultured neurons were scraped from the dish and collected by centrifugation (1000g for 10 minutes at 4ºC), followed by homogenization in ice cold buffer (pH 6.8, 50 mM Tris-HCl and 2 % SDS). The amount of protein was determined by BCA protein assay. The indicated amount of protein was loaded and separated using 7.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer (5 % non-fat dry milk in Tris-buffered saline containing 0.1 % Tween-20) for 1 hour at room temperature, and then incubated in blocking buffer with subunit-specific antibodies directed against NR2A subunits and respectively Synapsin I overnight at 4ºC. After washing for 30 minutes with four changes of Tris-buffered saline containing Tween-20, the membranes were incubated with horseradish peroxidase-conjugated antimouse antibody or antirabbit antibody (Amersham Life Science) for 1 hour at room temperature in blocking buffer. Following several washes with Tris-buffered saline containing Tween-20, proteins were visualized with enhanced chemiluminiscence (Pierce).

2.9 Electrophysiological recordings

Cultured cortical neurons at 14 and 21 DIV were visualized with a fixed-stage microscope (Axioskop 1, Zeiss, Jena, Germany), equipped with a 5× objective (Plan-NEOFLUAR, Zeiss), a 60× objective (LUMPlanFl/IR, Olympus, Tokyo, Japan) and an infrared-sensitive video camera (C2400 07, Hamamatsu, Japan). The preamplifier headstage holding the patch pipette was mounted on a motorized micromanipulator (Mini 25, Luigs&Neumann, Ratingen, Germany). Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal pipette puller (P87, Sutter Instruments, Novato, CA, USA) and had resistances between 5 and 7 MΩ when filled with intracellular solution. For fast application experiments a double-barreled pipette made of borosilicate theta glass (Hilgenberg) was controlled by a piezo translator (P270, Physik Instrumente,

Waldbronn, Germany) and perfused by a peristaltic pump (IPC-8, Ismatec, Glattbrugg, Switzerland). The optical and mechanical components were mounted to a vibration-isolation table (Physik Instrumente) and surrounded by a Faraday cage. Electrophysiological recordings were performed with an EPC-9 amplifier (HEKA, Lambrecht, Germany) in voltage clamp mode. All recordings were performed at room temperature on neurons with a characteristically pyramidal morphology.

2.9.1 Somatic recordings

Nucleated, whole-soma patches were obtained and 1 mM glutamate was fast-applied to activate AMPAR- and NMDAR-mediated currents at a holding potential of –70 mV. Patch pipettes were filled with (in mM): 140 CsCl, 2 MgATP, 10 EGTA, 10 HEPES (pH 7.25, 290-305 mOsM). Extracellular solution contained (in mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 5 HEPES (pH 7.25). AMPAR-mediated currents were activated in the presence of the NMDAR antagonist D-AP5 (50 µM). NMDAR-mediated currents were activated in the presence of the NMDAR coagonist glycine (10 µM) and the AMPAR antagonist NBQX (5 µM).

2.9.2 Synaptic recordings

Miniature excitatory postsynaptic currents (mEPSCs) were recorded at –70 mV in the same extracellular solution as used for nucleated-patch recordings, additionally supplied with 10 µM bicuculline methiodide (BMI, $GABA_A$ antagonist), 1 µM tetrodotoxin (TTX, sodium channel blocker) and 10 µM glycine. Patch pipettes were filled with (in mM): 120 Cs-gluconate, 20 CsCl, 1 $CaCl_2$, 4 MgATP, 0.3 NaGTP, 10 HEPES, 10 BAPTA, 2 TEA (pH 7.25, 290-305 mOsM).First, composite AMPA and NMDA mEPSCs were recorded, then D-AP5 was washed in to record pure AMPA mEPSCs. Events were analyzed off-line with the event detection program Peakscan (kindly provided by Prof. Misgeld, Institute of Physiology, University of Heidelberg). Around 100 mEPSC events were averaged for each condition. To obtain pure NMDAR-mediated currents, the averaged trace of the pure AMPA component was digitally subtracted (Igor Pro WaveMetrics, Lake Oswego, OR, USA) from the averaged trace of the composite AMPA and NMDA mEPSCs.

Data are presented as mean ± SEM. Statistical significance was evaluated by unpaired Student’s t-test.

3. Results

3.1 Introduction

NMDA-induced neurotoxicity in primary cultures of forebrain regions (neocortex and hippocampus) is a commonly used model of neuronal injury; in these cultures, NMDA receptors clustered in dendritic spines (O'Brien et al., 1998) mediate the most relevant pathophysiological processes, such as those that initiate ischaemia (Sattler et al., 2000). However, it is still unclear how the two major NMDAR subtypes expressed in the cultured cortical neurons NR1/NR2A and NR1/NR2B participate in NMDA-mediated excitotoxicity.

To investigate the respective contribution of the NMDA receptor subtypes NR1/NR2A and NR1/NR2B to excitatory cell death, NMDA-induced excitotoxicity experiments were performed in cortical cell cultures. One strategy used to investigate this issue was to induce neuronal cell death in gene-manipulated cultures prepared from mice expressing NR2A or NR2B subunits with altered signaling properties.

NMDA receptors possess extended intracellular C-terminal domains by which they interact with diverse proteins of the postsynaptic densities (PSD). This interaction plays a critical role in synaptic localization, clustering and signal transduction (Sheng et al., 1996; Kennedy, 1997, 1998; Kornau et al., 1997; Kim and Huganir, 1999).

In the cortical cultures prepared from $NR2A^{\Delta C/\Delta C}$ and $NR2B^{\Delta C/\Delta C}$ mice, neurons express NR2A and NR2B subunits with impaired signaling, due to the C-teminal truncation. Primary cell culture is an excellent system to study neurons from mice with lethal phenotype, such as $NR1^{-/-}$ or $NR2B^{\Delta C/\Delta C}$ mice.

Another way of manipulation of the NMDA receptor subunits was a pharmacological approach in wild-type cultures, using NR2A and NR2B subunit-specific antagonists in NMDA-induced excitotoxicity protocol.

3.2 The cortical cell culture system and excitotoxicity

Since the NR2A and NR2B subunits show a distinct developmental expression profile *in vitro* (Li et al., 1998), excitotoxicity experiments were performed at 14 and 21 DIV. At 14 DIV, the NR1 and NR2B subunits are fully expressed whereas the NR2A subunit is expressed at a low level. At three

weeks in culture, neurons become mature and express NR1, NR2A and NR2B subunits at their highest level (Li et al., 1999).

In the first set of experiments, the preparation of cortical neurons was established and conditions were optimized in order to keep the cultures up to three weeks *in vitro*.

Dissociated wild-type cortical neurons, plated at a density of $3x10^5$ cells/ ml, were prepared from E16 mouse embryos. Mature cultures consist of approximately 10 to 15 % neurons, as quantified by immunocytochemistry analysis using antibodies against neuronal marker proteins such MAP2 and NeuN (Fig. 7).

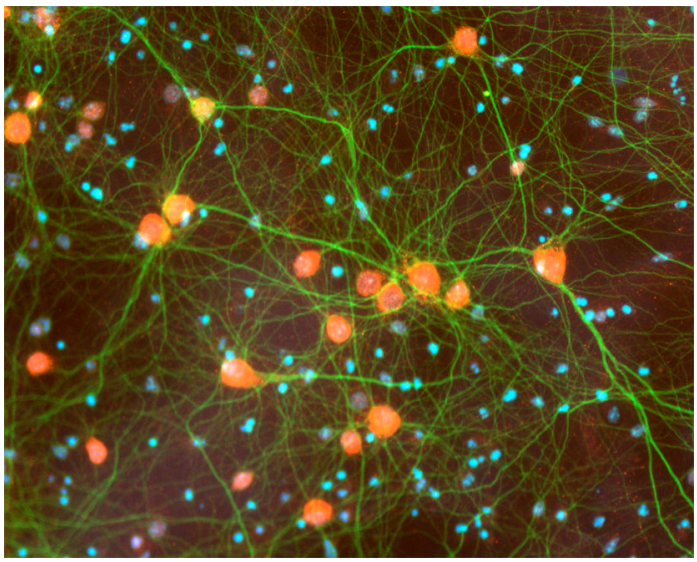

Fig. 7: *High-density cortical cell culture.*
Immunocitochemistry of primary cortical culture, 21 DIV. Neurons represent ~ 10 % of the entire cell population. MAP2 (green) stained the neurites, NeuN (red) labeled the neuronal cell body and DAPI (blue) marked the nuclei of all the cells in culture. Neurons lie on the top of the astrocyte bed.

Dissociated cortical neurons from single embryos were plated in one 24 well plate, giving the advantage to perform each excitotoxicity experiment in quadruplicate. Also, this protocol was excellent to study NMDA-induced toxicity in cultures prepared from heterozygous mice, where the preparation of the cells was done for each embryo, resulting in wild-type, heterozygous and homozygous sister cultures.

Increasing concentrations of NMDA applied for 10 minutes on cortical cells in culture produced a dose-dependent increase of neuronal loss (Fig. 8). Application of 100 µM NMDA in wild-type cultures led to 100 % neuronal loss at 14 DIV. 50 % cell death was induced by 50 µM NMDA (IC_{50}). At 21 DIV, mature cultures responded in a similar dose-response manner to NMDA exposure as young cultures. The IC_{50} was consistent among sister cultures of a given plating.

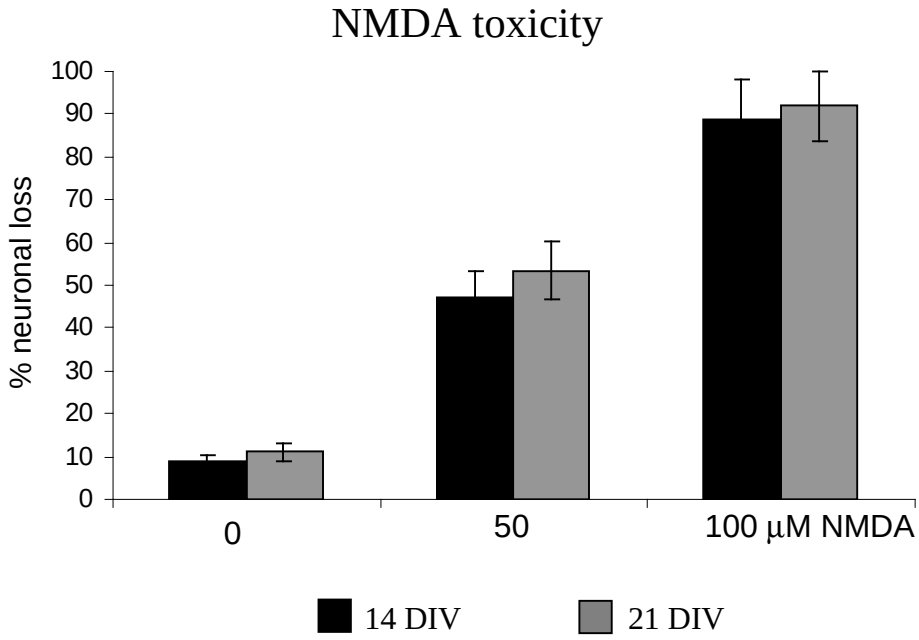

Fig. 8: *NMDA toxicity in wild-type culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA. Experiments were performed on sister cultures at two developmental stages, 14 and respectively 21 DIV. Values represent STDEV of 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

In additional exitotoxicity experiments, cultured neurons were exposed for 5 minutes to increasing concentrations of NMDA. The cells were affected by the NMDA application in a similar dose-response manner as after 10 minutes of NMDA bath application, indicating that a shorter exposure to NMDA does not diminish the percentage of neuronal loss.

After NMDA-induced excitotoxicity, the assessment of cell viability was performed using the LDH assay and Trypan blue staining (Koh and Choi, 1987). Both methods gave the same percentage of neuronal loss.

LDH is a colorimetric assay used for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase activity released from the cytosol of damaged neurons into the bath medium (Koh and Choi, 1987). A potentially important advantage of the LDH assay over many other assays for cytotoxicity is that it is completely non-invasive. The biological environment of the cultures is not altered and performance of the assay does not preclude subsequent or serial measurements.

The trypan blue assay was mostly used for a morphological analysis of the cultures after the NMDA-induced excitotoxicity. In this assay, neuronal loss is quantified based on the ability of healthy cells to exclude trypan blue, a very hydrophilic dye. However, LDH analysis was preferred, being a very reliable and fast method to investigate the percentage of cell death induced by NMDA application.

3.3 Excitotoxicity in NR1$^{-/-}$ cortical cultures

Once NMDA-induced toxicity was established in wild-type cultures, a positive control experiment was performed: embryos with gene ablation of the NR1 subunit (NR1$^{-/-}$) were used to prepare cortical cultures. (Forrest et al., 1994).

NR1 is the obligatory subunit for the functional NMDA receptor channel, thus mouse mutants without the NR1 subunit have no functional NMDARs, leading to perinatal death in the absence of obvious morphological brain abnormalities (Forrest et al., 1994; Li et al., 1994), thereby indicating important functions for NMDARs in brain physiology.

To prepare neuronal cultures from NR1$^{-/-}$ mice, heterozygous parents (NR1$^{+/-}$) were bred and cortex from single embryos was used for cell preparation. After plating the cells, individual embryos were genotyped by PCR analysis to distinguish between wild-type, heterozygous and homozygous sister cultures.

NMDA application showed no toxic effect in cultures prepared from the NR1$^{-/-}$ mice compared to the wild-type sister cultures (Fig. 9). These observations suggest that functional NMDA receptors are necessary for triggering receptor-dependent excitotoxicity, but actually cannot indicate whether the NR1 subunit plays a more distinct role in mediating neuronal death or survival.

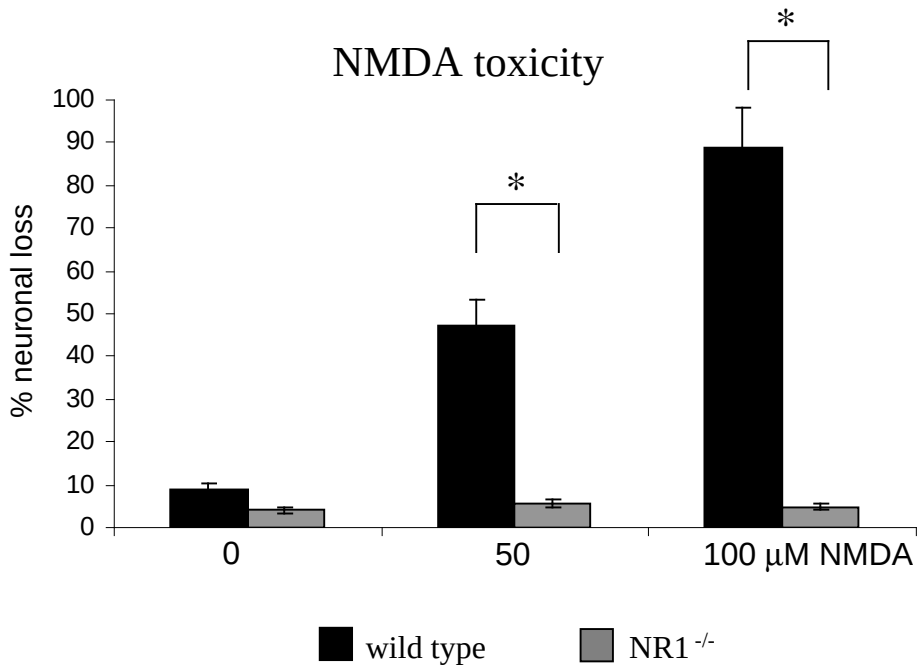

Fig. 9: *No NMDA toxicity in NR1$^{-/-}$ culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA. Experiments were performed at 21 DIV on NR1$^{-/-}$ and wild-type sister cultures. Results were normalized to the 100 % cell death produced by 300 µM NMDA exposure on wild-type sister cultures. Values represent STDEV of 2 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

3.4 NMDA-induced toxicity in gene-manipulated cultures

Direct interactions occur between NMDA receptor subunits and members of the postsynaptic density (PSD) protein family. In mice expressing NR2 subunits in a C-terminal truncated form, the interactions with constituents of the PSD are occluded and the intracellular signaling of these subunits is impaired.

NMDA-induced toxicity in gene-manipulated cultures is a very useful tool to investigate the contribution of the NR2A and NR2B subunits to excitatory cell death.

The gene-manipulated mice used in this study express C-terminally truncated NR2A (NR2A$^{\Delta C/\Delta C}$ mice) and NR2B (NR2B$^{\Delta C/\Delta C}$ mice) subunits.

3.4.1 NMDA-induced toxicity in NR2A$^{\Delta C/\Delta C}$ cultures

In the first set of experiments, NMDA toxicity was induced in cultures prepared from NR2A$^{\Delta C/\Delta C}$ mice. In these mice, the C-terminal tail of the NR2A subunit is truncated. The intracellular NR2A receptor domain that may mediate interaction of the ion channel with components transducing the synaptically evoked Ca^{2+} signal is functionally impaired.

In cultures prepared from NR2A$^{\Delta C/\Delta C}$ embryos, the neurons expressed all the NR2A subunits in C-terminally truncated form with altered signal properties.

NMDA application in young (14 DIV) NR2A$^{\Delta C/\Delta C}$ cultures triggered neuronal cell death in a similar dose-response manner as in wild-type sister cultures (Fig. 10).

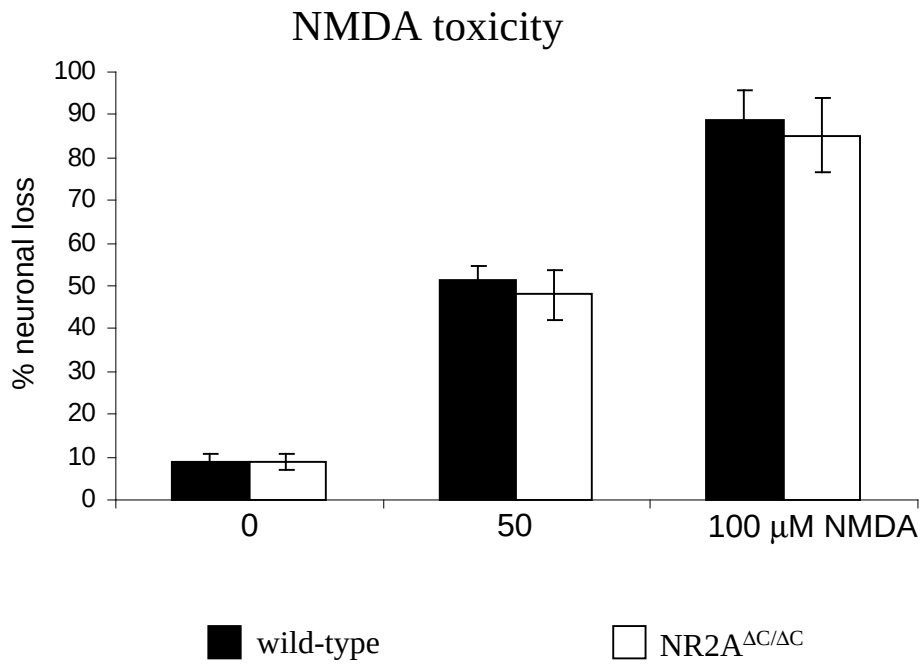

Fig. 10: *NMDA toxicity in NR2A$^{\Delta C/\Delta C}$ culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA. Experiments were performed at 14 DIV on NR2A$^{\Delta C/\Delta C}$ and wild-type sister cultures. The NMDA toxicity is similar in wild-type and NR2A$^{\Delta C/\Delta}$C cultures. Values represent STDEV of 5 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

This result can be explained by the low expression level of the NR2A subunit at 14 DIV. Interestingly, in mature NR2A$^{\Delta C/\Delta C}$ cultures (21 DIV), NMDA had a similar toxic effect as that observed in wild-type cultures (Fig. 11).

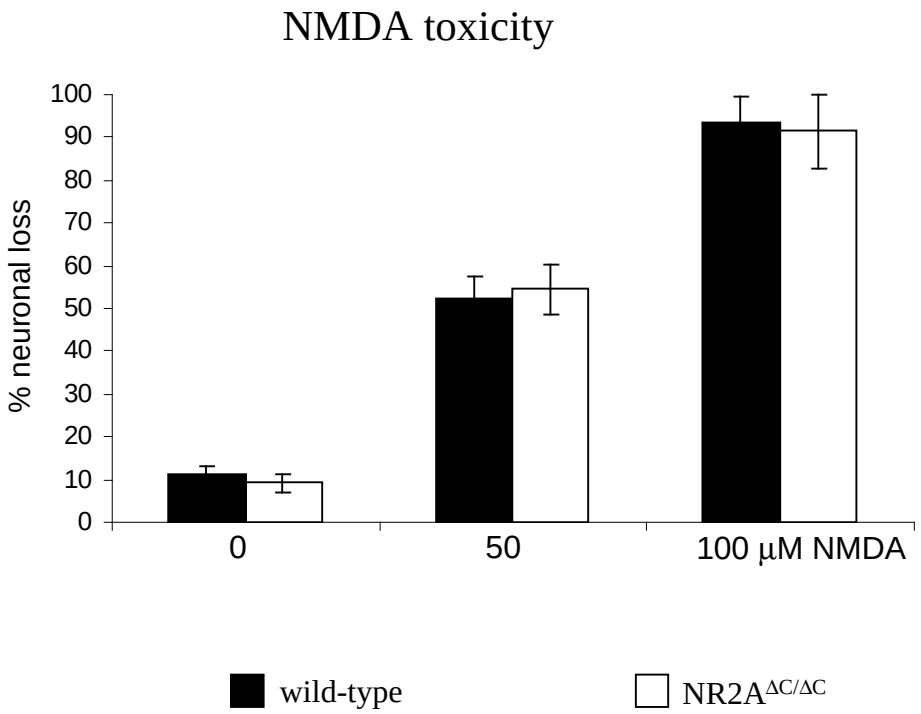

Fig. 11: *NMDA toxicity in NR2A$^{\Delta C/\Delta C}$ culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA. Experiments were performed at 21 DIV on NR2A$^{\Delta C/\Delta C}$ and wild-type sister cultures. The NMDA toxicity is similar in wild-type and NR2A$^{\Delta C/\Delta C}$ cultures. Values represent STDEV of 5 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

NR2A$^{\Delta C/\Delta C}$ cultures showed NMDA-induced excitotoxicity similar to wild-type cultures. Short application of increasing NMDA concentration (10 minutes) triggered visible neuronal cell death (Fig. 12).

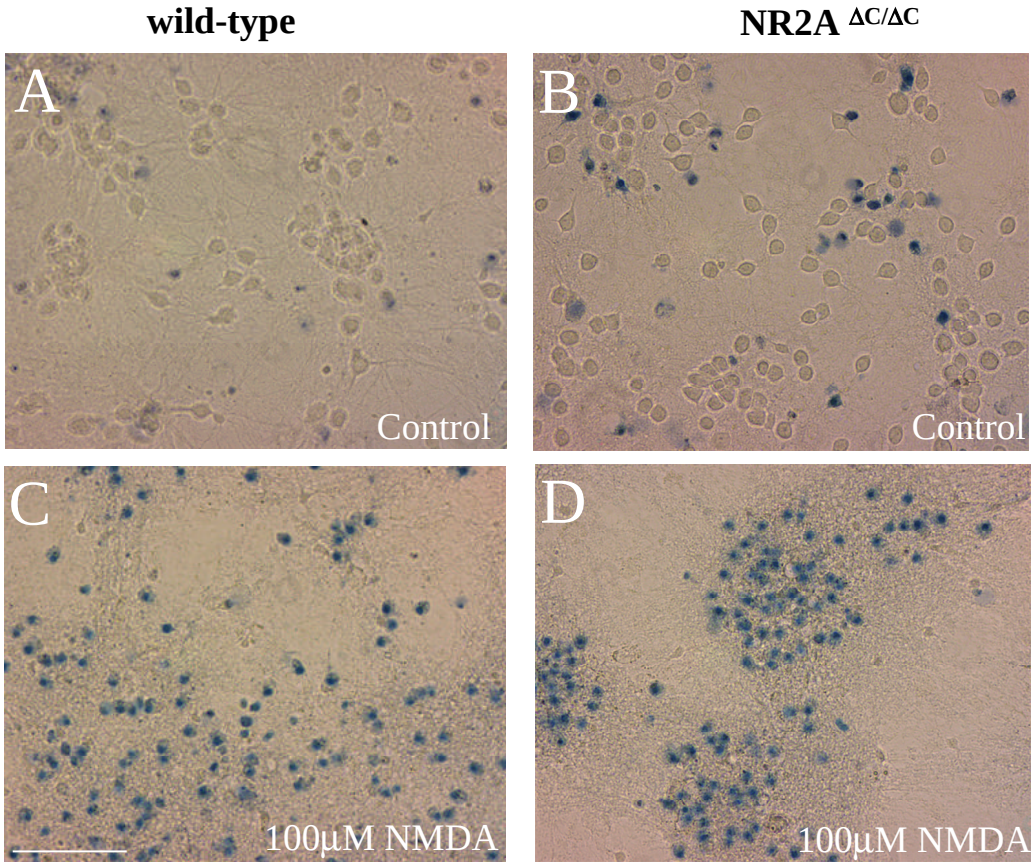

Fig. 12: *NMDA-induced toxicity in NR2A$^{\Delta C/\Delta C}$ culture*

Phase-contrast micrographs of identified fields of cortical cells taken one day after a 10 minutes exposure to 100µM NMDA (bright field after trypan blue incubation) on wild-type (A, C) and NR2A$^{\Delta C/\Delta C}$ cultures (B, D). NMDA application had a similar toxic effect on wild-type and NR2A$^{\Delta C/\Delta C}$ cultures. Scale bar 50µm.

So the impairment in the interaction of the NR2A subunits with the PSD proteins did not change the sensitivity to NMDA-induced cell death. One explanation is that in NR2A$^{\Delta C/\Delta C}$ cultures excitotoxicity is mediated mainly via the NR2B subunit.

To investigate if the truncated NR2A subunits are located at synaptic sites, an immunocytochemistry approach was used.

At synaptic sites in the NR2A$^{\Delta C/\Delta C}$ cultures, the intensity of NR2A-containing synapses was reduced when compared with NR2A in wild-type cultures. In mature NR2A$^{\Delta C/\Delta C}$ cultures a strong reduction was observed in the NR2A receptor localization at synaptic sites (Fig.13).

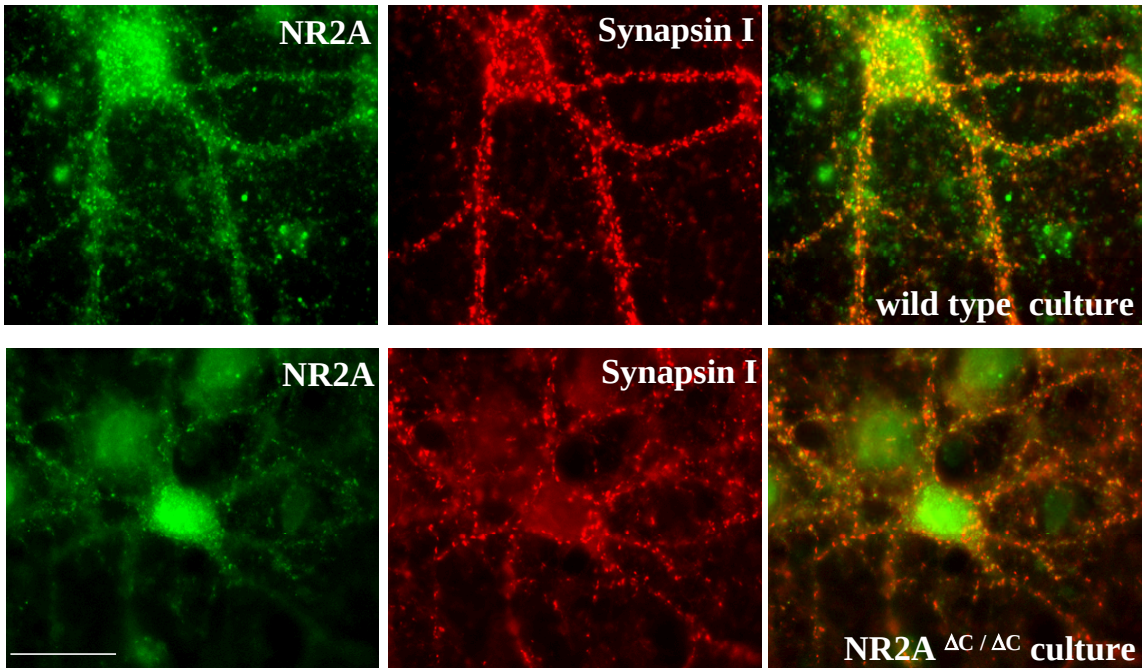

Fig. 13: *Immunofluorescent staining of NR2A subunits and Synapsin in wild-type and NR2A$^{\Delta C/\Delta C}$ cultures*
Double labeling for NR2A subunits (anti-mouse C-terminus antibody, 1µg/ml) and Synapsin I (anti-rabbit, 1 µg/ml) in cortical cells at 21 DIV. Scale bar 10 µm.

Colocalization analysis indicated a reduction of the NR2A subunit in the synapse of about 1/3 in the NR2A$^{\Delta C/\Delta C}$ cultures compared to the wild-type (Fig 14).

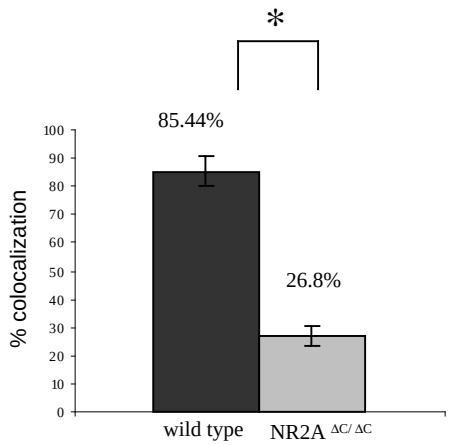

Fig. 14: *Colocalization of NR2A subunits with Synapsin in wild-type and NR2A$^{\Delta C/\Delta C}$ culture*
Colocalization analysis of NR2A subunits and Synapsin I (MetaMorph InVisitron System). Strong reduction of NR2A-containing synapses in NR2A$^{\Delta C/\Delta C}$ cultures compared to wild-type. Values represent STDEV of quantified colocalizations in 4 to 5 cells from 3 independent experiments. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

To investigate the contribution of the NR2B receptor subtype to excitotoxicity in NR2A$^{\Delta C/\Delta C}$ cultures, an NR2B subunit-specific antagonist ifenprodil was used during NMDA exposure. In the presence of ifenprodil, young cultures were protected from the NMDA-induced cell death (Fig.15). These data confirm that in young NR2A$^{\Delta C/\Delta C}$ cultures, excitotoxicity is mediated via the NR1/NR2B receptors, the most abundant NMDA receptor subtype at 14 DIV.

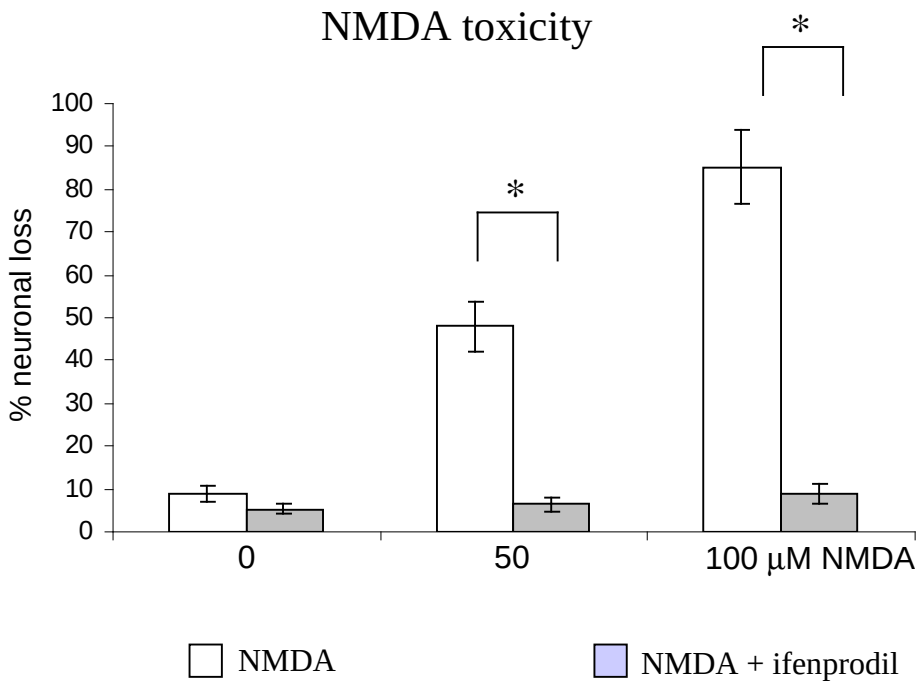

Fig. 15: *NMDA-induced toxicity in young NR2A$^{\Delta C/\Delta C}$ culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA in the presence of ifenprodil (10 µM). Experiments were performed at 14 DIV on NR2A$^{\Delta C/\Delta C}$ cultures. NMDA toxicity is mediated by the NR2B subunit. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

Interestingly, mature cultures (21DIV) were also protected from NMDA-toxicity in the presence of NR2B antagonist (Fig. 16).

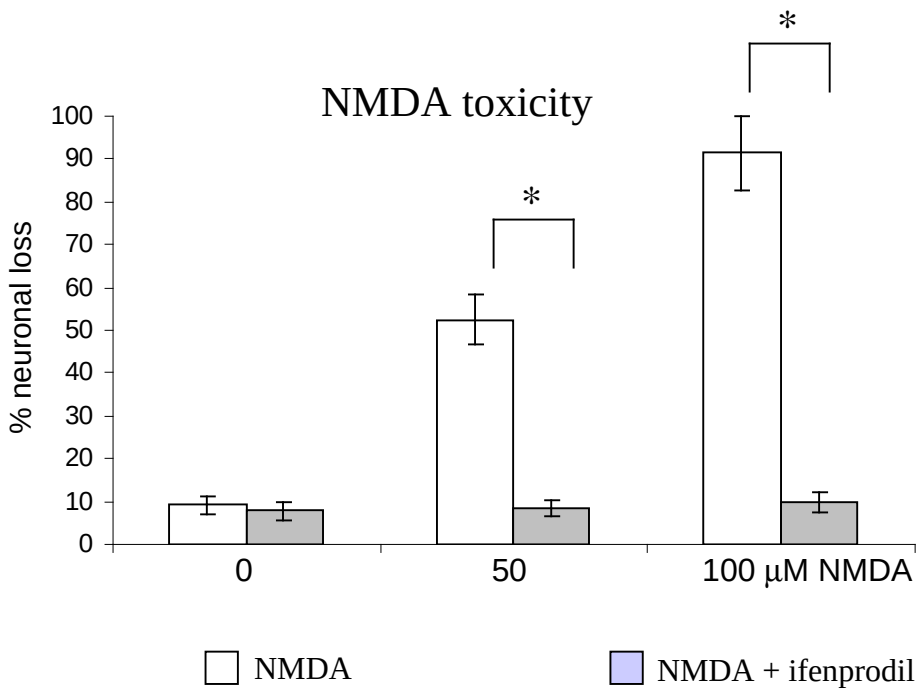

Fig. 16: *NMDA-induced toxicity in mature NR2A$^{\Delta C/\Delta C}$ cultures*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA in the presence of ifenprodil (10 µM). Experiments were performed at 21 DIV on NR2A$^{\Delta C/\Delta C}$ cultures. NMDA toxicity is mediated by the NR2B subunit. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

So at a mature stage, when NR2A subunit function is abolished, the NR2B subtype is the only mediator of the NMDA-induced toxicity.

This may reflect the inability of truncated NR2A channels to access or trigger the "death program" and probably due to a compensatory mechanism, NR2B subunits are the only mediators in the excitatory cell death.

Western blot analysis (performed by Jakob von Engelhardt) indicated that in the NR2A$^{\Delta C/\Delta C}$ cultures, the NR2B subunit is expressed at levels comparable to wild-type. The alteration of the intracellular signaling of the NR2A subunits did not affect the expression level of the NR2B subtype (Fig 17).

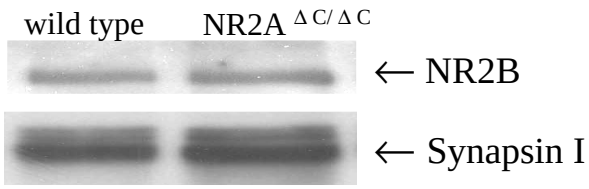

Fig. 17: *Western blot analysis of NR2B subunit protein in wild-type and NR2A$^{\Delta C/\Delta C}$ cultures*

Cells at 21 DIV were harvested and membrane homogenates were separated by SDS-PAGE. After transferring to nitrocellulose membrane, the expression profile of NR2A subunits and Synapsin I (presynaptic marker used as positive control) was determined using specific antibodies against NR2A and Synapsin I poplypeptides. NR2B subunit is expressed in NR2A$^{\Delta C/\Delta C}$ cultures at levels comparable to wild-type cultures. Experiments were performed in duplicate by Jakob von Engelhardt.

There is a strong relationship between NMDA-triggered excitotoxicity and excessive Ca^{2+} influx through NMDA receptors. There is undoubtedly a large diversity of pathways for Ca^{2+} entry into neurons, but the NMDA pathway plays the key role in neuronal death.

It is still controversial whether excitotoxicity itself is a truly significant player in the stroke process or the additional mechanisms of excitotoxicity are more important. Ca^{2+}-dependent neurotoxicity, as with many physiological events, occurs through distinct intracellular signaling pathways, likely through physical interactions of cell membrane receptors with specialized submembrane molecules.

Because the key mediators of excitotoxic damage are Ca^{2+}, an important issue was to investigate the ^{45}Ca influx through NMDA receptors in the NR2A$^{\Delta C/\Delta C}$ cultures.

At 21 DIV, application of NMDA in NR2A$^{\Delta C/\Delta C}$ cultures triggered a similar influx of Ca^{2+} as in wild-type cultures (Fig.18). Jokob von Engelhardt performed NMDA-evoked ^{45}Ca influx experiments.

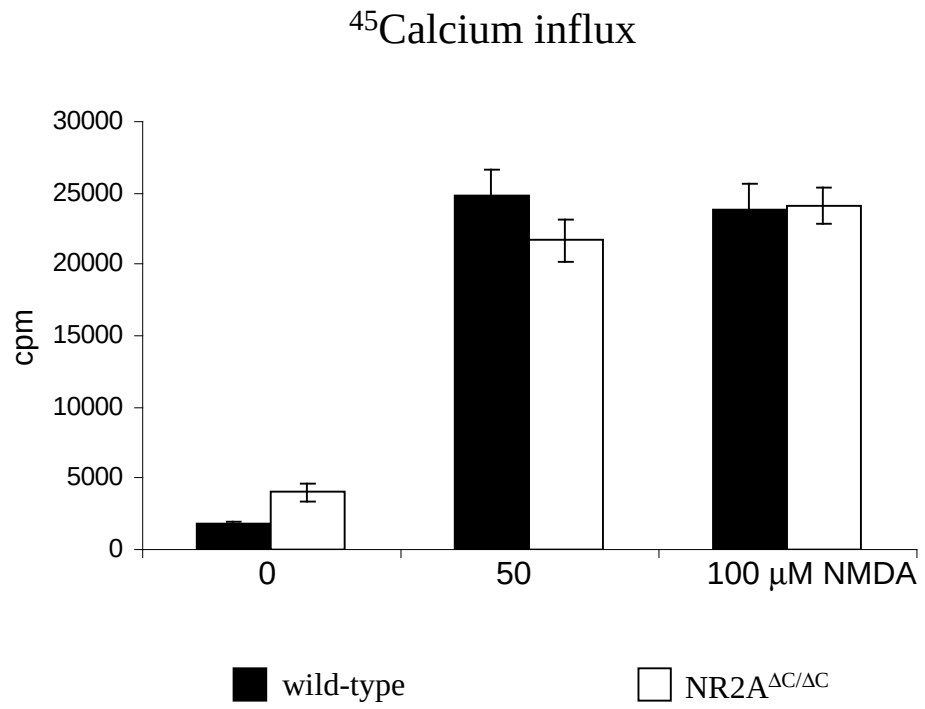

Fig. 18: ***45Ca influx in NR2AΔC/ΔC culture***

At 21 DIV in NR2A$^{\Delta C/\Delta C}$ cultures the ^{45}Ca influx is similar with wild-type. For measurement of Ca^{2+} influx, ^{45}Ca was added during the NMDA exposure for 5 minutes; cultures were then washed thoroughly and ^{45}Ca activity was measured. Values represent mean ± SEM of 6 to 9 independent experiments performed by Jakob von Engelhardt. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

Moreover, NMDA-induced Ca^{2+} influx was suppressed by ifenprodil application, indicating that in these cultures Ca^{2+} enters only through NR1/NR2B channels (Fig.19).

In summary, excitotoxicity experiments, immnunocytochemistry, Western blot analysis and Ca^{2+} influx indicated that in cultured cortical neurons expressing NR2A subtype only in its C-terminally truncated form, NMDA-mediated killing occurs efficiently via the NR2B channels, both at 14 DIV and 21 DIV.

Electrophysiological experiments were further performed by Verena Pawlak (MPI for Medical Research, Heidelberg) to investigate if there is any change in the synaptic and extrasynaptic currents in the NR2A$^{\Delta C/\Delta C}$ cultures.

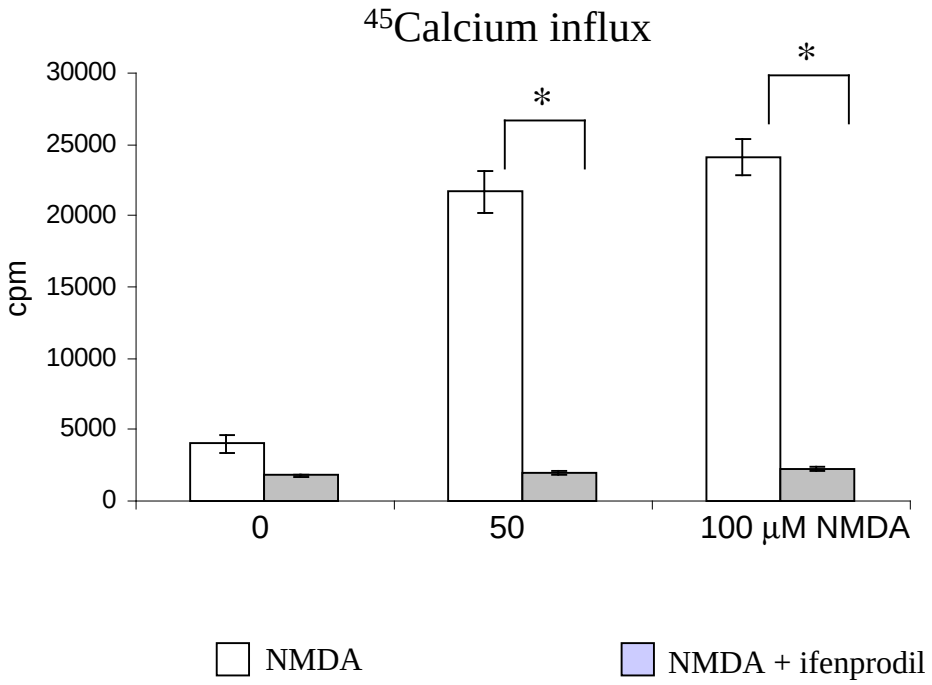

Fig. 19: ***45Ca influx occurs through NR2B channels in NR2A$^{\Delta C/\Delta C}$ culture***

At 21 DIV in NR2A$^{\Delta C/\Delta C}$ cultures the Ca^{2+} influx occur mainly via the NR2B subunit. For measurement of Ca^{2+} influx, ^{45}Ca was added during the NMDA exposure for 5 minutes; cultures were then washed thoroughly and ^{45}Ca activity was measured. Values represent mean ± SEM of 6 to 9 independent experiments performed by Jakob von Engelhardt. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

Nucleated patches indicated no difference in the extrasynaptic currents in mature NR2A$^{\Delta C/\Delta C}$ cultures compared with wild-type sister cultures (Table 1A). Furthermore, the synaptic currents indicated by mEPSC recordings were also unchanged (Table 1B).

A	**Somatic currents (-70 mV)**	
	wild-type	**NR2A ΔC/ΔC**
NMDA Amplitude (pA/pF)	-108 ± 18	-96 ± 31
Deactivation (ms)	65 ± 13	42 ± 6
AMPA Amplitude	-296 ± 51	-553 ± 150
NMDA / AMPA	**0.44 ± 0.08**	**0.30 ± 0.08**

B	**Synaptic currents (-70 mV)**	
	wild-type	**NR2A ΔC/ΔC**
NMDA Amplitude (pA)	-6.9 ± 1.3	-4.3 ± 0.7
NMDA Charge (pC)	-0.28 ± 0.05	-0.18 ± 0.04
AMPA Amplitude (pA)	-36 ± 2	-25 ± 2
Rise time (ms)	0.5 ± 0.0	0.6 ± 0.0
Deactivation (ms)	2.9 ± 0.2	3.1 ± 0.3
Charge (pC)	-0.15 ± 0.01	-0.15 ± 0.02
NMDA / AMPA	**0.18 ± 0.03**	**0.17 ± 0.02**

Table 1: *Synaptic and extrasynaptic currents in NR2A$^{\Delta C/\Delta C}$ culture*
The table summarizes data obtained from three-week-old (DIV21) primary cortical cultures prepared from wild-type and NR2A$^{\Delta C/\Delta C}$ mice: NMDA or AMPA peak currents, charges and kinetics. Extrasynaptic AMPA/NMDA ratios were unchanged in NR2A$^{\Delta C/\Delta C}$ cultures compared to wild-type sister cultures, as indicated by the nucleated patches (A). mEPSCs recordings indicate comparable AMPA/NMDA amplitude ratios between wild-type and NR2A$^{\Delta C/\Delta C}$ cultures (B). Experiments were performed by Verena Pawlak, MPI for Medical Research, Heidelberg.

All the investigations in the NR2A$^{\Delta C/\Delta C}$ cultures suggested that in the absence of fully functional NR2A subunits, due to a compensatory mechanism, the NR1/NR2B receptor subtype became the only Ca^{2+} influx mediator and also the only NMDA receptor subtype responsible for excitatory cell death.

3.4.2 NMDA-induced toxicity in the NR2B $^{\Delta C/\Delta C}$ cultures

The NMDA-induced toxicity analysis in cultures that express the C-terminally truncated NR2B subunit is particularly interesting, because the NR1/NR2B receptors are most likely the first ionotropic glutamate receptors expressed at nascent, functionally immature synapses (Durand et al., 1996; Malenka and Nicoll, 1997).

NMDA toxicity was induced in cultures prepared from NR2B $^{\Delta C/\Delta C}$ embryos. In these cultures, the neurons express the NR2B subunit in the C-terminally truncated form with impaired signal properties.

Interestingly, the application of different concentrations of NMDA had no toxic effect in the young NR2B$^{\Delta C/\Delta C}$ cultures (Fig. 20) compared with wild-type sister cultures.

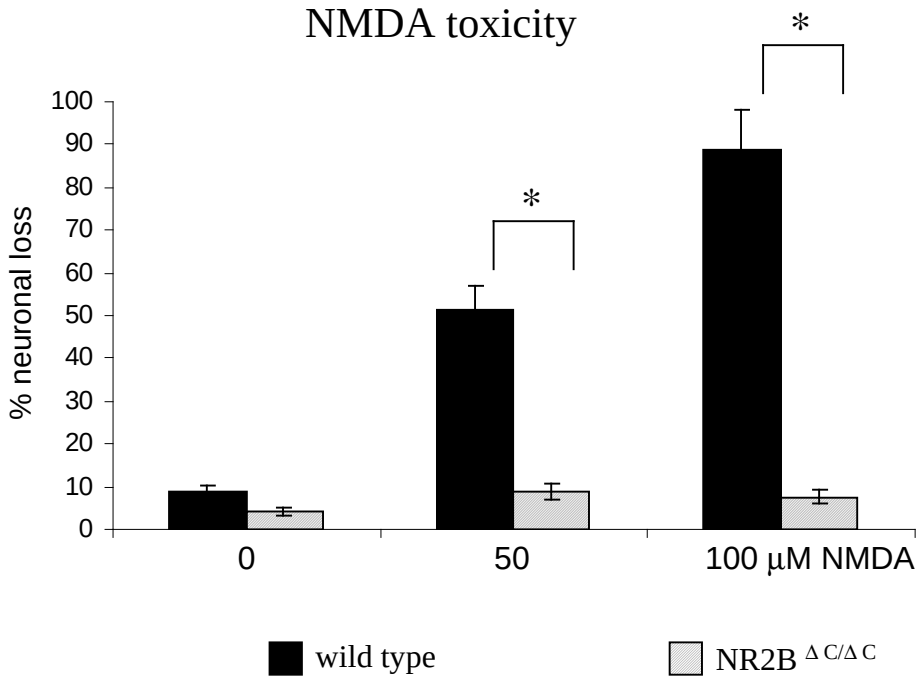

Fig. 20: *NMDA-induced toxicity in young NR2B$^{\Delta C/\Delta C}$ culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA. Experiments were performed at 14 DIV on NR2B$^{\Delta C/\Delta C}$ cultures. NMDA application had no toxic effect. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

Furthermore, mature NR2B$^{\Delta C/\Delta C}$ neurons in culture were also protected from the toxic effect of NMDA (Fig. 21).

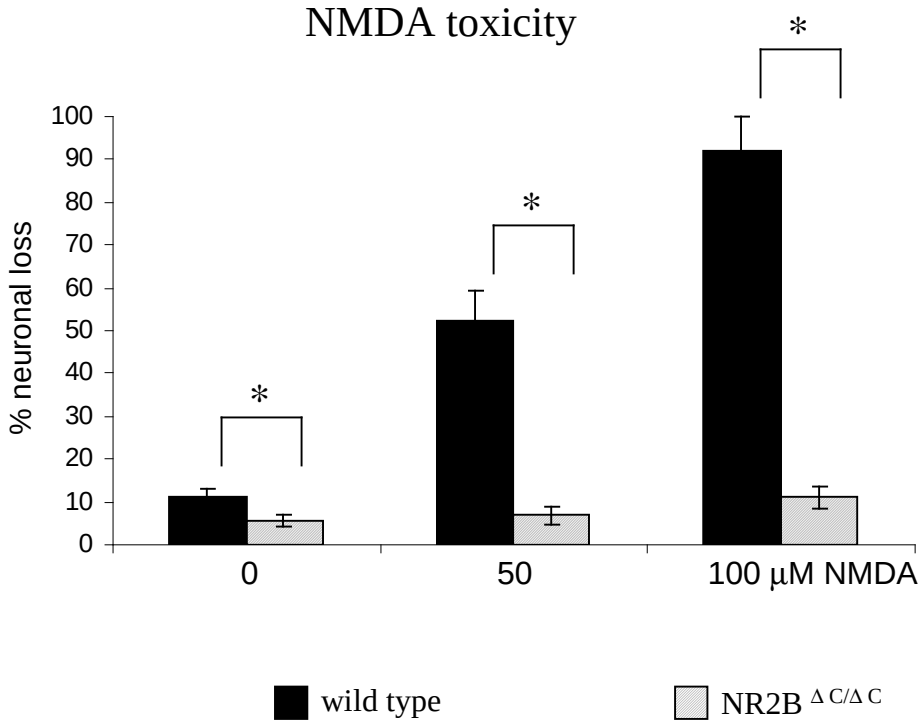

Fig. 21: *NMDA-induced toxicity in mature NR2B$^{\Delta C/\Delta C}$ culture*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA. Experiments were performed at 21DIV on NR2B$^{\Delta C/\Delta C}$ cultures. NMDA application had no toxic effect. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

NR2B$^{\Delta C/\Delta C}$ cultures showed a strong protection to NMDA-induced excitotoxicity. Short application (10 minutes) of a high NMDA concentration (1mM) did not trigger any visible neuronal cell death. Almost all neurons could be found intact one day after exposure. These surviving cells excluded trypan blue and remained morphologically stable for at least several more days (Fig 22). These experiments show that the NR2B$^{\Delta C/\Delta C}$ cultures are as resistant as NR1$^{-/-}$ cultures to NMDA-induced excitotoxicity.

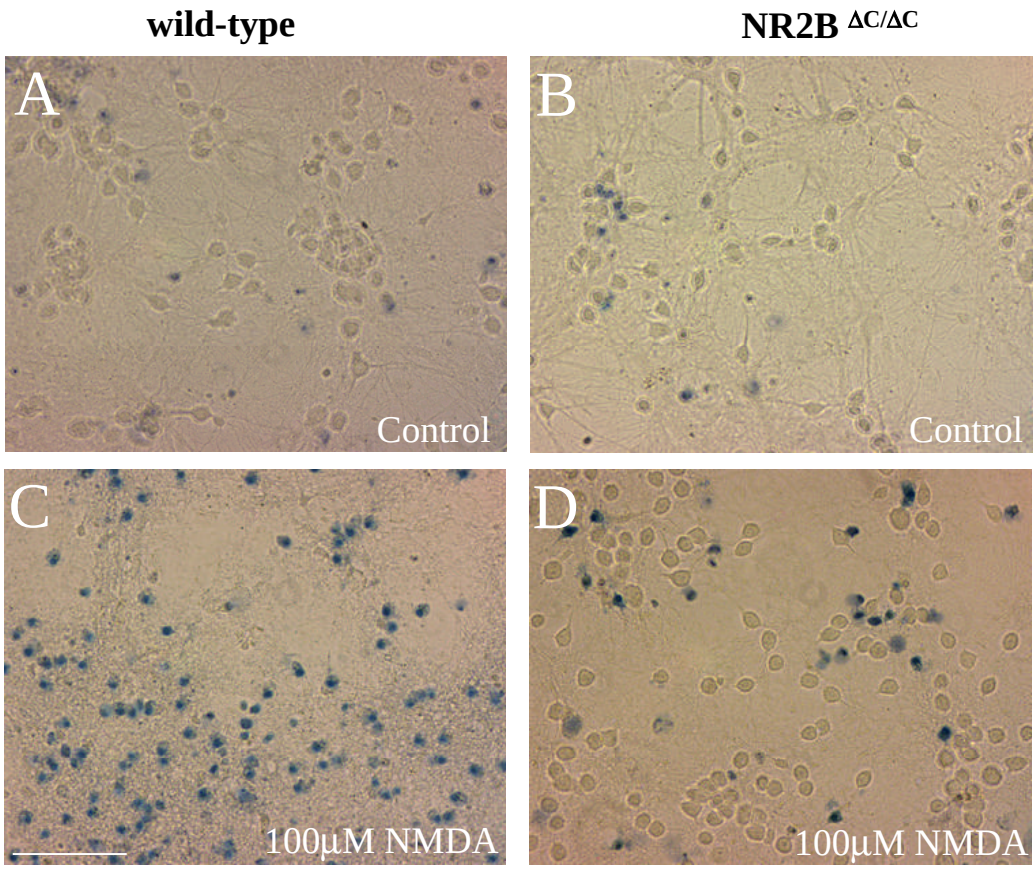

Fig. 22: *NMDA-induced toxicity in NR2B$^{\Delta C/\Delta C}$ culture*

Phase-contrast micrographs of identified fields of cortical cells taken one day after a 10 minutes exposure to 100µM NMDA (bright field after trypan blue incubation) on wild-type (A, C) and NR2B$^{\Delta C/\Delta C}$ cultures (B, D). NMDA application had no toxic effect on NR2B$^{\Delta C/\Delta C}$ cultures. Scale bar 50 µm.

NMDA receptor activation leads rapidly to Ca^{2+} influx and the accumulation of high levels of intracellular Ca^{2+} triggers the cell death cascade. Could the C-terminal truncation of the NR2B subunit impair the Ca^{2+} influx through NMDAR channels in the NR2B$^{\Delta C/\Delta C}$ cultures?

^{45}Ca analysis indicated no Ca^{2+} influx through NMDA receptors in the NR2B$^{\Delta C/\Delta C}$ mature cultures, compared with wild-type sister cultures (Fig.23). These results suggested that the NR2A subunit could not substitute or provide any compensation for the impaired functionality of the NR2B subunit.

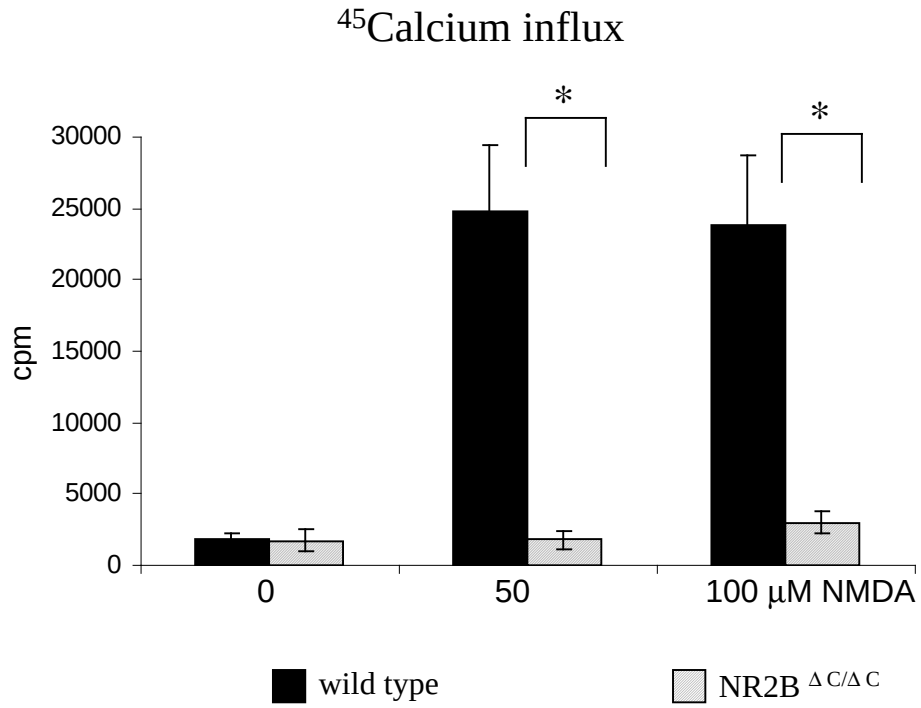

Fig. 23: ***^{45}Ca influx through NMDA receptor channels in NR2B$^{\Delta C/\Delta C}$ culture***

At 21 DIV in NR2B$^{\Delta C/\Delta C}$ cultures there is no NMDA-induced calcium influx. For measurement of Ca^{2+} influx, ^{45}Ca was added during the NMDA exposure for 5 minutes; cultures were then washed thoroughly and ^{45}Ca activity was measured. Values represent mean ± SEM of 5 to 6 independent experiments performed by Jakob von Engelhardt. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

Since NMDA applications did not trigger any cell death and ^{45}Ca analysis shows no Ca^{2+} influx in NR2B$^{\Delta C/\Delta C}$ cultures, an important issue was to investigate the NR2A subunit expression and localization in these cultures, using Western blot analysis and elecrophysiological recordings.

Western blot analysis in homogenates obtained from mature NR2B$^{\Delta C/\Delta C}$ cultures (21 DIV) indicated that the NR2A subunit was expressed at levels comparable with those observed in wild-type cultures (Fig.24). This experiment suggested that the functional impairment of NR2B subunit did not affect the level of the NR2A subunit expression. The NR2A NMDA receptor subtype was also normally transported to the cell membrane surface, as indicated by the Western blot analysis.

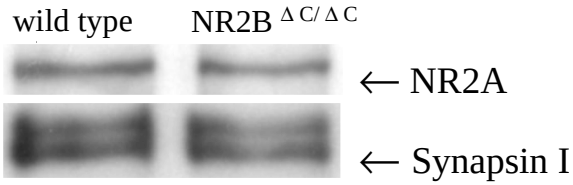

Fig. 24: *Western blot analysis of NR2A subunit protein in wild-type and NR2B$^{\Delta C/\Delta C}$ cultures*

Cells at 21 DIV were harvested and membrane homogenates were separated by SDS-PAGE. After transferring to nitrocellulose membrane, the expression profile of NR2A subunits and Synapsin I (presynaptic marker used as positive control) was determined using specific antibodies against NR2A and Synapsin I poplypeptides. NR2A subunit is expressed in NR2B$^{\Delta C/\Delta C}$ cultures at levels comparable to wild-type cultures. Experiments were performed in duplicate by Jakob von Engelhardt.

Interestingly, electrophysiological measurements showed a reduction in the synaptic and somatic NMDA currents. Nucleated patches in NR2B$^{\Delta C/\Delta C}$ cultures indicated that somatic currents were reduced by one-third compared with those seen in wild-type sister cultures (Table 2A). Synaptic currents, indicated by mEPSC recordings, were however reduced by two-thirds in NR2B$^{\Delta C/\Delta C}$ cultures compared with wild-type (Table 2B).

A	**Somatic currents (-70 mV)**	
	wild-type	**NR2A ΔC/ΔC**
NMDA Amplitude (pA/pF)	-108 ± 18	-73 ± 21
Deactivation (ms)	65 ± 13	34 ± 5
AMPA Amplitude	-296 ± 51	-580 ± 178
NMDA / AMPA	**0.44 ± 0.08**	**0.18 ± 0.05**

B	**Synaptic currents (-70 mV)**	
	wild-type	**NR2A ΔC/ΔC**
NMDA Amplitude (pA)	-6.9 ± 1.3	-1.3 ± 0.5
NMDA Charge (pC)	-0.28 ± 0.05	-0.04 ± 0.01
AMPA Amplitude (pA)	-36 ± 2	-25 ± 4
Rise time (ms)	0.5 ± 0.0	0.6 ± 0.0
Deactivation (ms)	2.9 ± 0.2	3.8 ± 0.5
Charge (pC)	-0.15 ± 0.01	-0.14 ± 0.04
NMDA / AMPA	**0.18 ± 0.03**	**0.06 ± 0.03**

Table 2: *Synaptic and extrasynaptic currents in NR2B$^{\Delta C/\Delta C}$ culture*

The table summarizes data obtained from three-week-old (DIV21) primary cortical cultures prepared from wild-type and NR2B$^{\Delta C/\Delta C}$ mice: NMDA or AMPA peak currents, charges and kinetics. Extrasynaptic AMPA/NMDA ratios were reduced by half in NR2B$^{\Delta C/\Delta C}$ cultures compared to wild-type sister cultures, as indicated by the nucleated patches (A). mEPSCs recordings indicate reduced by one third AMPA/NMDA amplitude ratios NR2B$^{\Delta C/\Delta C}$ cultures compared to wild-type (B). Experiments were performed by Verena Pawlak, MPI for Medical Research, Heidelberg.

Western blot analysis and elecrophysiological recordings clearly indicate that in NR2B$^{\Delta C/\Delta C}$ cultures, in which the NR2B subunit has impaired signaling properties, the NR2A subtype is expressed at normal levels; it is transported to the cell membrane but does not reach the synaptic sites, remaining extrasynaptically located. These findings suggest that normal NR2B signaling is required for the postsynaptic presence of NR2A-containing channels.

3.5 NMDA-induced toxicity in wild-type cultures

To investigate the contribution of NR2A and NR2B subtypes to excitatory cell death, NMDA toxicity experiments were performed in wild-type cultures. A pharmacological approach using the NR2B and NR2A subunit-specific antagonists offered the advantage to study these receptor subtypes in wild-type high-density cultures. In this system both NR2A and NR2B subunits are normally expressed with unaltered functional properties. By blocking any of these two NMDA receptor

subtypes, excitotoxicity experiments indicated the contribution to neuronal cell death of the remaining "available" NR1/NR2 subunit combination.

3.5.1 NMDA-induced toxicity in the presence of the NR2B subunit-specific antagonist

Application of NMDA in the presence of the NR2B subunit-specific blocker (ifenprodil) had a protective effect in young wild-type cultures (Fig. 25). This neuronal protection indicated that at 14 DIV, when the NR2A subunit is expressed at low levels, the NR2B subtype is the only mediator of the NMDA-induced excitotoxicity.

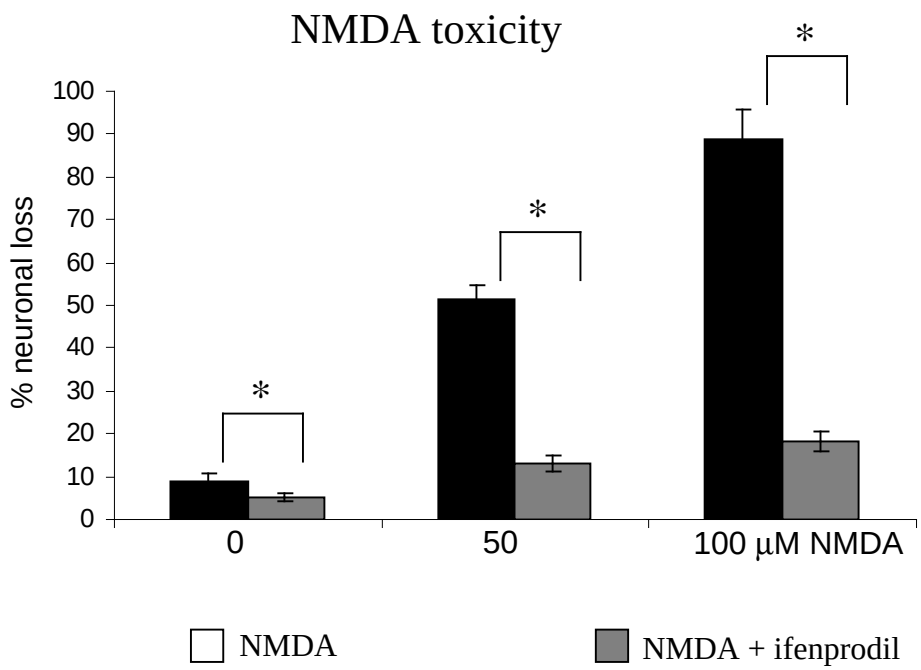

Fig. 25: *NMDA-induced toxicity in young wild-type culture in the presence of ifenprodil*
Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 μM NMDA in the presence of ifenprodil (10 μM). Experiments were performed at 14 DIV on wild-type cultures. In the presence of ifenprodil, NMDA toxicity is mediated by the NR2B subunit. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

At 21 DIV, when the NR2A subunit reaches the highest expression levels, cultures exposed to NMDA in the presence of ifenprodil were not protected anymore from the toxic effect. This result indicates that in mature cultures, by blocking the NR2B subunit, toxicity becomes mediated mainly via the NR2A-containing channels (Fig. 26).

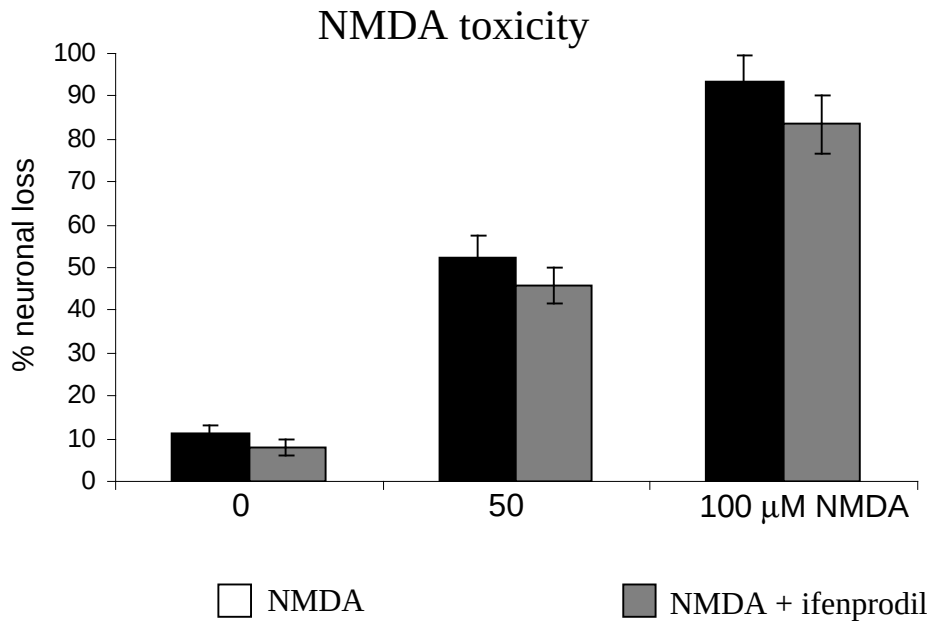

Fig. 26: *NMDA-induced toxicity in mature wild-type culture in the presence of ifenprodil*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA in the presence of ifenprodil (10 µM). Experiments were performed at 21 DIV on wild-type cultures. In the presence of ifenprodil, NMDA toxicity is mediated via the NR2A subunits. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

In summary, NMDA application triggers neuronal cell death mainly due to the NR2B subunits in young wild-type cultures. At a later stage *in vitro*, when the NR2B subunit is blocked by ifenprodil, excitotoxicity is mainly mediated via the NR2A subtype.

3.5.2 NMDA-induced toxicity in the presence of the NR2A subunit-specific antagonist

A big advantage to study the NR1/NR2A subunit contribution to toxicity was to use a newly developed drug, NVP-AAM077, which is an NR2A subunit-specific antagonist (Novartis Pharma AG, Switzerland).

In young cultures NMDA application in the presence of NR2A blocker triggered neuronal loss in a normal dose-response manner, since at this age *in vitro* the NR2A subunit is expressed at low levels (Fig 27).

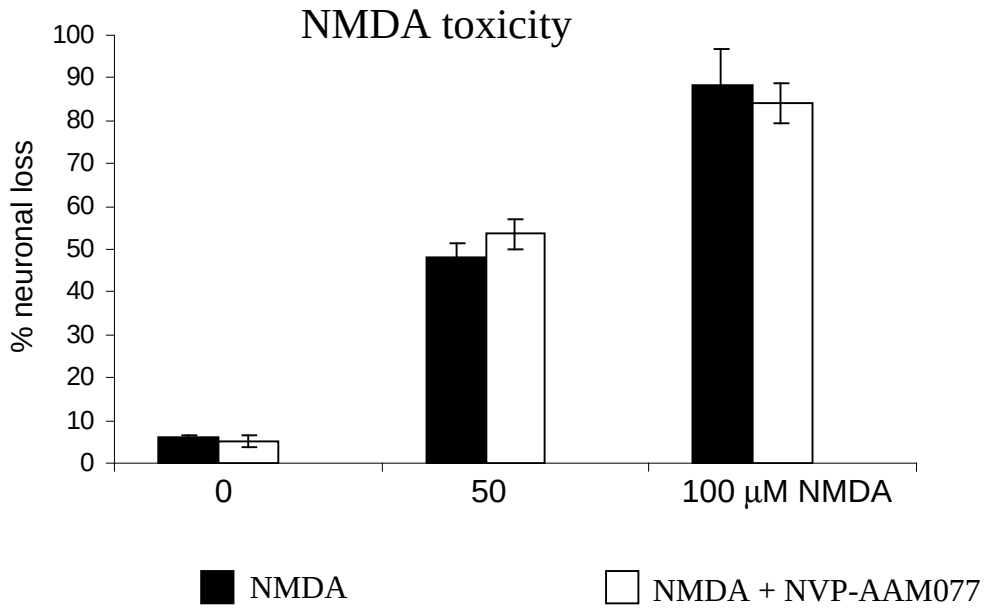

Fig. 27: *NMDA-induced toxicity in young wild-type culture in the presence of NVP-AAMO77*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 μM NMDA in the presence of NVP-AAM077 (0.05 μM). Experiments were performed at 14 DIV on wild-type cultures. The presence of NR2A subunit antagonist had no protective effect to NMDA-induced toxicity at 14DIV. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

In contrast, at 21 DIV, application of NMDA in the presence of NR2A subunit-specific antagonist led to a strong neuronal cell death (Fig 28). Interestingly, 50 μM NMDA led to almost 100 % cell

death, suggesting that the NR2A blocker application during NMDA exposure triggers probably a more “aggressive” cell death pathway.

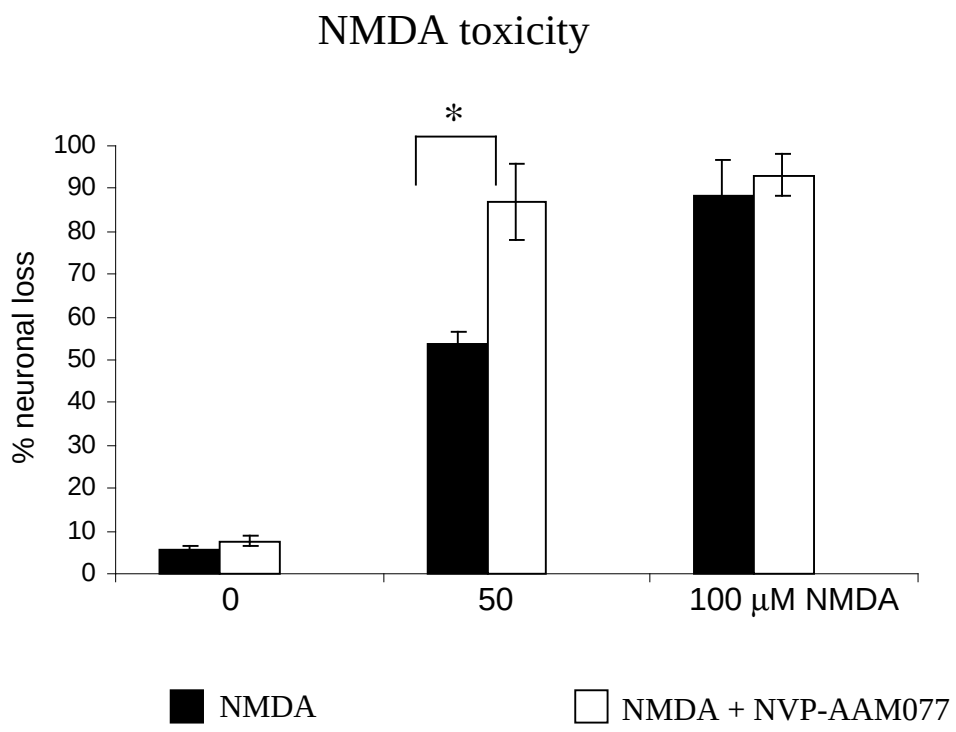

Fig. 28: *NMDA-induced toxicity in mature wild-type culture in the presence of NVP-AAMO77*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 μM NMDA in the presence of NVP-AAM077 (0.05 μM). Experiments were performed at 21 DIV on wild-type cultures. The presence of NR2A subunit antagonist had no protective effect to NMDA-induced toxicity. Values represent STDEV of 5 to 6 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student’s t-test, $p<0.05$.

Notably, the application of the NR2A subunit-specific blocker, NVP-AAM077, has no cytotoxic effect. A series of experiments were performed using different concentrations of NR2A blocker: 0.5 μM, 0.1 μM and 0.05 μM. In all the experiments the results were similar.

In summary, in mature cultured neurons, neither the NR2A antagonist NVP-AAM077 nor the NR2B antagonist ifenprodil protected the cells from NMDA-mediated toxicity, indicating that both NR2A

and NR2B subtypes are responsible for excitotoxicity. Notably, the cells were protected when both NR2A and NR2B subunits were blocked during NMDA application (Fig. 29).

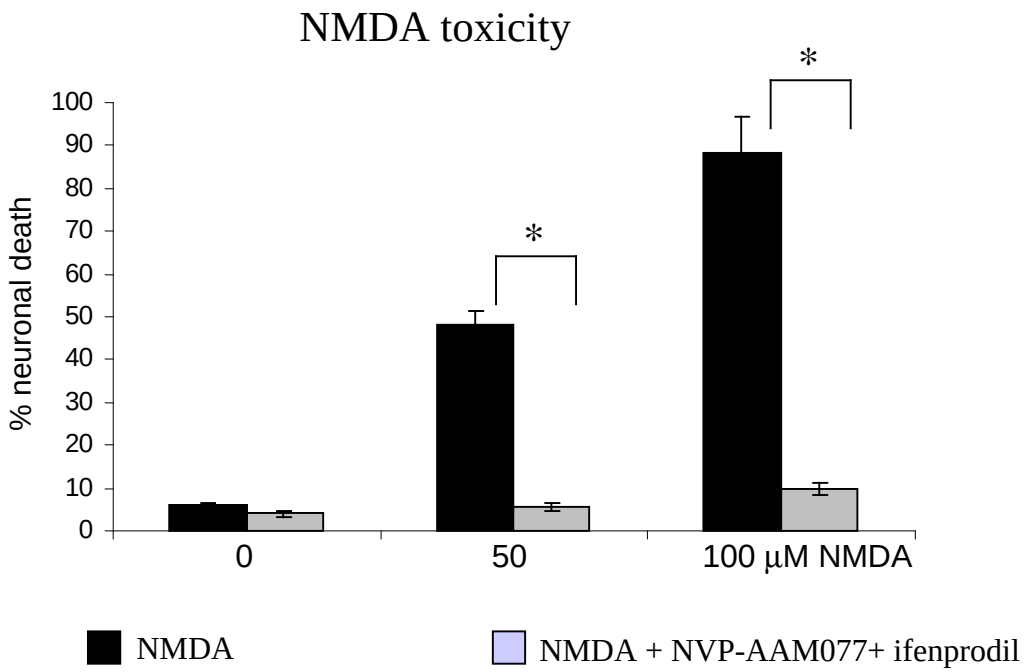

Fig. 29: *NMDA-induced toxicity in wild-type culture in the presence of NVP-AAMO77 and ifenprodil*

Neuronal viability of cortical neurons *in vitro* 24 hours after 10 minutes exposure to either 50 or 100 µM NMDA in the presence of NVP-AAM077 (0.05 µM) and ifenprodil (10µM). Experiments were performed 21 DIV on wild-type cultures. The presence of NR2A and NR2B subunit antagonists protected cells from NMDA-induced toxicity. Values represent STDEV of 4 to 5 independent experiments, each performed in quadruplicate. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

Control experiments were performed with the NR2A subunit specific antagonist on $NR2A^{\Delta C/\Delta C}$ cultures. Application of NMDA in the presence of NVP-AAM077 induced a toxic response as did NMDA application alone, indicating that the NR2A antagonist has not side effects and is not toxic itself when applied to neurons in culture. Furthermore, at 21 DIV, ^{45}Ca analysis indicated that in the presence of the NR2B subunit-specific blocker, Ca^{2+} influx became reduced by half, suggesting that

in mature cultures NR2A and NR2B are roughly equally contributing to NMDA-induced Ca^{2+} influx (Fig. 30).

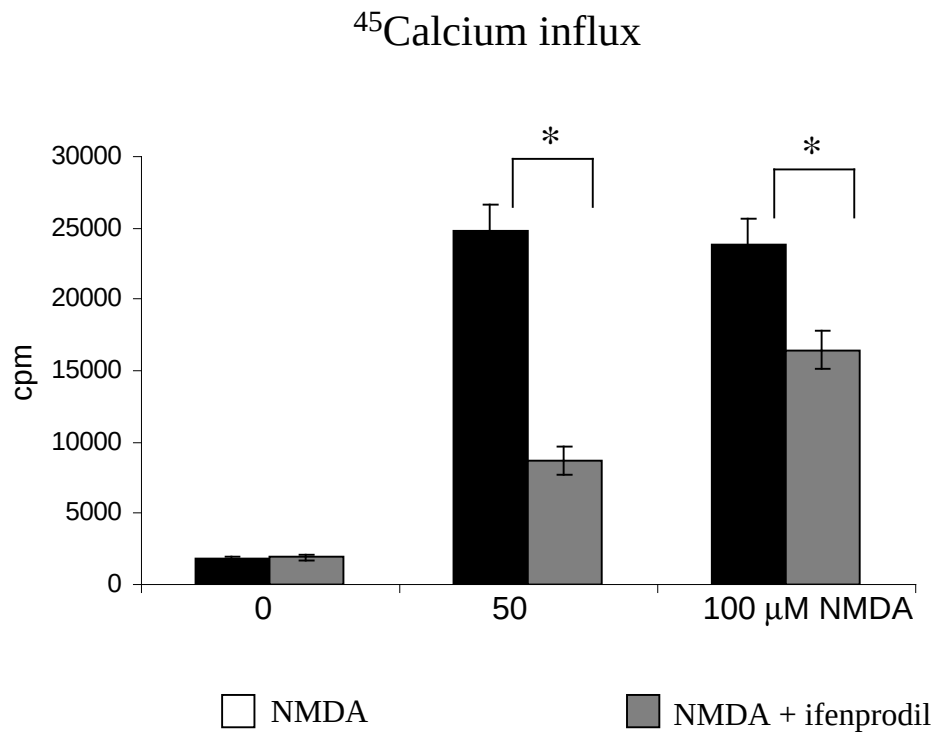

Fig. 30: ***45Ca influx through NMDA receptor channels in wild-type cultures in the presence of ifenprodil***

At 21 DIV in wild-type cultures the NR2A and NR2B subunis equally mediated NMDA-induced Ca^{2+} influx. For measurement of Ca^{2+} influx, ^{45}Ca was added during the NMDA exposure for 5 minutes; cultures were then washed thoroughly and ^{45}Ca activity was measured. Values represent mean ± SEM of 5 to 6 independent experiments performed by Jakob von Engelhardt. Statistical significance was evaluated by unpaired Student's t-test, $p<0.05$.

These results indicate that the differences in the developmental expression pattern of NR2A and NR2B might play a critical role in NMDA-evoked toxicity. In young cultures, the NR2B subtype is responsible for excitotoxicity. In mature cultures, both NR2A and NR2B are roughly equally contributing to NMDA-induced Ca^{2+} influx and excitotoxicity. Only the combined action of the NR2A and NR2B subunit-specific antagonists could protect the cells from NMDA-induced excitotoxicity.

4. Discussion

Neurological diseases affect the humans in memory, cognition, language, personality and skilled movements. Cerebrovascular diseases (stroke syndromes) are a major cause of morbidity and mortality in middle and later life.

Inappropriate activation of NMDARs has been implicated in the etiology of several disease states. In particular, excessive Ca^{2+} influx through NMDARs can cause excitotoxic neuronal death, and thus blockade of NMDARs is neuroprotective in animal models of both stroke and seizure (Li et al., 1999). Stroke was, therefore, the first clinical indication considered for NMDARs antagonists, but the usefulness of most drugs was limited by their actions on normal synaptic transmission or by additional side effects. Many of NMDAR antagonists lacking psychotic side-effects that have been considered for the treatment of stroke failed to live up to preclinical expectations, showing little or no therapeutic benefit.

4.1 NMDA receptor subtypes in excitotoxicity

In recent years, the study of excitotoxicity has been carried out in a variety of simplified *in vitro* preparations, including cell culture, tissue culture, retina and brain slice. Information gathered in such simplified systems has generally been consistent with *in vivo* observations, and has produced additional information about underlying mechanisms. However, it is clear that important differences can occur by studying the nature of excitotoxicity using different neuronal preparations.

NR1/NR2A and NR1/NR2B are the two major NMDA receptor subtypes expressed in principal neurons. These receptors play an important role in synaptic plasticity and memory formation, but the contribution to excitotoxicity by these two major NMDA receptor subtypes, NR2A and NR2B, has remained enigmatic.

Both NR2A- and NR2B-type NMDA receptors activate signaling pathways that lead to LTP formation (Köhr et al., 2003). The NR2B subunit predominates early in development and then gradually decreases, whereas expression of NR2A is low shortly after birth but continues to increase. Therefore, NR2B is the major subunit during the early period of a neuron's life, whereas NR2A is predominant in the later stages, suggesting that the NR2B to NR2A switch is responsible for the transition of a synapse from a more plastic to a less plastic state. In young animals, both NR2A and

NR2B contribute to LTP, whereas in adult animals, NR2A-mediated signaling is dominating (Köhr et al., 2003). The importance of having the correct number and composition of NMDA receptor subunits is demonstrated by genetic studies. Targeted disruption of the NR2A subunits produces mice with a reduction in LTP and deficiences in some learning tests (Sakimura et al., 1995). The contribution of the NR2B-type pathway to learning and memory was demonstrated in mice overexpressing the NR2B subunit (Tang et al., 1999; Wong et al., 2002). Overexpression of the NR2B subunit in the forebrain produces a so-called “smart mouse” that shows improved performance on memory tasks and larger NMDA-mediated currents in the hippocampus. However, it is not known if this is due to the overexpression of the NR2B subunits or due to an increased total number of NMDA receptors.

The development of mutant mice provides further insights into the role of distinct NR2 subunits in neurotoxicity. Morikawa (Morikawa et al., 1998) investigated the role of the NR2A and NR2B subunits in brain ischemia using mutant mice deficient in NR2A and double mutants deficient in NR2A and NR2B (Sakimura et al., 1995; Kiyama et al., 1998). NR2A deficiency results in a pronounced reduction in infarct volume. Infarct volume in mice lacking both NR2A and NR2B was not different compared to the infarct volume in NR2A deficiency alone. The authors suggested that NR2A subunits play an important role in glutamate neurotoxicity. However, an alternative suggestion is that the observed protective effect could also result from a reduced number of functional NMDA receptors, as NR2A knock-out mice show decreased NMDA receptor channel activity (Sakimura et al., 1995; Kiyama et al., 1998). Alternatives to knock-out mice are gene-manipulated mice which express C-terminally truncated NR2 subunits. Most of these mice express gateable receptors that are synaptically activated, but are defective in intracellular signaling and synaptic localization.

The intracellular, C-terminal domains of the NR2 subunits have been shown to interact with putative scaffolding proteins of the postsynaptic density, such as PSD-95 (Kornau et al., 1995, 1997; Kim et al., 1996; Mueller et al., 1996; Niethammer et al., 1996; Kim and Huganir, 1999). This type of interaction has been suggested to be critical for synaptic targeting, clustering and signal transduction. The importance of the C-termini of NMDA receptor subunits is further emphasized by the $NR2^{\Delta C/\Delta C}$ mice expressing truncated NR2 subunits (Sprengel et al., 1998), which phenotypically resemble the respective knock-out mice.

In order to investigate the contribution of the NR1/NR2A and NR1/NR2B NMDA receptor subtypes to excitatory cell death, NMDA-induced toxicity experiments were performed using high-density primary cortical cell cultures. This *in vitro* cell culture system simplifies the direct pharmacological approach of the NR2A and NR2B subunits. By using NR2 subunit-specific antagonists, NMDA receptors could be directly manipulated during relatively fast and reproducible excitotoxicity experiments. The use of neuronal cultures from mice with impaired functions of the NR2 subunits ($NR2^{\Delta C/\Delta C}$ mice) made it possible to study separately the involvement of NR2A and NR2B subtypes during excitatory cell death.

Immunoblot analysis, using antibodies against distinct N-methyl-D-aspartic acid (NMDA) receptor subunits, illustrated that the NR2A and NR2B subunit proteins have developmental profiles in cultured cortical neurons similar to those seen *in vivo* (Li et al., 1998). NR1 and NR2B subunits display high levels of expression within the first week. In contrast, the NR2A subunit is barely detectable at 7 days in vitro (DIV) and then gradually increased to mature levels at 21 DIV. Immunocytochemical analysis indicated that NMDA receptor subunits cluster in the dendrites and soma of cortical neurons in culture (Li et al., 1998). Clusters of NR1 and NR2B subunits were observed as early as 3 DIV, while NR2A clusters were rarely observed before 10 DIV. At 18 DIV, NR2B clusters partially co-localize with those of NR2A subunits, but NR2B clusters always co-localize with those of NR1 subunits. Li's studies indicate that excitatory synapse formation occurs when NMDA receptors comprise NR1 and NR2B subunits, and that NR2A subunits cluster preferentially at synaptic sites.

To accurate investigate the role of the two major NMDA receptor subtypes NR2A and NR2B to excitatory cell death, all the NMDA-induced toxicity experiments were therefore in this study performed in young (14 DIV) as well as in mature (21 DIV) cultures.

4.1.1 In $NR2A^{\Delta C/\Delta C}$ cultures excitotoxicity is mediated via NR1/NR2B containing NMDA receptors

In the first set of experiments, NMDA-induced toxicity experiments were performed on cultures prepared from $NR2A^{\Delta C/\Delta C}$ mice. By blocking NR2B subunits during NMDA application, it was observed that young and mature neurons were entirely protected from NMDA-induced toxicity.

Furthermore, $^{45}Ca^{2+}$ analysis indicated that in the presence of a NR2B subunit-specific blocker, no Ca^{2+} influx through NMDA receptor could be observed. These results suggest that in the NR2A$^{\Delta C/\Delta C}$ cultures in which the function of the NR2A subunit is impaired, the NR2B subunit is the only key mediator of Ca^{2+} influx and excitatory cell death.

Electrophysiological recordings indicated no change in the somatic and synaptic NMDA currents in the NR2A$^{\Delta C/\Delta C}$ cultures compared to those recorded in wild-type sister cultures. Western blot analysis proved furthermore that the NR2B subunit is expressed at normal levels, suggesting that the C-terminal truncation of the NR2A subunit had no consequences on the expression level of the NR2B subunit. Colocalization analysis of the immunoflourescently labeled NR2A subunit and the synaptic marker protein Synapsin I indicated a reduction in the number of NR2A-containing synapses in mature NR2A$^{\Delta C/\Delta C}$ cultures. This suggests that the C-terminal truncation of the NR2A subunit led to an impaired synaptic localization of NMDA receptors containing the NR2A subunit. These results are consistent with the data obtained by Steigerwald et al. (2000) analyzing the function of the NR2A$^{\Delta C}$ subunit in hippocampal slices and hippocampal cultures.

In hippocampal slices from adult wild-type mice, the NR2A subunit is primarily responsible for the LTP induction at hippocampal CA1-to-CA3 synapses, independent of the used tetanization paradigm (Köhr et al., 2003). However, in hippocampal slices from NR2A$^{\Delta C/\Delta C}$ mice, a lack in NR2A-mediated LTP is observed; a strong LTP induction protocol leads to the formation of an exclusively NR2B-mediated LTP. Excitotoxicity experiments performed on cultures prepared from NR2A$^{\Delta C/\Delta C}$ mice were consistent with these LTP studies. When the intracellular pathway was eliminated by removal of the C-terminal domain of the NR2A subunit, NR2B-type channels were the only mediators of the signaling that access the “cell death program” pathway.

4.1.2 No NMDA-mediated excitatory cell death in neuronal NR2B$^{\Delta C/\Delta C}$ cultures

Studies on cultured neocortical neurons prepared from NR2B$^{\Delta C/\Delta C}$ mice indicated that the synaptic NMDA receptor fraction is drastically reduced, suggesting that the C-terminal domain of the NR2B subunit plays a major role in synaptic targeting of NMDA receptors at nascent synapses (Mohrmann et al., 2002). Furtehrmore, the analyisis of the biophysical properties of NMDA receptor channels

revealed a reduced peak open probability and a reduced mean open time in neurons from $NR2B^{\Delta C/\Delta C}$ mice. This might reflect the loss of tonic modulation of NMDA receptor channels that is mediated by protein phosphorylation of the C-terminal domain of NR2 subunits (Mohrmann et al., 2002).

It is particularly interesting to study the effect of the C-terminal truncation of the NR2B subunit in excitotoxicity, since NR1/ NR2B receptors represent the "young" form of NMDA receptors in immature synapses.

Therefore in a second set of experiments NMDA-induced toxicity experiments were performed on cortical cultures prepared from $NR2B^{\Delta C/\Delta C}$ mice. Interestingly, cultured neurons from $NR2B^{\Delta C/\Delta C}$ embryos showed a strong protection against NMDA-induced toxicity. $^{45}Ca^{2+}$ analysis indicated no Ca^{2+} influx through NMDA receptors in cultures prepared from $NR2B^{\Delta C/\Delta C}$ mice compared to wild-type sister cultures. These results suggest that the NR2A subunit can not be part of any compensatory mechanism contributing to excitatory cell death in cortical cultures in which the intracellular NR2B subunit function is impaired by the truncation of the C-terminus.

Western blot analysis indicated a normal expression level of the NR2A subunit, comparable to those observed in wild-type cultures. Electrophysiological recordings from nucleated patches showed about one-third reduced somatic currents. Analysis of mEPSC recordings revealed that synaptic currents are reduced one third compared to those recorded in wild-type sister cultures.

These results lead to the conclusion that the NR2A subunit is not able to compensate the functional impairment of the NR2B subunit. Furthermore, when the NR2B subunit is expressed in the C-terminal truncated form, the localization of the NR2A subunit is abolished at synaptic sites. As indicated by the Western blot analysis, the NR2A subunit is expressed at a level comparable to that observed in wild-type sister cultures; the subunit is transported to the cell membrane but it can not be functionally integrated into synaptic sites.

4.1.3 In wild-type cultures excitotoxicity is mediated by NR2A and NR2B containing NMDA receptors

The investigation of the contribution of NMDA receptors with different subunit compositions to excitotoxicity has been difficult without the availability of pharmacological agents that can reliably distinguish between the different NR2 subunits. The only ones available have been ifenprodil and CP101606, two polyamine site antagonists. These antagonists are selective for the NR2B subunit.

However, the recent introduction of NVP-AAM077, a NR2A selective antagonist, has allowed in this study the further investigation of the role this major subunit plays in excitotoxicity. Recently that have been published two studies, using the newly developed NR2A subunit specific antagonist, that focus on the roles of NR2A- and NR2B-containing NMDA receptors in both LTP and LTD in slices from adult and juvenile rats. The results of these two studies demonstrate that NR2A receptors are involved in LTP and depotentiation while NR2B receptors are involved in formation of *de novo* LTD (LTD induced at synapses that have not been previously altered experimentally) (Liu et al., 2004; Massey et al., 2004).

In order to study the particular contribution of the NR2A and NR2B subtypes to NMDA-induced excitotoxicity, a pharmacological approach was performed using cortical wild-type cultures.

Young cultures were protected from neuronal loss when the NR2B subunit-specific blockers ifenprodil or CP-101606 was added during NMDA application. But when the NR2B subunits were blocked by ifenprodil the mature wild-type cultures were vulnerable to NMDA mediated excitatory cell death. These contrasting results can be explained by the different developmental expression profile of the two subunits: in young cultures excitotoxicity is mediated mainly via receptors containing at this stage NR2B subunit, as a dominant subtype. The higher expression level of the NR2A-subunit in mature neurons leads to the vulnerability in the presence of the NR2B-specific antagonist. These experiments lead to the conclusion that in mature cultures in the presence of a NR2B-specific blocker NMDA toxicity is mediated via the NR2A-containing receptors.

As could be expected from the developmental expression profile of the NR2A subunit and the previously described results, the application of a newly developed NR2A-specific antagonist called NVP-AAM007 can not protect young neuronal cultures to NMDA-induced excitotoxicity. But surprisingly the antagonist is also not able to prevent neuronal cell death in the mature cultures. Only the combined application of a NR2A and a NR2B-specific antagonists can rescue the neurons at 21 DIV. Blocking any of the two subtypes is not reducing toxicity at a mature stage, due to the ability of both subtypes, NR2A-containing or NR2B-containing NMDA receptors, to compensate with no apparent time delay.

4.2 NMDA receptor localization and excitotoxicity

Functional and immunocytochemical studies have shown that NMDA receptors are present at both synaptic and extrasynaptic sites. It is possible that the extrasynaptic population simply represents receptors that have been delivered to the plasma membrane and await incorporation into the synapse. This idea is supported by recent work that shows that NMDARs can rapidly move between synaptic and extrasynaptic sites, possibly by lateral diffusion of the receptor (Tovar and Westbrook, 2002). However, the possibility remains that there is a distinct population of extrasynaptic receptors with a specific function. Furthermore, synaptic and extrasynaptic receptor pools have different subunit compositions and form distinct receptor populations: NR2A-containing NMDA receptors are mainly localized at synaptic sites, whereas NR2B-containing NMDA receptors are mainly at extrasynaptic sites (Tovar and Wesbrook, 1999).

Recent studies indicate that synaptic and extrasynaptic NMDA receptors have opposite effects on CREB (cAMP response element binding protein) function, gene regulation and neuronal survival (Hardingham et al., 2002). Ca^{2+} entry through synaptic NMDA receptors induces CREB activity and BDNF (brain-derived neurotrophic factor) gene expression. In contrast, Ca^{2+} entry through extrasynaptic NMDA receptors activates a dominant CREB shut-off pathway that blocks BDNF gene expression and promotes neuronal cell death. The results obtained by Hardingham et al. actually suggest that the NR2B NMDA receptor subtype, located extrasynaptically, is the main mediator of the cell death pathway.

Neuronal cultures prepared from $NR2B^{\Delta C/\Delta C}$ embryos exhibited no vulnerability against NMDA-mediated excitotoxicity. As indicated by Western blot analysis the NR2A subunit is expressed in these cultures at levels comparable with those observed in wild-type neurons. Furthermore the recordings of extrasynaptic currents prove that the NR2A subunits are transported to the cell surface membrane and stay localized at extrasynaptic sites. This leads to the conclusion that the activation of extrasynaptic NMDA receptors is not the main determinant for the induction of the neuronal cell death program. These results are contradictory with the studies described by Hardingham et al. One possible explanation is that all the investigations done by Hardingham et al. were performed in low-density hippocampal cultures, at 10-12 days *in vitro*. By using relatively young neuronal cultures (14 DIV) we were also able to conclude that excitotoxicity is indeed mainly mediated via the NR2B subunit, since neurons are not mature and express only low levels of the NR2A subunit. In addition our results obtained from mature $NR2A^{\Delta C/\Delta C}$ cultures are consistent with the excitotoxicity seen in

wild-type cultures, but, in contrast in the cultures with the C-terminally truncated form of the NR2A subunit, NR2B-containing receptors are responsible for the neuronal cell death. Our data suggest that in the $NR2A^{\Delta C/\Delta C}$ cultures, in which the function of the NR2A subunit is impaired, the NR2B subunit localized at synaptic sites is the only mediator of Ca^{2+} influx and excitatory cell death.

4.3 Ca^{2+} influx, intracellular signaling and excitotoxicity

Ca^{2+} -mediated neurotoxicity requires distinct signaling pathways to be triggered in cells, and such pathways are more efficiently triggered when Ca^{2+} ions enter neurons at specific entry points, particularly at Ca^{2+} -permeable glutamate receptors (Timianski et al., 1993; Sattler et al., 1998). This has been called the “source-specificity” hypothesis of Ca^{2+} neurotoxicity.

A previous study (Sattler et al., 1998) reveals that Ca^{2+} loading through L-type voltage-sensitive Ca^{2+} channels (VSCCs) is not harmful, whereas similar Ca^{2+} loads produced via NMDA receptors are highly neurotoxic. The source-specificity hypothesis proposes that rate-limiting enzymes or substrates responsible for excitotoxicity must be co-localized with NMDA receptors.

Current knowledge of the molecular organization of the PSD provides a blueprint for explaining the toxicity of Ca^{2+} ions that enter neurons through NMDA receptors. It suggests that NMDA receptor-mediated Ca^{2+} signals are linked to downstream neurotoxic signaling pathways through protein-protein interactions.

Recent studies show that by suppressing the expression of PSD-95 in cultured cortical neurons using antisense oligonucleotides, the function of NMDA receptors is not altered and the receptor expression, NMDA currents and Ca^{2+} loading via NMDA receptors are unchanged (Sattler et al., 1999). However, suppressing PSD-95 selectively attenuated Ca^{2+}-activated NO (nitric oxide) production by NMDA receptors. The authors conclude that PSD-95 is required for the efficient coupling of NMDA receptor activity to NO toxicity, and imparts specificity to NMDA receptor-mediated excitotoxic Ca^{2+} signaling.

In the present study, no specific experiments were performed to investigate any component of the intracellular signaling pathway. But by using cultures prepared from $NR2^{\Delta C/\Delta C}$ mice it was possible to study the importance of the C-termini of NR2A and NR2B to Ca^{2+} influx and excitatory cell death.

Cultures prepared from NR2B$^{\Delta C/\Delta C}$ mice were resistant against NMDA-induced excitotoxicity, and no obvious Ca^{2+} influx could be measured. In contrast, in NR2A$^{\Delta C/\Delta C}$ cultures Ca^{2+} influx and NMDA-induced neuronal loss were similar with wild-type cultures. These results indicate that in the absence of functional NR2A subunits, the NR2B subtype is able to compensate and mediate Ca^{2+} influx alone, thereby inducing the cell death pathway. However, when NR2B subunit function is impaired, the NR2A subtype is not able to participate in any compensatory mechanism and Ca^{2+} influx is occluded. Electrophysiological recordings indicated reduced extrasynaptic currents in NR2B$^{\Delta C/\Delta C}$ cultures. These results do not exclude that in the NR2B$^{\Delta C/\Delta C}$ cultures a very low Ca^{2+} influx through extrasynaptic NMDA receptors occurs, but these concentrations are to small to be detected with the $^{45}Ca^{2+}$ assay. The amount of Ca^{2+} ions that enter the cells is not sufficient to trigger the cell death cascade.

4.4 From molecular interactions to clinical treatments

The idea that the NMDA receptor/PSD-95 interaction might constitute a therapeutic target for diseases that involve excitotoxicity was recently investigated (Aarts et al., 2002). This concept is illustrating the importance of intracellular interactions between the C-terminus of NR2 subunits with other proteins. NMDA receptors mediate ischaemic brain damage, but their main function is the transmission of neuronal excitation, which is essential for normal CNS function. Thus, blocking NMDA receptors in order to treat stroke is problematic. Targeting PSD-95 protein therefore represents an alternative therapeutic approach that may circumvent the negative consequences of blocking NMDA receptor function. However, mutation or suppression of PSD-95 is impractical as a therapy for brain injury and can not be applied after an injury has occurred.

The question was whether interfering with the NMDA receptor/PSD-95 interaction rather than altering PSD-95 expression would be a better approach in order to suppress excitotoxicity *in vitro* and ischemic brain damage *in vivo*. To treat stroke without blocking NMDA receptors, neurons *in vitro* and *in vivo* are transfected with peptides that bind to the modular domains governing interactions of NMDA receptors with PSD-95. The peptide treatment protects cultured neurons from excitotoxicity. In addition it reduces dramatically cerebral infarction in rats subjected to transient focal cerebral ischemia, while improving neurological functions. The treatment is effective when applied either before, or one hour after, the onset of excitotoxicity *in vitro* and cerebral ischemia *in*

vivo (Aarts et al., 2002). This and similar strategies based on a molecular understanding of excitotoxic mechanisms may amount to practical future treatments for human neurological disorders.

4.5 NMDA subunits antagonits and clinical trials

Among CNS disorders, the preclinical rationale for the acute treatment of brain ischemia is the strongest (Kemp et al., 1999), and nonselective NMDA antagonists are the most consistently neuroprotective agents in animal models of stroke. Nevertheless, clinical trials in stroke and traumatic brain injury with NMDA antagonists have failed so far (Kemp et al., 1999; Lees et al., 2000; Sacco et al., 2001). NMDA antagonists have a number of adverse CNS effects, including hallucinations, a centrally mediated increase in blood pressure and, at high doses, catatonia and anesthesia (for which the NMDA channel blockers phencyclidine and ketamine were initially developed).

Subtype selective compounds of those with improved biochemical mechanisms are further being developed and studied. A number of selective NR2B subunit antagonists have been described, most of which are neuroprotective in animal models and produce minimal side effects at maximally neuroptotective doses (for example ifenprodil, CP-101606, Ro 25-6981 and Ro 63-1908). Encouragingly, in humans, CP-101606 did not induce the usual side effects seen with nonselective NMDA antagonists. However, enthusiasm for this approach has been tempered by its reported failure in a clinical trial of traumatic brain injury (press release from Pfitzer, October 2001).

There is big hope for further trials with different compounds, and the new NR2A subunit antagonist NVP-AAM077 has recently been developed by Novartis Pharma (AG, Switzerland). In the present study, this NR2A subunit specific antagonist showed no protective effect to NMDA-induced excitotoxicity using mouse cortical cell cultures. Blocking any of the two NR2 subtypes did not reduce excitotoxicity in a mature neuronal culture system, due to the ability of both studied NR2 subunits to compensate for the functional loss of NMDA receptors with the other subunit without apparent time delay.

4.6 Ischemia – still a challenge

The past decade has produced a plethora of negative neuroprotective trial results. This is frustrating because all of these agents seemed effective in animal trials. The ischemic cascade appears to be so complex that targeting a single pathway may be ineffective.

A "good" *in vivo* animal model of stroke must reproduce the etiology, anatomical, functional and metabolic consequences of human pathology and must also permit the study of anti-ischemic drugs in conditions pertinent to the clinical therapeutics. As stroke is a very heterogeneous clinical entity, such a model could only mimic a limited part of stroke. Animal data are usually collected in healthy laboratory rodents of the same age, in which a standardized amount of cerebral ischemia is induced by a reproducible intervention. In contrast, aetiology, location and severity of ischaemic stroke in patients is very heterogeneous.

A large number of compounds have been shown to be active using animal models, but unfortunately, none of them was found to be active in clinical trials. Various factors could be responsible for this major discrepancy, and some of them are not related to pre-clinical studies, but to the complexities of the clinical problem of stroke. Failure in the translation of results from animal models to humans implicates potential limitations of the current drug development process. Retrospective analysis of studies suggests possible improvements at several stages during pre-clinical studies.

Standardized guidelines for preclinical evaluation of neuroprotective drugs may improve chances of success.

5. Abbreviations

α	Alpha
A	Ampere
ACPD	aminocyclopentane-1S, 3R-dicarboxylic acid, mGluR agonist
AMP	adenosine monophosphate
AMPA	L-α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid
AMPAR	AMPA receptor
D-AP5	D-5-amino phosphonate pentanoic acid
ARL 15896	[(+)-alpha-phenyl-2-pyridineethanamine], NMDAR channel antagonist
ATP	adenosine triphosphate
BAPTA	1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetracacetic acid
BDNF	brain-derived neurotrophic factor
BMI	bicuculline methiodide
BSA	bovine serum albumin
bp	baise pairs
C	Celsius
CA	Cornu ammonis
CaMKII	Ca^{2+}/calmodulin-dependent proteinkinase II
cDNA	copy DNA
CNS	central nervous system
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione, AMPA antagonist
Δ	Delta
DIV	days *in vitro*
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
IC50	concentration required for half maximal response
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
EPSC	excitatory postsynaptic current
et al.	et alii
Fig.	Figure

FITC	Fluorescein isothiocyanate
G-protein	guanosine nucleotide-binding protein
GABA	γ-Aminobutiric acid
γ	gamma
GluR	glutamate receptor
GTP	guanosine triphosphate
h	hour
HCSS	HEPES-buffered control salt solution
HEPES	N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid
IPSP	inhibitory postsynaptic current
KA	kainate
LTD	long-term depression
LTP	long term potentiation
µ	micro
m	milli
m	miniature
M	membrane (domain)
M	Mol
mGluR	metabotropic glutamate receptor
min	minute
mRNA	messenger RNA
MS-153	[R]-[-]-5-methyl-1-nicotinoyl-2-pyrazoline, NMDAR antagonist
n	nano
n	number (of experiments, animals, etc.)
N	asparagine
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NBQX	2,3-dihidroxy-6-nitro-7-sulfmoyl-benzo-(F)-quinoxalone
NO	nitric oxide
NOS	nitric oxide synthase
NR1	NMDAR subunit 1

NR1-1a	NMDAR subunit 1 splice variant 1a
NR2A/B/C	NMDAR subunit 2A/B/C
NR3A/B	NMDAR subunit 3A/B
NTD	N-terminal domain
NVP-AAM077	(1RS,1'S)-PEAQX, NR2A subunit specific antagonist
Ω	Ohm
Osm	Osmol
%	percentage
p	pico
p	probability
P	postnatal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95, Disc-large, Zona-occludens-1, proteins in which PDZ domains were originally identified
PFA	paraformaldehyde
PKA	cyclic AMP-dependent protein kinase A
PKC	protein kinase C
PSD	postsynaptic density
Q	glutamine
R	arginine
RNA	ribonucleic acid
s	second
S	segment
S	serine
S	Siemens
SDS-PAGE	SDS-PolyAcrylamide Gel Electrophoresis
SDS	sodium dodecylsulfate
SEM	standard error of the mean
STDEV	Standard deviation
Taq	Therminus aquaticus
Tris	Tris(hydroxymethyl)aminomethan

TTX	tetrodoxin
U	unit
V	voltage
V	volts
VDCCs	voltage-dependent calcium channels
vs	versus
W	Watt
wt	wild-type

6.References

Aarts, M., Liu, Y., Liu, L. et al. (2002). Treatment of ischemic brain damage by perturbing NMDA receptor. *Science* 298, 846-850.

Akazawa, C., Shigemoto, R., Bessho, Y., Nakanishi, S., and Mizuno, N. (1994). Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J Comp Neurol* 347, 150-160.

Anson, L. C., Chen, P. E., Wyllie, D J., Colquhoun, D., and Schoepfer, R. (1998). Identification of amino acid residues of the NR2A subunit that control glutamate potency in recombinat NR1/NR2A NMDA receptors. *J Neurosci* 18, 581-589.

Araki, K., Meguro, H., Kushiya, E., Takayama, C., Inoue, Y., and Mishina, M. (1993). Selective expression of the glutamate receptor channel delta 2 subunit in cerebellar Purkinje cells. *Biochem Biophys Res Commun* 197, 1267-1276.

Araque, A., Sanzgiri, R. P., Parpura, V., and Haydon, P. G. (1998). Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci* 18, 6822-6829.

Armstrong, N., Sun, Y., Chen, G. Q., and Gouaux, E. (1998). Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature* 395, 913-917.

Audinat, E., Lambolez, B., Rossier, J., Crepel, F. (1994). Activity-dependent regulation of N-methyl-D-aspartate receptor subunit expression in rat cerebellar granule cells. *Eur J Neurosci* 6, 1792-1800.

Ault, B., and Hildebrand, L. M. (1993). Activation of nociceptive reflexes by peripheral kainate receptors. *J Pharmacol Exp Ther* 265, 927-932.

Ayalon, G., and Stern-Bach, Y. (2001). Functional assembly of AMPA and kainate receptors is mediated by several discrete protein-protein interactions. *Neuron* 31, 103-113.

Barish, M. E., Mansdorf, N. B. (1991). Development of intracellular calcium responses to depolarization and to kainate and N-mathyl-D-aspartatein cultured mouse hippocampal neurons. *Brain Res Dev Brain Res* 63, 53-61.

Barria, A., and Mallinow, R. (2002). Subunit-specific NMDA receptor trafficking to synapses. *Neuron* 35, 345-353.

Barry, M. F., and Ziff, E. B. (2002). Receptor trafficking and the plasticity of excitatory synapses. *Curr Opin Neurobiol* 12, 279-286.

Behe, P., Colquhoun, D., and Wyllie, D. J. A. (1999). Activation of single AMPA- and NMDA-type glutamate-receptor channels. Jonas P and Monyer H (eds) *Ionotropic Glutamate Receptors in the CNS*, pp. 175–218. London: Springer.

Behe, P., Stern, P., Wyllie, D. J., Nassar, M., Schoepfer, R., and Colquhoun, D. (1995). Determination of NMDA NR1 subunit copy number in recombinant NMDA receptors. *Proc R Soc Lond B Biol Sci* 262, 205-213.

Belayev, L., Busto, R., Zhao, W., and Ginsberg, M. D. (1995). HU-211, a novel non-competitive N-methyl-D-aspartate antagonist, improves neurological deficit and reduces infarct volume after reversible focal ischaemia in the rat. *Stroke* 26, 2313-2319.

Benveniste, H., Drejer, J., Schousboe, A. and Diemer, N. H. (1984). Elevation of the extracellular concentrations of glutamate in rat hippocampus during transient cerebral ischaemia monitored by intracerebral microdialysis. *J Neurochem* 43, 1369–1374.

Blanpied, T. A., Boeckmann, F. A., Aizenmann, E., and Johnson, J. W. (1997). Trapping channel block of NMDA-activated responses by amantadine and memantine. *J Neurophysiol* 77, 309-323.

Burnashev, N., Monyer, H., Seeburg, P. H., and Sakmann, B. (1992). Divalent ionpermeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8, 189-198.

Calver, A. R., Robbins, M. J., Cosio, C., Rice, S. Q., Babbs, A. J., Hirst, W. D., Boyfield, I., Wood, M. D., Russel, R. B., Price, G. W., *et al*. (2001). The C-terminal domains of the GABA(b) receptor subunits mediate intracellular trafficking but are not required for receptor signaling. *J Neurosci* 21, 1203-1210.

Carmignoto, G., and Vicini, S. (1992). Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science* 258, 1007-1011.

Chatterton, J. E. (2002). Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* 30 , 715-719.

Chazot, P. L., and Stephenson, F. A. (1997a). Biochemical evidence for the existence of a pool of unassembled C2 exon-containing NR1 subunits of the mammalian forebrain NMDA receptor. *J Neurochem* 68, 507-516.

Chazot, P. L., and Stephenson, F. A. (1997b). Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A and NR2B subunits whithin the same complex. *J Neurochem* 69, 2138-2144.

Chenu, C., Serre, C. M., Raynal, C., Burt-Pichat, B., and Delmas, P. D. (1998). Glutamate receptors are expressed by bone cells and are involved in bone resorption. *Bone* 22, 295-299.

Choi, D. W. (1985). Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci Lett* 58, 293–297.
Choi, D. W. (1987). Ionic dependence of glutamate neurotoxicity in cortical cell culture. *J Neurosci* 7, 369–379.

Choi, D. W. (1988a). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischaemic damage. *Trends Neurosci* 11, 465–469.

Choi, D. W. (1988b). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1, 623–634.

Choi, D. W., Maulucci-Gedde, M., Kriegstein, A. R. (1987). Glutamate neurotoxicity in cortical cellculture. *J Neurosci* 7, 357-368.

Coyle, J. T., Bird, S. J., Evans, R. H., Gulley, R. L., Nadler, J. V., Nicklas, W. J., and Olney, J. W. (1981). Excitatory amino acid neurotoxins: selectively and mechanism of action. *Neurosci Res Prog Bulletin* 19, 331-427.

Crair, M. C., and Malenka, R. C. (1995). A critical period for long-term potentiation at thalamocortical synapses. *Nature* 375, 325-328.

Cregan, E. F., Peeling, J., Corbett, D., Buchan, A. M., Saunders, J., Auer, R. N., Gao, M., McCarthy, D. J., Eisman, M. S., Campbell, T. M., Murray, R. J., Stagnitto, M. L., and Palmer, G. C. (1997). [(S)-α-Phenyl-2-pyridine-ethanamine dihydrochloride], a low affinity uncompetitive N-methyl-D-aspartic acid antagonist, is effective in rodent modelsof global and focal ischaemia. *J Pharmacol Exp Ther* 283, 1412-1424.

Cull-Candy, S., Brickley, S., and Farrant, M. (2001). NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol* 11, 327-335.

Dani, J. W., Chernjavsky, A., and Smith, J. S. (1992). Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* 8,429-440.

Das, S., Sasaki, Y. F., Rothe, T., Premkumar, L. S., Takasu, M., Crandall, J. E., Dikkes, P., Conner, D. A., Rayudu, P. V., Cheung, W. (1998). Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature* 393, 377-381.

Davenport, R. (2002). Glutamate receptors in plants. *Ann Bot (Lond)*, 90 (5), 549-557.

Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacol Rev* 51, 7-61.

Dunah, A. W., Luo, J., Wang, Y. H., Yasuda, R. P., and Wolfe, B. B. (1998). Subunit composition of N-methyl-D-aspartate receptors in the central nervous system that contain the NR2D subunit. *Mol Pharmacol* 53, 429-437.

Durand, G. M., Kovalchuk, Y., and Konnerth, A. (1996). Long-term potentiation and functional synapse induction in developing hippocampus. *Nature* 381, 71-75.

Dzubay, J. A. and Jahr, C. E. (1996). Kinetics of NMDA channel opening. *Journal of Neuroscience* 16: 4129–4134.

Favaron, M., Manev, H., Siman, R., Bertolino, M., Szekely, A. M., De Erausquin, G., Guidotti, A. and Costa, E. (1990). Down-regulation of protein kinase C protects cerebellar granule neurons in primary culture from glutamate-induced neuronal death. *Proc Natl Acad Sci USA* 87, 1983–1987.

Forrest, D., Yuzaki, M., Soares, H. D., Ng, L., Sheng, M., Stewart, C. L., Morgan, J. I., Connor, J. A., and Curran, T. (1994). Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. *Neuron* 13, 325-338.

Garner, C. C., Nash, J., and Huganir, R. L. (2000). PDZ domains in synapse assembly and signaling. *Trens Cell Biol* 10, 274-280.

Gill, S. S., Pulido, O. M., Mueller, R. W., and McGuire, P. F. (1998). Molecular and immunochemical characterization of the ionotropic glutamate receptors in the rat heart. *Brain Res Bull* 46, 429-434.

Hayashi, Y., Shi, S.H., Esteban, J. A., Piccini, A., Poncer, J. C., and Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* 287, 2262-2267.

Higuki, M., Single, F. N., Kohler, M., Sommer, B., Sprengel, R., and Seeburg, P. H. (1993). RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. *Cell* 75, 1361-1370.

Hollmann M (1999). Structure of ionotropic glutamate receptors. *Ionotropic glutamate receptors in the CNS*. Edited by Jonas P, Monyer H. Berlin: Springer 1-98.

Hollmann, M., and Heinemann, S. (1994). Cloned glutamate receptors. *Annu Rev Neurosci* 17, 31-108.

Huh, K. H., and Wenthold, R. J. (1999). Turnover analysis of glutamate receptors identifies rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *J Biol Chem* 274, 151-157.

Hume, R. I., Dingledine, R., and Heinemann, S. F. (1991). Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* 253, 1028-1031.

Iacopino, A., Christakos, S., German, D., Sonsalla, P. K. and Altar, C. A. (1992). Calbindin-D28K-containing neurons in animal models of neurodegeneration: possible protection from excitotoxicity. *Brain Re. Mol Brain Re.* 13, 251–261.

Inagaki, N., Kuromi, H., Gonoi, T., Okamoto, Y., Ishida, H., Seino, Y., Kaneko, T., Iwanaga, T., and Seino, S. (1995). Expression and role of ionotropic glutamate receptors in pancreatic islet cells. *Faseb J* 9, 686-691.

Innocenti, B., Parpura, V., and Haydon, P. G. (2000). Imaging extracellular waves of glutamate during calcium signaling in cultured astrocytes. *J Neurosci* 20, 1800-1808.

Johnson, J. W., and Ascher, P. (1990). Voltage-dependent block by intracellular Mg^{2+} of N-methyl-D-aspartate-activated channels. *Biophys J* 57, 1085-1090.

Johnston, D., Magee, J. C., Colbert, C. M., and Cristie, B. R. (1996). Active properties of neuronal dendrites. *Annu Rev Neurosci* 19, 165-186.

Kashiwagi, K., Pahk, A. J., Masuko, T., Igarashi, K., and Williams, K. (1997). Block and modulation of N-methyl-D-aspartate receptors by polyamines and protons: role of amino-acid residues in the transmembrane and pore-forming regions of NR1 and NR2 subunits. *Mol Pharmacol* 52, 701-713.

Kemp, J. A., Kew, J. N., and Gill, R. (1999). *Handbook of Experimental Pharmacology*, vol. 141 (eds. Jonas, P., and Monyer, H.) 495-527 (Springer, Berlin).

Kennedy, M. B. (1997). The postsynaptic density at glutamatergic synapses. *Trends Neurosci* 20, 264-268.

Kennedy, M. B. (1998). Signal transduction molecules at the glutamatergic postsynaptic membrane. *Brain Res Brain Res Rev* 26, 243-257.

Kennedy, M. B. (2000). Signal-processing machinery at the postsynaptic density. *Science*, 750-754.

Kim, E., Cho, K. O., Rothschild, A, and Sheng, M. (1996). Heteromultimerization and NMDA receptor-clustering activity of chapsyn-110, a member of the PSD-95 family of proteins. *Neuron* 17, 103-113.

Kim, J. H., and Huganir, R. L. (1999). Organization and regulation of proteins at synapses. *Curr Opin Cell Biol* 11: 248-254.

Kiyama, Y., Manabe, T., Sakimura, K., Kawakmi, F., Mori, H., Mishina, M. (1998). Increased thresholds for long-term potentiation and contextual learning in mice lacking the NMDA-type glutamate receptor epsilon 1 subunit. *J Neurosci* 18, 6704-6712.

Koh, J. Y., and Choi, D. W. (1987). Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Meth* 20, 83-90.

Köhr, G., and Seeburg, P. H. (1996). Subtype-specific regulation of recombinant NMDA receptor channels by protein tyrosine kinases of the src family. *J Physiol (Lond)* 492, 445-452.

Köhr, G., Jensen, V., Koester, H. J., Mihaljevic, A. L. A., Utvik, J. K., Kvello, A., Ottersen, O. P., Seeburg, P. H., Sprengel R., and Hvalby Ø (2003). Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. *J Neurosci* 24, 10791-10799.

Kohr, G., Jensen, V., Koester, H. J., Mihaljevic, A. L., Utvik, J. K., Kvello, A.,Ottersen, O. P., Seeburg, P. H., Sprengel, R., and Hvalby, O. (2003). Intracellular domains of NMDA receptor subtypes are determinats for long-term potentiation induction. *J Neurosci* 23, 10791-10799.

Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain intercation between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269, 1737-1740.

Kornau, H. C., Seeburg, P. H., and Kennedy, M. B. (1997). Interaction of ion channels and receptors with PDZ domain proteins. *Curr Opin Neurobiol* 7, 368-373.

Kuryatov, A., Laube, B., Betz, H., and Kuhse, J. (1994). Modulational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. *Neuron* 12, 1291-1300.

Laube, B., Kuhse, J., and Betz, H. (1998). Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci* 18, 2954-2961.

Laurie, D. J., and Seeburg, P. H. (1994). Regional and developmental heterogenity in splicing of the rat brain NMDAR1 mRNA. *J Neurosci* 14, 3180-3194.

Lee, J. M., Zipfel, G. J., and Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanism. *Nature* 399, A7-14.

Lees, K. R., et al. (2000). Glycine antagonist (gavestinel) in neuroprotection (GAIN International) in patients with acute stroke : a randomized controlled trial. GAIN International Investigators. *Lancet* 355, 1949-1954.

Leonard, A. S., and Hell, J. W. (1997). Cyclic AMP –dependent protein kinase and protein kinase C phosphorylate N-methyl-D-aspartatereceptors at different sites. *J Biol Chem* 272, 12107-12115.

Li, J. H., Wang, Y. H., Wolfe, B. B., Krueger K. E., Corsi, L., Stocca, G., and Vicini S. (1998). Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. *J Neurosci* 10, 1704-1709.

Li, Y., Erzurumlu, R. S., Chen, C., Jhaveri, S., and Tonegawa, S. (1994). Whisker-related neuronal patterns fail to develop in the trigeminal brainstem nuclei of NMDAR1 knockout mice, *Cell* 76, 427-437.

Lindlbauer, R., Mohrmann, R., Hatt, H., Gottmann, K. (1998). Regulation of kinetic and pharmacological properties of synaptic NMDA receptors depends on presynaptic exocytosisin rat hippocampal neurons. *J Physiol* 508 (Pt. 2), 495-502.

Li-Smerin, Y., and Johnson, J. W. (1996). Kinetics of the block by intracellular Mg^{2+} of the NMDA-activated channel in cultured rat neurons. *J Physiol* 491 (Pt 1), 121-135.

Liu,L., Wong, T. P., Pozza, M. F., Lingenhoehl, K., Wang, Y., Sheng, M., Auberson, Y., and Wang, Y. T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science* 304: 1021-1024.

Lolmeli, H., Sprengel, R., Laurie, D. J., Kohr, G., Herb,A., Seeburg, P. H., and Wisden, W. (1993). The rat delta-1 and delta-2 subunits extend the excitatory amino acid receptor family. *FEBS Lett* 315, 318-322.

Lukas, W. and Jones, K. A. (1994). Cortical neurons containing calretinin are selectively resistant to calcium overload and excitotoxicity in vitro. *Neuroscience* 61, 307–316.

Luo, J., Wang, Y., Yasuda, R. P., Dunah, A. W., and Wolfe, B. B. (1997). The majority of N-methyl-D-aspartate receptor complex in the adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). *Mol Pharmacol* 51, 79-86.

Maccaferri, G., and Lacaille, J. C. (2003). Interneuron Diversity series: Hippocampal interneuron classifications-making things as simple as possible, not simpler. *Trends Neurosci* 26, 564-571.

Malenka, R. C., and Nicoll, R. A. (1997). Silent synapses speak up. *Neuron* 19, 473-476.

Marks, J. D., Friedman, J. E., Haddad, G. G. (1996). Vulnerability of CA1 neurons to glutamate is developmentally regulated. *Brain Res Dev Brain Res* 97, 194-206.

Massey, P. V., Johnson, B. E., Moult, P. R., Auberson, Y. P., Brown, M. W., Molnar, E., Collingridge, G. L., and Bashir, Z. I. (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J of Neurosci* 24 (36): 7821-7828.

Matsuda, K., Kamiya, Y., Matsuda, S., and Yuzaki, M. (2002). Cloning and characterization of a novel NMDA receptor subunit NR3B: a dominant subunit that reduces clcium permeability. *Mol Brain Res* 100, 43-52.

Mayer, M. L., Westbrook, G. L. and Guthrie, P. B. (1984). Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurons. *Nature* 309, 261-263.

McGeer, P. L., and McGeer, E. G.(1982). Kainic acid: the neurotoxic breakthrough. *CRC Crit Rev Toxicol* 10, 1-26.

Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., and Mishina, M. (1992). Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature* 357, 70-74.

Mills, L. R. and Kater, S. B. (1990). Neuron-specific and state-specific differences in calcium homeostasis regulate the generation and degeneration of neuronal architecture. *Neuron* 2, 149–163.

Miyamoto, Y., Yamada, K., Noda, Y., Mori, H., Mishina, M., and Nabeshima, T. (2001). Hyperfunction of dopaminergic and serotonergic neuronal systems in mice lacking the NMDA receptor epsilon 1 subunit. *J Neurosci* 21, 750-757.

Mizuta, I., Katayama, M., Watanabe, M., Mishina, M., Ishii, K. (1998). Developmental expression of NMDA receptor subunits and the emergence of glutamte neurotoxicity in primary cultures of murine cerebral cortical neurons. *Cell Mol Life Sci* 54, 721-725.

Mohn, A. R., Gainetdinov, R. R., Caron, M. G., and Koller, B. H. (1999). Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. *Cell* 98, 427-436.

Monyer, H., Burnashev, N., Laurie, D., Sakmann, B., and Seeburg, P. H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12, 529-540.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., and Seeburg, P. H. (1992). Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256, 1217-1221.

Moon, I. S., Apperson, M. L., Kennedy, M. B. (1994). The major tyrosine-phosphorylated protein in the postsynaptic density fraction in N-methyl-D-aspartate receptor subunit 2B. *Proc Natl Acad Sci USA* 91, 3954-3958.

Mori, H., Manabe, T., Watanabe, M., Satoh, Y., Suzuki, N., Toki, S., Nakamura, K., Yagi, T., Kushiya, E., Takahashi, T., *et al*. (1998). Role of the carboxy-terminal region of the GluR epsilon 2 subunit in synaptic localization of the NMDA receptor channel. *Neuron* 21, 571-580.

Morikawa, E., Mori, H., Kyiama, Y., Mishina, M., Asano, T., Kirino, T. (1998). Attenuation of focal ischaemic brain injury in mice deficient in the epsilon (NR2A) subunit of NMDA receptor. *J Neurosci* 18, 9727-9732.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., Nakanishi, S. (1991). Molecular cloning and characterization of the rat NMDA receptor. *Nature* 354, 31-37.

Mueller, B. M., Kistner, U., Kindler, S., Chung, W. J., Kuhlendahl, S., Fenster, S. D., Lau, L. F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996). SAP 102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. *Neuron* 17, 255-265.

Nakanishi, N., Axel, R., and Shneider, N. A. (1992). Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proc Natl Acad Sci USA* 89, 8552-8556.

Niethammer, M., Kim, E., and Sheng, M. (1996). Interaction between the C-terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. *J Neurosci* 16, 2157-2163.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., and Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* 307, 462-465.

O'Brien, R., Lau, L. F., and Huganir, R. L. (1998). Molecular mechanism of glutamate receptor clustering at excitatory synapses. *Curr Opin Neurobiol* 8, 364-369.

Olney, J. W. (1969). Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. *Science* 164, 719-721.

Olney, J. W. (1986). Inciting excitotoxic cytocide among central neurons. *Adv Exp Med Biol* 203, 631-645.

Olney, J. W., Sharpe, L. Gl., and Feigin, R. D. (1972). Glutamate-induced brain damage in infant primates. *J Neuropathol Exper Neurol* 31, 464-488.

Omkumar, R. V., Kiely, M. J., Rosenstein, A. J., Min, K. T., and Keneddy, M. B. (1996). Identification of a phosphorylation site for calcium/calmodulin-dependent protein kinase II in the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* 271, 31670-31678.

Orrenius, S., McConkey, D. J., Bellomo, G. and Nicotera, P. (1989). Role of Ca21 in toxic cell killing. *Trends Pharmacol Sci* 10, 281–284.

Osten, P., Khatri, L., Perez, J. L., Kohr, G., Giese, G., Daly, C., Schulz, T. W., Wensky, A., Lee, L. M., and Ziff, E. B. (2000). Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor. *Neuron* 27, 313-325.

Pagano, A., Rovelli, G., Mosbacher, J., Lohmann, T., Duthey, B., Stauffer, D., Risting, D., Schuler, V., Meigel, I., Lampert, C., *et al.* (2001). C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA(b) receptors. *J Neurosci* 21, 1189-1202.

Parpura, V., and Haydon, P. G. (2000). Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. *Proc Natl Acad Sci U S A* 97, 8629-8634.

Passafaro, M., Piech, V., and Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. *Nat Neurosci* 4, 917-926.

Perez-Otano, I., Schulteis, C. T., Contractor, A., Lipton, S. A., Trimmer, J. S., Sucher, N. J., and Heineman, S. F. (2001). Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. *J Neurosci* 21, 1228-1237.

Premkumar, L. S., and Auerbach, A. (1997). Stoichiometry of recombinant N-methyl-D-aspartate receptor channels inferred from single-channel current patterns. *J Gen Physiol* 110, 485-502.

Roche, K. W., Standley, S., McCallum, J., Dune Ly, C., Ehlers, M. D. and Wenthold, R. J. (2001). Molecular determinants of NMDA receptor internalization. *Nat Neurosci* 4, 794-802.

Ronsenmund, C., Stern-Bach, Y., and Stevens, C. F. (1998). The tetrameric structure of glutamate receptor channel. *Science* 280, 1596-1599.

Rothman, S. M. (1985). The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J Neurosci* 5, 1483–1489.

Rowley, M., Bristow, L. J., and Hutson, P. H. (2001). Current and novel approaches to the drug treatment of schizophrenia. *J Med Chem* 44, 477-501.

Rumbaugh, S., and Vicini, S. (1999). Distinct synaptic and extrasynaptic NMDA receptors in developing cerebellar granule neurons. *J Neurosci* 19, 10603-10610.

Ruppersberg, J. P., Mosbacher, J., Gunther, W., Schoepfer, R., and Fakler, B. (1993).Studying block in cloned N-methyl-D-aspartate (NMDA) receptors. *Biochem Pharmacol* 46, 1877-1885.

Sacco, R. L. (2001). Glycine antagonist in neuroprotection for patients with acute stroke: GAIN Americas: a randomized controlled trial. *JAMA* 285, 1719-1728.

Sakimura, K., Kutsuwada, T., Ito, I., Manabe, T., Takayama, C., Kushiya, E., Yagi, T., Aizawa, S., Inoue, Y., Sugiyama, H. and et al. (1995). Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. *Nature* 373, 151-155.

Sakurada, K., Masu, M., and Nakanishi, S.(1993). Alteration of Ca^{2+} permeability and sensitivity to Mg^{2+} and channel blockers by a single amino acid substitution in the N-methyl-D-aspartate receptor. *J Biol Chem* 268, 410-415.

Sattler, R., Charlton, M. P., Hafner, M., and Timianski, M. (1998). Distinct influx pathways,not calcium lod, determine neuronal vulnerability to calcium neurotoxicity. *J Neurochem* 71, 2349-2364.

Sattler, R., Xiong, Z., Lu, W. Y., Hafner, M.,MacDonald, J. F., and Timianski, M. (1999). Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 284, 1845-1848.

Sattler, R., Xiong, Z., Lu, W. Y., MacDonald, J. F., Tymianski, M.(2000). Distincte roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. *J Neurosci* 20, 22-33.

Sheng, M. (1996). PDZs and receptor/channel clustering: rounding up the latest suspect. *Neuron* 17, 575-578.

Sheng, M. (2001). Molecular organization of the postsynaptic specialization. *Proc Natl Acad Sci USA* 98, 7058-7061.

Sheng, M., and Kim, M. J. (2002). Postsynaptic signaling and plasticity mechanisms. *Science* 298, 776-780.

Sheng, M., and Pak, D. T. (2000). Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. *Annu Rev Physiol* 62, 755-778.

Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N., and Jan, L. Y. (1994). Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* 368, 144-147.

Shi, J., Aamodt, S. M., Townsend, M., and Constantine-Paton, M. (2001b). Developmental depression of glutamate neurotransmission by chronic low-level activation of NMDA receptors. *J Neurosci* 21, 6233-6244.

Shi, S., Hayashi, Y., Esteban, J. A., and Malinow, R. (2001a). Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* 105, 331-343.

Siman, R., Noszek, J. C. and Kegerise, C. (1989). Calpain I activation is specifically related to excitatory amino acid induction of hippocampal damage. *J Neurosci* 9, 1579–1590.

Single, F. N., Rozov, A., Burnashev, N. (2000). Dysfunctions in mice by NMDA receptor point mutations NR1(N598Q) and NR1(N598R). *J Neurosci* 20, 2558-2566.

Sobolevsky, A. I., Yelshansky, M. V., and Wollmuth, L. P. (2004). The outer poreof the glutamate receptor channel has 2-fold rotational symmetry. *Neuron* 41, 367-378.

Sommer, B., Kohler, M., Sprengel, R., and Seeburg, P. H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67, 11-19.

Sprengel, R., and Single, F. N. (1999). Mice with genetically modified NMDA and AMPA receptors. *Ann N Y Acad Sci* 868, 494-501.

Sprengel, R., Suchanek, B., Amico, C., Brusa, R., Burnashev, N., Rozov, A., Hvalby, O., Jensen,V., Paulsen, O., Andersen, P., *et al*. (1998). Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell* 92, 279-289.

Stece-Collier, K., Chambers, L. K., Jaw-Tsai, S. S., Menniti, F. S.,and Greenamyre, J. T. (2000). Antiparkinsonian actions of CP-101,606, an antagonist of NR2Bsubunit-containing N-methyl-D-aspartate receptors. *Exp Neurol* 163, 239-243.

Steigerwald, F., Schultz, T. W., Schenker, L. T., Kennedy, M. B., Seeburg, P. H. and Kohr, G. (2000). C-terminal truncation of NR2A subunits impairs synaptic but not extrsynaptic localization of NMDA receptors. *J Neurosci* 20, 4573-4581.

Stern-Bach, Y., Bettler, B., Hartley, M., Sheppard, P. O., O´Hara, P. J., and Heinemann, S. F. (1994). Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron* 13, 1345-1357.

Stocca, G., and Vicini, S. (1998).Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. *J Phisiol* 507 (Pt 1), 13-24.

Stuart, G. J., and Sakmann, B. (1994). Active propgation of somatic action potentials into neocortical pyramidal cell dendrites. *Nature* 367, 69-72.

Sugihara, H., Moriyoshi, K., Ishii, T., Masu, M., Nakanishi, S. (1992). Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem Biophys Res Commun* 185, 826-832.

Swanson, G. T., Feldmeyer, D., Kaneda, M., and Cull-Candy, S. G. (1996). Effect of RNA editing and subunit co-assembly single-channel properties of recombinant kainate receptors. *J.Physiol* 492, 129-142.

Tang, Y. P., Shimizu, E., Dube, G. R., Rampon, C., Kerchner, G. A., Zhuo, M., Liu, G., and Tsien, J. Z. (1999). Genetic enhancement of learning and memory in mice. *Nature* 401, 63-69.

Timianski, M., Charlton, M. P., Carlen, P. L., Tator, C. H. (1993). Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. *J Neurosci* 13, 2085-2104.

Tingley, W. G., Ehlers, M. D., Kameyama, K., Doherty, C., Ptak, J. B., Riley, C. T. and Huganir, R. L. (1997). Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J Biol Chem* 272, 5157-5166.

Tokita, Y., Bessho, Y., Masu, M., et al. (1996). Characterization of excittory amino acid neurotoxicity in N-methyl-D-aspartate receptor-deficient mouse cortical neuronal cells. *Eur J Neurosci* 8, 69-78.

Tomita, S., Nicoll, R. A., and Bredt, D. S. (2001). PDZ protein interactions regulating glutamate receptor function and plasticity. *J Cell Biol* 153, F19-F24.

Tovar, K. R., and Westbrook, G. L. (1999). The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19, 4180-4188.

Tovar, K., R., Westbrook, G., L. (2002). Mobile NMDA receptors at hippocampal synapses. *Neuron* 34:255–64.

Umemura, K., Gemba, T., Mizuno, A., and Nakashima, M. (1996). Inhibitory effect of MS-153 on elevated brain glutamate level induced by rat middle cerebral artery occlusion. *Stroke* 27, 1624-1628.

Vicini, S., Wang, J. F., Li, J. H., Zhu, W. J., Wang, Y. H., Luo, J. H., Wolfe, B. B., and Grayson, D. R. (1998). Functional and pharmacological differences between recombinat N-methyl-D-aspartate receptors. *J Neurophysiol* 79, 555-566.

Wahl, P., Schousboe, A., Honore, T., Drejer, J. (1989). Glutamate-induced increase in intracellular Ca2+ in cerebral cortex neurons is transient in immature cells but permanent in mature cells. *J Neurochem* 53, 1316-1319.

Wahlestedt, C., Golanov, E., Yamamoto, S., et al. (1993).Antisense oligonucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infractions. *Nature* 363, 260-263.

Wang, Y. T. and Salter, M. V. (1994). Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* 369, 233-235.

Wei, F., Wang, G. D., Kerchner, G. A., Kim, S. J., Xu, H. M., Chen, Z. F., and Zhuo, M. (2001). Genetic enhancement of inflammatory pn by forebrain NR2B overexpression. *Nat Neurosci* 4, 164-169.

Wenthold, R. J., Prybylowski, K., Standley, S., Sans, N., and Petralia, R. S. (2003). Trafficking of NMDA receptors. *Annu Rev Pharmacol Toxicol* 43, 335-358.

Wenzel, A., Fritschy, J. M.,Mohler, H., Benke, D. (1997). NMDA receptor heterogenity during postnatal development of the rat brain: differential expression of the NR2A, NR2B and NR2C subunit proteins. *J Neurochem* 68, 469-478.

Wisden, W., and Seeburg, P. H. (1993) Mammalian ionotropic glutamate receptors. *Curr Opin Neurobiol* 3, 291-298.

Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A. H., Craig, A. M., Sheng, M. (1997). Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* 385, 439–442.

Yuste, R., and Tank, D. W. (1996). Dendritic integration in mammalian neurons, a century after Cajal. *Neuron* 16, 701-716.

Zou, J., De Jager, P. L., Takahashi, K. A., Jiang, W., Linden, D. J., and Heintz, N. (1997). Neurodegeneration in Lurcher mice caused by mutation in delta2 glutamate receptor gene. *Nature* 388, 769-773.