MODULATION OF GLU N3B-CONTAINING EXCITATORY GLYCINE RECEPTORS BY ZINC

by

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TWO HUNDRED TWENTY-TWO DAYS OF LIGHT
WILL BE DESIRED BY A NIGHT

Nightwish – Sleeping Sun
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Abbreviations

7-CKA  7-chlorokynurenic acid
A/D  analog/digital
ALS  amyotrophic lateral sclerosis
AMPA  α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR  AMPA receptor
AP5  (2R)-amino-5-phosphonovaleric acid
ATP  adenosine 5’-triphosphate
ATD  amino terminal domain
APP  amyloid precursor protein
BiFC  bimolecular fluorescence complementation
bp  base pairs
BSA  bovine serum albumin
CaMKII  Ca^{2+}/calmodulin dependent protein kinase II
cds  coding sequence
CTD  carboxy-terminal domain
CNS  central nervous system
CSF  cerebrospinal fluid
Dam  DNA adenine methylase
DCKA  5,7-dichlorokynurenic acid
Dcm  DNA cytosine methylase
DEPC  diethylpyrocarbonate
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
E. coli  Escherichia coli
eCFP  enhanced cyan fluorescent protein
eGFP  enhanced green fluorescent protein
EDTA  ethylenediaminetetraacetic acid
ER  endoplasmatic reticulum
EtBr  ethidium bromide
eYFP  enhanced yellow fluorescent protein
FAS  fetal alcohol syndrome
FRET  fluorescence resonance energy transfer
GABA  γ-aminobutyric acid
GFP  green fluorescent protein
GlyR  glycine receptor
HEK  human embryonic kidney
KA  kainate
Abbreviations

KAR  kainate receptor
LAOBP lysine-arginine-ornithine-binding protein
LB  Luria-Bertani growing medium
LBD  ligand binding domain
LIVBP leucine-isoleucine-valine-binding protein
LTD  long-term depression
LTP  long-term potentiation
MCS  multiple cloning site
MK-801 [5R,10S]-\(+\)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
MOPS  3-(N-morpholino)propanesulfonic acid
NaOH  sodium hydroxide
NFR  normal frog Ringer
NMDA  N-methyl-Daspartate
NMDAR  NMDA receptor
NTP  nucleoside triphosphate
ori  origin of replication
orf  open reading frame
PCR  polymerase chain reaction
PDZ  PSD-95/Dig/ZO-1
PEG  polyethylene glycol
PKA  protein kinase A
PKC  protein kinase C
PNK  polynucleotide kinase
PNS  peripheral nervous system
PSD  postsynaptic density
RNA  ribonucleic acid
RNase  ribonuclease
SDS  sodium dodecyl sulfate
SEM  standard error of the mean
SP  signal peptide
SRP  signal recognition particle
TARP  transmembrane AMPA receptor regulatory protein
TBE  Tris-borat-EDTA
TE  Tris-EDTA
TEVC  two-electrode voltage clamp
TMD  transmembrane domain
UTR  untranslated region
UV  ultraviolet
YT  yeast-tryptone growing medium

For units and amino acids standard abbreviations were used.
Abstract

In the central nervous system, the NMDA receptor subfamily of the ionotropic glutamate receptors plays a crucial role in mechanisms underlying learning and memory. Most abundant are receptors containing GluN1 and GluN2 subunits, activated by the binding of glycine to the GluN1 and glutamate to the GluN2 subunit. This subunit combination is strongly blocked by zinc. The remaining members of the family, the glycine-binding GluN3 subunits, were found a few years later. The GluN3 subunits were soon recognized to modulate all functional characteristics of the conventional GluN1/GluN2-containing receptor when incorporated into the receptor complex: the calcium permeability is lowered significantly, the block by magnesium ions is reduced, and the current responses are decreased. When coexpressed with GluN1 alone, GluN3 gives rise to receptors that are activated by the binding of glycine alone, are barely permeable for calcium and not blocked by magnesium. This subunit combination, for which the name “excitatory glycine receptor” was coined, has been shown to exist in vivo (Piña-Crespo et al., 2010).

Concerning the GluN3A-containing excitatory glycine receptor, it is known that zinc can coactivate and potentiate the glycine-induced current response, and that this potentiation can be further enhanced by the coapplication of a GluN1 glycine binding site antagonist. However, for the GluN3B-containing receptor variant no such information exists.

The aim of this thesis was to find out how the GluN3B-containing excitatory glycine receptor behaves towards zinc, and if this behavior is comparable to that of GluN1/GluN3A-containing or conventional GluN1/GluN2-containing receptors. Also, if there were any modulatory effects of zinc, it would be important to identify the specific sequence elements that mediate these effects.

The GluN3B-containing excitatory glycine receptor combination shows a differential behavior towards zinc as both blocking action and potentiation can be observed, a pattern that is in general independent of the N-terminal splice variant of the GluN1 subunit, GluN1-a or GluN1-b. In addition, the potentiating effect has two components that reside in different parts of the receptor. Though the overall potentiation remains, one of these elements can be influenced by exon 5: when exon 5 is present, the receptor is potentiated to a lesser degree. Mutational analysis suggests a binding site in the vicinity of the amino terminal domain’s (ATD) dimer interface between GluN1 and GluN3B.

With regard to the second element with a weaker potency it is clear that it involves structural elements within the ligand binding domain (LBD), but not directly at the ligand binding site: zinc competes with MDL-29951, a highly efficient GluN1 glycine binding site antagonist that blocks the conventional GluN2-containing receptors, but potentiates current responses in GluN3-containing receptors. Yet when the binding of MDL-29951 is abolished, the zinc potentiation remains.
To investigate the comparability of GluN3A- and GluN3B-containing receptors, also GluN3A-containing receptors were tested with the same combinations of concentrations of zinc and MDL-29951. Interestingly, it was found that at the glycine concentrations effective at GluN3B-containing receptors only zinc potentiates the glycine-induced current response, while MDL-29951 has no effect. Furthermore, the proposed “supralinear” potentiation of the glycine-induced current seen upon coapplication of MDL-29951 and zinc (Madry et al., 2008) was not observed. Surprisingly, the removal of the 5’ untranslated region (UTR) of the GluN3A subunit led to the manifestation of such a “supralinear” effect, although MDL-29951 when applied alone still showed no effect. Whereas the GluN3B-containing receptors were found to be sensitive to protons regardless of the GluN1-a or GluN1-b splice variant (Cavara et al., 2009), here the GluN3A-containing receptors showed no sensitivity towards protons.

Additionally, it could be shown that the plasma membrane export of the GluN1-3 variant of the GluN1 subunit is not as pronounced as previously thought. The endoplasmatic reticulum (ER) retention, mediated by an ER retention motif, is supposed to be overriden by a PSD-95/Dig/ZO-1 (PDZ) binding motif, which resides in the additional C2’ sequence in the carboxy-terminal domain (CTD) of this GluN1 variant (Xia et al., 2001; Standley et al., 2000; Scott et al., 2001). However, the small degree of plasma membrane export seen here suggests only a rather weak reduction of ER retention.
1. Introduction

1.1. The Central Nervous System

The central nervous system (CNS) is the control unit of all higher organisms in nature. Whereas the CNS consists of the brain and the spinal cord, the peripheral nervous system (PNS) contains all other neurons. In the organism, the CNS integrates and processes all input information from, e.g., the optic, olfactory, acoustic, and sensory systems. Also, the CNS steers the adequate reactions to the information gained through the input. Furthermore, it controls motor activity and learning processes. All the signals within the CNS are conducted via neurons. Neurons thereby form manifold connections among each other and with specialized cells such as muscle cells or the different sensory cells. The signal conduction between two neurons always happens at the axon of one neuron that connects to a second neuron. This contact is called synapse, of which one neuron can form up to 1,000 to other neurons.

1.2. Excitatory Signal Transduction

The basis of an excitatory signal in the CNS is the generation and transduction of an action potential. An action potential is a change of the resting membrane potential of $-70 \text{ mV}$ over a certain threshold. As a result, $\text{Na}^+$ channels open, sodium ions flux into the neuron, and the membrane is depolarized to a level of $+30 \text{ mV}$. This depolarization opens voltage-gated potassium channels that in turn lead to a $\text{K}^+$ outflux, repolarizing the cell. Nearby channels are subsequently activated which leads to a signal propagation along the membrane. To transmit the signal at a synapse, nature has come up with two different variants: The electrical and the chemical synapse. At an electrical synapse gap junctions form a direct connection between the two adjacent neurons. One advantage of this system is the immediate transduction of the signal without delay, the major disadvantage is the lack of the possibility to modulate the signal.

At a chemical synapse the two neurons are divided by the synaptic cleft, a small gap between the cells. When an action potential reaches the synapse, it triggers the release of neurotransmitters from vesicles into the synaptic cleft via inducing the fusion of the vesicles with the plasma membrane. The neurotransmitters diffuse across the synaptic cleft and reach the postsynaptic membrane that contains appropriate receptors. These receptors – e.g., glutamate receptors – open upon agonist binding, allowing ions to flux and thereby the propagation of the signal. The disadvantage of this sort of signal transduction is a short delay of about 2 ms. Nevertheless, the major advantage is the possibility of modulating the signal. This can happen via the amount of postsynaptically expressed membrane receptors.
that can increase the strength of the signal or the corelease of modulators together with the neurotransmitter. Besides excitatory synapses that propagate a signal, also inhibitory synapses exist. At inhibitory synapses the membrane potential is hyperpolarized in order to hinder the excitation. The most important inhibitory neurotransmitter is γ-aminobutyric acid (GABA).

1.3. The Ionotropic Glutamate Receptors

In the CNS the most important excitatory neurotransmitter is the amino acid L-glutamate. The ionotropic glutamate receptors are ligand-gated excitatory receptors that form unspecific cation channels when activated. They comprise four different subclasses. Beside the ionotropic receptors also metabotropic glutamate receptors exist that activate different signal cascades and are seven-transmembrane receptors.

1.3.1. The Different Subclasses

The ionotropic glutamate receptors are divided into different subclasses and were firstly categorized according to their different pharmacological properties: The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), the kainate (KA), and the N-methyl-D-aspartate (NMDA) receptor subclasses.

![Figure 1.1: Overview over the different classes of the ionotropic glutamate receptors.](image)

The AMPA receptor (AMPAR) subclass comprises the four subunits GluA1 through GluA4. AMPARs mediate fast synaptic transmission and they desensitize in the short millisecond range. Their amount in the postsynaptic density (PSD) depends on synaptic activity (Derkach et al., 2007), therefore they play an important part in long-term potentiation (LTP)(see also chapter 1.4). The kainate receptor (KAR) subclass is further divided into the high affinity (GluK1, GluK2, and GluK3) and the low affinity (GluK4 and GluK5) subunits. Both AMPARs and KARs are taken together as non-NMDA receptors. Within the NMDA receptor (NMDAR) subfamily, three different groups of subunits exist: GluN1, GluN2A through GluN2D, and GluN3A plus GluN3B. They mediate the slow
1.3. The Ionotropic Glutamate Receptors

part of synaptic signal transduction. Additionally, the so-called “orphan” receptors exist, namely GluD1 and GluD2. These receptor subunits are not able to form a functional ion channel and no ligand could be identified so far (Schmid and Hollmann, 2008). Only one functional exception is known so far, the constitutively open, so called lurcher mutant (Zuo et al., 1997; Schmid et al., 2009). The function of the two GluD subunits is so far not fully understood.

1.3.2. The Structure

A functional glutamate receptor has a tetrameric structure (Sobolevsky et al., 2009; Karakas and Furukawa, 2014; Lee et al., 2014). The initial step in forming the tetrameric receptor is the assembly of two subunits into a dimer (Ayalon and Stern-Bach, 2001; Schüler et al., 2008). This dimer can then, together with a second dimer, form the functional tetrameric receptor. All AMPARs and the low affinity KARs (GluK1 through GluK3) are able to form functional homomeric receptors. The high affinity KARs (GluK4 and GluK5) as well as the NMDARs are capable of forming functional receptors only as heteromers (Wenthold et al., 1992; Brose et al., 1994; Schmidt and Hollmann, 2008).

All different subunits of the glutamate receptors share the same topology, consisting of four distinct domains: An extracellular ATD and ligand binding domain (LBD), a membrane segment with three transmembrane domains (TMD) A, B, C, and the poreloop P, and the intracellular CTD. The poreloop is fit between the transmembrane domains A and B. The first crystal structure of a complete ionotropic glutamate receptor was solved for the GluA2 homomer in 2009 by Sobolevsky et al. In 2014 the crystal structure for an NMDAR containing GluN1 and GluN2B was published (rat GluN1/GluN2B receptor by Karakas and Furukawa, shortly afterwards followed by the XenGluN1/GluN2B receptor by Lee et al.).

The ATD is posttranslationally glycosylated, this affects desensitization and maximal currents of both AMPARs and KARs (Hollmann et al., 1994; Everts et al., 1997). The ATD plays an important role for the initial dimerisation of two subunits (Everts et al., 1997; Kuusinen et al., 1999; Ayalon and Stern-Bach, 2001; Ayalon et al., 2005). In the NMDAR subclass the ATD contains various binding sites for different modulators, e.g. zinc (in conventional, GluN2-containing receptors) or ifenprodil (Westbrook and Mayer, 1987; Williams, 1993; Karakas et al., 2009). Interestingly, the ATD shows sequence similarity to the bacterial leucine-isoleucine-valine-binding protein (LIVBP)(O’Hara et al., 1993; Stern-Bach et al., 1994).

Right after the ATD, the LBD follows. The LBD is formed by two domains that form a clam shell-like structure, D1 and D2. Between those two domains the agonist binding site is located. The domains D1 and D2 are formed by the segment S1 that links the ATD and the TMD A, and the segment S2, which forms a loop connecting the TMDs B and C. These domains S1 and S2 show sequence similarity to the lysine-arginine-ornithine-binding protein (LAOBP)(Nakanishi et al., 1990; Kuryatov et al., 1994).

The membrane segment is formed by the TMDs A, B, C, and the poreloop P. These domains form the ion pore that shares sequence similarity with the ion pore of potassium channels (Wo and Oswald, 1995; Kuner et al., 2003). The pore loop, forming the ion
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Figure 1.2.: General structure of ionotropic glutamate receptors. Left: Schematic representation of a single glutamate receptor subunit, beginning with the extracellular ATD. After the ATD the LBD follows. Connected via short linkers, the TMDs A, B, and C as well as the pore loop follow. Intracellularly the receptor ends with the CTD. Right: Crystal structure of a single GluN1-1a receptor subunit including a glycine molecule. The other three subunits (GluN1-1a, 2x GluN2B) are depicted in grey (4PE5, Karakas and Furukawa, 2014), lacking the CTD.

pore, does not span the membrane (Hollmann et al., 1994; Sobolevsky et al., 2009). The membrane domains are extracellularly connected to the LBD via short linkers. These linkers translate the closure of the LBD upon agonist binding into the opening of the ion pore. The pore is closed again either via diffusion and release of the agonist or by closure of the pore while the ligand is still bound. The latter process is also called desensitization (Sobolevsky et al., 2009; Karakas and Furukawa, 2014; Lee et al., 2014).

The CTD contains various phosphorylation sites that are important for interaction with auxiliary subunits and the export of the receptor from the ER to the plasma membrane (Lau and Huganir, 1995; Malinow and Malenka, 2002).

1.4. The NMDA Receptor

The NMDAR class comprises the three different subclasses GluN1, GluN2, and GluN3. They mediate the slow compound of synaptic signaling and maintain the modulation of synaptic strength (Monyer et al., 1992). Through many different genes and splice variants encoding these subunits, the NMDARs build a large, heterogenous family: For GluN1 only one gene but eight different functional splice variants have been found (Sugihara et al.,
1.4. The NMDA Receptor

1992; Hollmann et al., 1993), for GluN2 four different genes that also undergo alternative
splicing are known (Monyer et al., 1992; Ishii et al., 1993), and GluN3 adds another two
different genes that are also alternatively spliced (Sun et al., 1998; Sasaki et al., 2002;
de Jesus Domingues et al., 2011).

Contrary to the non-NMDA receptors (except GluK4 and GluK5) the different NMDAR
subunits are not able to form a functional homomeric channel. One receptor always com-
prises two GluN1 subunits together with GluN2 and/or GluN3 subunits to form the
tetrameric receptor (Monyer et al., 1992; Laube et al., 1998; Ulbrich and Isacoff, 2007;
Schüler et al., 2008). Whereas GluN2 binds glutamate, GluN1 and GluN3 bind glycine
(Kuryatov et al., 1994; Laube et al., 1997). Therefore, conventional NMDAR subunit com-
binations comprising two GluN1 and two GluN2 subunits are only fully activated by simulta-
nous binding of both glycine and glutamate. Combining GluN1 and GluN3 leads to the
formation of receptors that are fully activated only by glycine, thus representing excitatory
glycine receptors (Chatterton et al., 2002). These unusual receptors were formerly thought
to be artifacts of heterologous expression systems and it was believed that the GluN3 sub-
unit in vivo has only regulatory functions. However, the in vivo formation of this excitatory
glycine receptors has recently been proven in optic nerve myelin (Piña-Crespo et al., 2010).

NMDARs provide the molecular basis for synaptic plasticity, and they are essential for
mechanisms of learning as they mediate LTP and long-term depression (LTD). Upon pro-
nounced NMDAR activation, a strong influx of calcium ions into the neuron is mediated.
This leads via calmodulin to the activation of Ca$^{++}$/calmodulin dependent protein kinase
II (CaMKII) and further on to the phosphorylation of AMPARs, enhancing their conduc-
tance. Also, subsequently more AMPARs are inserted into the synapse. Vice versa, less
activation of NMDARs leads to a lesser influx of calcium, leading to a dephosphorylation
of receptors (Leonard et al., 1999; Nicoll and Malenka, 1999; Bayer et al., 2001; Barria and
Malinow, 2005).

As correct NMDAR function is important for the maintenance of synaptic strength,
it is not surprising that NMDARs are also linked to excitotoxicity and subsequent cell
death. Pronounced activation of NMDARs by excessive agonist release in the synaptic
cleft can lead to a strong calcium influx into the cell (Tymianski et al., 1993). These
elevated amounts of calcium ions can then mediate cell death via various mechanisms.
The most important of these mechanisms are the induction of apoptosis mediated by activation
of caspases and the production of radicals (Dykens, 1994; Du et al., 1997; Tenneti et al.,
1998). Excitotoxicity is linked to a multitude of neurodegenerative diseases: amyotrophic
lateral sclerosis (ALS), multiple sclerosis, Huntington’s disease (there enhancing NMDAR
sensitivity and reducing Mg$^{2+}$ sensitivity), morbus Parkinson, morbus Alzheimer, stroke,
and epilepsy (Choahan and Iqbal, 2006; Hardingham, 2009; Lau and Tymianski, 2010).

Also, decreased or blocked NMDAR activity can lead to diseases, e.g. the fetal alcohol
syndrome (FAS), with apoptosis of neuronal cells during the synaptogenesis in the develop-
ing embryonal brain (overview in Olney et al. 2001). NMDAR hypofunction is also linked
to schizophrenia (Bennett, 2009). Further, the ethanol-mediated block of NMDA-evoked
currents in primary neurons presents a link to ethanol addiction (Wright et al., 1996).

Although there is much known about the contribution of NMDARs to the various dis-
1. Introduction

- long time potentiation
- long time depression
- fetal alcohol syndrome
- schizophrenia
- amyotrophic lateral sclerosis
- Huntington’s disease
- Parkinson’s disease
- Alzheimer’s disease
- epilepsy

Hypo

Hyper

NMDA receptor activity

Figure 1.3.: Physiological importance of NMDAR function. Whereas correct NMDAR function is essential for mechanisms like LTP and LTD, malfunction is linked to a variety of diseases.

Promising approaches, however, emerged, e.g. for the GluN2B channel blocker memantine. In Alzheimer’s disease, where memantine blocked only aberrant NMDARs, it showed some benefits (Danysz et al., 2000; Reisberg et al., 2003). In addition, NMDAR antagonists may have antidepressant effects (Paul and Skolnick, 2003). Nevertheless, many clinical trials for promising drugs failed due to side effects or negative outcome (Muir, 2006).

1.4.1. The GluN1 Subunit

The GluN1 subunit was the first NMDAR subunit molecularly identified (Moriyoshi et al., 1991; Sugihara et al., 1992; Hollmann et al., 1993). Though it is the only NMDAR subunit that is encoded by just one gene, it gains functional diversity through alternative splicing that ultimately leads to eight different functional variants. Also, a truncated ninth variant was found, yet no function could be shown for it so far (Sugihara et al., 1992; Hollmann et al., 1993). The coding sequence of the GluN1 subunit is distributed over 22 exons. C-terminal splicing within exons 21 and 22 leads to four different variants.

Following the exon 20, the inclusion of exon 21 (cassette C1) leads to the variants GluN1-1 and GluN1-3, whereas GluN1-2 and GluN1-4 lack exon 21. Exon 22 generates an alternative C-terminus: The alternative splicing leads to two different versions with different lengths. GluN1-1 and GluN1-2 contain a long version, the C2 cassette. In a short version, where a part including the original stop codon is deleted, a new stop codon arises in the so called C2’ cassette. This C2’ cassette is present in GluN1-3 and GluN1-4 subunits (Sugihara et al., 1992; Hollmann et al., 1993).

The presence or absence of the alternatively spliced N-terminal exon 5 that incorporates 63 bp and applies to all four C-terminally spliced variants further enhances the diversity. Receptor subunits without the exon 5 are called the “a” variants, receptor subunits carrying this exon are the so-called “b” variants. This 21 amino acid inset in the N-terminal part of the protein plays an important role in the pharmacological behavior. Whereas receptors containing GluN2 and GluN1 “a” variants are proton-sensitive, receptors containing exon 5, the GluN1 “b” variants, are proton-insensitive. The loop formed by these additional amino acids is thought to “shield” the region that mediates the proton block (Traynelis et al., 1995, 1998).
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The effects of the C-terminal splicing do not affect the pharmacological behavior of the receptor, instead, it influences the ER export of the receptor subunits. The C1 cassette (exon 21) contains an ER retention signal (RRR) that interferes with plasma membrane expression. Thus, only the GluN1-1 and GluN1-3 are affected. For GluN1-3, however, this ER retention signal can be overridden via the C2' cassette that carries a PSD-95/Dig/ZO-1 (PDZ) binding motif (STVV). This motif can interact with PSD-95, a MAGUK family protein, ultimately leading to a certain level of membrane expression. GluN1-2 and GluN1-4 do not contain this C1 cassette; accordingly, their membrane expression is not impaired (Standley et al., 2000; Scott et al., 2001; Xia et al., 2001).

In the *X. laevis* oocyte system, oocytes injected solely with GluN1 RNA showed agonist-inducible currents (Hollmann et al., 1993). This led to the interpretation that the GluN1 subunit is able to form functional homomeric receptors. However, the existence of such homomeric receptors was disproven: The GluN1 subunit is capable of forming functional heteromeric receptors with the *Xen*GluN2B subunit, the only *Xenopus* glutamate receptor subunit expressed endogenously in oocytes at the protein level (Schmidt and Hollmann, 2008).

All GluN1 subunits are generally expressed all over the CNS with especially high levels in the cerebellum, hippocampus, cerebral cortex, and olfactory bulb (Moriyoshi et al., 1991; Monyer et al., 1994). To a certain degree a splice variant-dependent expression pattern seems to exist: GluN1-1a and GluN1-2a seem to be expressed mainly in cortex and hippocampus, the GluN1-2b and GluN1-4b subunits in thalamus, cerebellum and brainstem. The GluN1-3 variant showed a generally low expression pattern, mainly in cortex and hippocampus (Laurie and Seeburg, 1994; Laurie et al., 1995).
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The GluN1 subunits normative agonist is glycine, the simplest of all amino acids. The isolated LBD of GluN1 has a ligand affinity for glycine of 26.4 \( \mu \)M. Interestingly, it can also bind D-Serine with a \( K_d \) value of 7.02 \( \mu \)M, lower than that of glycine (Furukawa and Gouaux, 2003). The agonist of GluN2 subunits, glutamate, cannot bind to the GluN1 LBD at all, with the apparent \( K_d \) being \( > 300 \)mM (Yao and Mayer, 2006). Potent antagonists are derivates of kynurenic acid like 7-chlorokynurenic acid (7-CKA) with a \( K_d \) of 2.6 \( \mu \)M or 5,7-dichlorokynurenic acid (DCKA)(\( K_d \) 0.54 \( \mu \)M). DCKA binding leads to a smaller degree of domain closure, stabilizing the open conformation (Furukawa and Gouaux, 2003; Yao and Mayer, 2006). Other potent blockers are MDL-100748 and MDL-29951 that were developed as anticonvulsants and showed positive effects in seizure-susceptible mice (Salituro et al., 1990; Baron et al., 1992). Mutation of a phenylalanine in position 466 in the ligand binding domain to an alanine raises the EC\( _{50} \) of glycine in GluN1/GluN2-containing receptors about 6500-fold (Kuryatov et al., 1994). This phenylalanine stabilizes glycine binding via \( \pi \) electron interaction and prevents glycine from leaving the closed LBD (Furukawa and Gouaux, 2003).

1.4.2. The GluN2 Subunit

The four GluN2 subunits, GluN2A through GluN2D, are encoded by four different genes (Monyer et al., 1992). Together with GluN1 they form the classical NMDAR. Alternative splicing occurs in all four variants, though the different variants do not seem to differ significantly in their function (Yamazaki et al., 1992; Ishii et al., 1993; Suchanek et al., 1995; Klein et al., 1998; Tabish and Ticku, 2004). Between the subunits substantial differences concerning their regional and temporal expression profiles exist. In rats, GluN2B and GluN2D are predominantly expressed prenatally, whereas GluN2A and GluN2C arise first near birth, leading to a postnatal switchover from GluN2B- to GluN2A-containing receptors (Monyer et al., 1994). GluN2B is mostly found in the hippocampus, but also in the striatum, cerebellum, and thalamus. The GluN2D subunit shows the highest expression in the midbrain and the thalamus. Perinatally, the GluN2A subunit is mainly expressed in the cortex and the hippocampus, whereas the GluN2C subunit is mostly restricted to the cerebellum (Monyer et al., 1992, 1994).

One special feature of the GluN1/GluN2-containing receptors is the voltage-dependent block of the ion pore by magnesium ions. In order to remove this block, an initial depolarization is required, e.g. mediated through AMPARs. GluN2A and GluN2B subunits are more efficiently blocked by Mg\(^{2+} \) already at membrane potentials below –25 mV compared to GluN2C and GluN2D. The latter are blocked at membrane potentials below –45 mV (Nowak et al., 1984; Sakurada et al., 1993; Monyer et al., 1994; Kuner and Schoepfer, 1996). The mandatory predepolarization and the required simultaneous binding of glutamate and glycine to open the ion pore renders GluN2-containing receptors to be coincidence detectors, a unique feature amongst the ionotropic glutamate receptors. The GluN2 subunits bind glutamate with an affinity ranging from 0.5 \( \mu \)M (GluN2D) to 3.3 \( \mu \)M (GluN2A)(Laube et al., 1997; Erreger et al., 2007).

Conventional GluN1- and GluN2-containing receptors exhibit a potent block by zinc ions (Westbrook and Mayer, 1987; Legendre and Westbrook, 1990; Yeh et al., 1990; Paoletti...
et al., 2000). This Zn$^{2+}$ sensitivity is thought to be mediated by enhancing the proton block in GluN2 (Choi and Lipton, 1999; Low et al., 2000) and stabilization of the closed conformation of the receptor (Rachline et al., 2005; Karakas et al., 2009). Interestingly, this effect is still partially mediated via the GluN1 subunit as the presence of exon 5 in receptors containing GluN1-1b and GluN2A or GluN2B renders them insensitive to zinc (Traynelis et al., 1998). The zinc binding site, however, is located in the ATD of the GluN2 subunits, where six residues control zinc binding (Fayyazuddin et al., 2000; Paolelli et al., 2000; Karakas et al., 2009). Besides this high affinity voltage-independent block with an IC$_{50}$ for GluN2B-containing receptors in the nanomolar range, the GluN2A subunit also undergoes a low affinity block in the µM range. This low affinity block is voltage-dependent and resembles the Mg$^{2+}$ block (Williams, 1996).

GluN2-containing receptors can be blocked by a variety of substances. Examples are the channel blocker [5R,10S]-[+]5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801)(Wong et al., 1986), ketamine and memantine (Kotermanski and Johnson, 2009), and (2R)-amino-5-phosphonovaleric acid (AP5)(Davies et al., 1981). So far, only few substances have been identified that show a strong selectivity between the different GluN2 subunits: Ifenprodil, for example, has a 100-fold higher affinity for GluN2B- over GluN2A-containing receptors (Williams, 1993). Ifenprodil binds at the GluN1/GluN2 ATD interface (Rachline et al., 2005; Karakas et al., 2011; Sirrieh et al., 2015). A promising approach for the future search for selective substances has been successfully conducted by Bettini et al. in 2010, where via a high throughput screening two sulfonamide derivates were identified that displayed a 500-fold higher affinity for GluN2A- over GluN2B-containing receptors.

1.5. The GluN3 Subunit

The GluN3 subunits were the last NMDAR subunits identified. GluN3A, found by Ciabarra et al. (1995) and Sucher et al. (1995), is a 1115 AS long protein with two known splice variants (GluN3A-1 and GluN3A-2), differing by a 20 AS insert in the CTD (Sun et al., 1998; Sasaki et al., 2002). This insert contains additional phosphorylation sites for CaMKII, protein kinase A (PKA), and protein kinase C (PKC). The second GluN3 subunit, firstly described as GluN3B by Nishi et al. (2001), as well as Chatterton et al. (2002) and Matsuda et al. (2002), generates a 1003 AS (rat) or 1002 AS (mouse) protein. For this subunit four different splice variants have been found, with deletions of 24 or 45 (N-terminal) and 600 or 1125 (C-terminally) bp, all of them functional (de Jesus Domingues et al., 2011). The GluN3B subunit does not possess PDZ binding sites, but several ER retention signals (Matsuda et al., 2002). GluN3A and GluN3B share 47% sequence similarity between each other and 17% respectively 26% to the GluN1 and GluN2 subunits (Chatterton et al., 2002).

1.5.1. Electrophysiology

The electrophysiological properties of GluN3-containing receptors are unique amongst the NMDARs, as the GluN3 subunit binds glycine rather than glutamate (Kuryatov et al.,
GluN3 subunits are not capable of forming functional receptors together with GluN2 subunits in the absence of a GluN1 subunit (Chatterton et al., 2002; Schüler et al., 2008). Also, they cannot form homomeric receptors (Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Chatterton et al., 2002). The combination with a GluN1 subunit – which also binds glycine – therefore generates an excitatory glycine receptor.

GluN3 subunits can modulate all three key characteristics of conventional, GluN1- and GluN2-containing receptors when they form a GluN1/GluN2/GluN3 triheteromeric receptor: In heterologous expression systems both GluN3A and GluN3B reduce the agonist-induced current responses (Ciabarra et al., 1995; Sucher et al., 1995; Chatterton et al., 2002). Consistently, cortical neurons of mice lacking the GluN3A subunit showed increased current responses (Das et al., 1998; Tong et al., 2008). Also, the Mg$^{2+}$ block is strongly reduced by coexpression of either GluN3A or GluN3B (Sasaki et al., 2002; Smothers and Woodward, 2007). These receptor combinations additionally show a reduced Ca$^{2+}$ permeability (Perez-Otano et al., 2001; Sasaki et al., 2002; Tong et al., 2008).

These triheteromeric combinations appear to exist also in vivo: In mouse hippocampal neurons overexpressing GluN3A, glycine alone was not able to elicit current responses. Still, the characteristic properties of the conventional, GluN1/GluN2-containing receptor such as Mg$^{2+}$ sensitivity and Ca$^{2+}$ permeability were altered, indicating the presence of GluN1/GluN2/GluN3A-containing receptors (Tong et al., 2008). One problem arising in heterologous expression systems is the possibility that two different populations of receptors might coexist, one containing GluN1/GluN2 subunits and a second containing GluN1/GluN3 receptors (Ulbrich and Isacoff, 2008). However, there is also evidence supporting the idea of triheteromeric receptor combinations in heterologous expression systems, as in human embryonic kidney (HEK) cells the expression of GluN3A-CFP together with GluN2B-YFP showed no fluorescence resonance energy transfer (FRET) signal until the GluN1 (wild type) subunit was coexpressed (Schüler et al., 2008).

Excitatory glycine receptors formed by GluN1 and GluN3 subunits are composed of two subunits each in an alternating 1-3-1-3 stochiometry (Ulbrich and Isacoff, 2007; Schüler et al., 2008). Interestingly, these combinations are almost Ca$^{2+}$-impermeable and insensitive to Mg$^{2+}$ (Chatterton et al., 2002; Matsuda et al., 2002; Smothers and Woodward, 2007). Contrary to the conventional receptor subunits, the pH dependency of GluN1/GluN3B-containing receptors is independent of the GluN1 splice variant (Cavara et al., 2009).

In HEK cells the GluN1-2, GluN1-3 and GluN1-4 splice variants were found to form functional excitatory glycine receptors with either GluN3A or GluN3B (Smothers and Woodward, 2009). The GluN1-1 splice variant was found to form functional receptors only upon coexpression of both GluN3A and GluN3B. Nevertheless, mutations in the GluN1 subunit known to enhance the glycine-induced current response reversed this effect. This suggests that the current response of GluN1-1/GluN3A-containing receptors under standard conditions is, due to desensitization, simply below the detection threshold in HEK cells (Smothers and Woodward, 2009).
1.5.2. Pharmacology

Like GluN1 subunits, the GluN3 subunits binds glycine. Although both subunits share this common feature, they also show a considerable number of distinct features. The first and most important is the affinity to glycine: The GluN3A subunit binds glycine with a 650-fold higher affinity than GluN1 ($K_d$ 40.4 nM; Yao and Mayer 2006). The binding pocket for glycine is a bit larger in GluN3A and GluN3B, which in part explains this different behavior (Yao and Mayer, 2006; Yao et al., 2008). Additionally, GluN3 subunits feature a loop that forms a capping helix in the LBD, stabilizing the closed conformation (Yao et al., 2008). The isolated GluN3A ligand binding domain can also bind glutamate, though not at physiological concentrations ($K_d > 9.6$ mM; Yao and Mayer 2006). GluN3A and GluN3B differ only by a factor of 2.5 regarding their glycine affinity ($K_d$ 40.4 nM for GluN3A and 16.4 nM for GluN3B), which is no surprise as the LBDs are almost identical (Yao and Mayer, 2006; Yao et al., 2008).

GluN1/GluN3-containing receptors show a bell-shaped concentration-dependent current response upon glycine application. This effect is suggested to be mediated via desensitization of the receptor by the GluN1 subunit at increasing glycine concentrations (Awobuluyi et al., 2007; Madry et al., 2007a): Glycine would have an agonistic effect on the GluN3 subunit and an inhibitory effect on the GluN1 subunit. A very recent study suggests that the mechanism underlying this effect is mediated via the NTD dimer-dimer interface, as the deletion of the NTDs abolishes this autoinhibition yet preserves receptor function (Mesic et al., 2016).

Contrary to GluN1/GluN2-containing receptors, when the phenylalanine in position 466 in GluN1 is mutated to an alanine in GluN1/GluN3-containing receptors, the agonist-induced current response is not decreased, but strongly increased. Within the ligand binding domain of GluN3, a tyrosine at position 482 also forms a π electron interaction, equivalent to the phenylalanine found in GluN1 subunits at position 466. However, when this aromatic residue is mutated to an alanine, the glycine-induced current response is strongly decreased. The potentiating effect of the GluN1(F466A) mutant on GluN3A-containing receptors can also be evoked pharmacologically by GluN1 glycine site antagonists such as MDL-29951 and DCKA (Awobuluyi et al., 2007; Madry et al., 2007a). A very interesting effect has been described for GluN1-1a/GluN3A-containing receptors: The coapplication of glycine, the GluN1 glycine binding site antagonist MDL-29951 and zinc led to an even higher potentiation than seen upon the coapplication of glycine and MDL-29951 (Madry et al., 2008). However, the mechanism of this effect remains elusive.

Triheteromeric receptors containing GluN1/GluN2A and GluN3A or GluN3B are, interestingly, almost resistant against blockers of the conventional receptor combinations like memantine and MK-801 and the inhibitor AP5 (Chatterton et al., 2002; Smothers and Woodward, 2007; McClymont et al., 2012). Towards GluN1/GluN3-containing excitatory glycine receptors D-serine acts, when coapplied with glycine, as an antagonist, contrary to conventional, GluN1/GluN2-containing receptors, where it has agonistic properties (see chapter 1.4.1). Yet D-serine applied alone is capable of inducing a small current response in GluN3-containing diheteromers (Chatterton et al., 2002; Smothers and Woodward, 2007). There also exists evidence for a participation of at least GluN3A in drug-evoked synap-
tic plasticity, e.g. with cocaine (Yuan et al., 2013). Due to the almost identical LBD, the pharmacological discrimination between GluN3A and GluN3B has proven itself to be very difficult and agonists or antagonists specific for the respective LBD are scarcely available. Lately, a diheterocyclic compound (TK80) was found to be highly selective for GluN3B over GluN3A (Kvist et al., 2013), yet this compound is not commercially available so far.

1.5.3. Physiology

The spatial expression of the GluN3 subunits during development undergoes intense alterations. Whereas the GluN3A subunit is expressed mainly pre- and perinatally, the GluN3B subunit shows prenatally only weak expression that rises postnatally and remains elevated in adulthood. The GluN3A mRNA expression level in rodents is highly region-specific. At E15, GluN3A mRNA can be found in the spinal cord, the medulla, hypothalamus, and thalamus. Levels then increase and expression spreads to the whole brain stem and the hippocampus, with a peak at P8 – P10. Towards adulthood the expression declines, only weak expression can be found in the olfactory bulb, the cerebellum, the retina, and the prefrontal cortex (Ciabarra et al., 1995; Sucher et al., 1995).

On the protein level, GluN3A peaks at P8 and can be found in cortex, amygdala, hippocampus, thalamus, brainstem, and spinal cord (Goebel and Poosch, 1999; Al-Hallaq et al., 2002; Wong et al., 2002). GluN3B appears first in motorneurons of the brainstem and the spinal cord at P14 and remains elevated in adulthood (Nishi et al., 2001; Chatterton et al., 2002; Fukaya et al., 2005). It is also found in the pons and the midbrain and at low levels in the cerebellum, cortex, hippocampus, olfactory tract, and forebrain (Matsuda et al., 2002). However, there is also evidence for a wider distribution of GluN3B protein expression in the CNS that can even reach the level of GluN1 (Wee et al., 2008). The GluN3B subunit contains an ER retention signal, yet when this is abolished the receptor is still not exported, which suggests the lack of a forward-trafficking signal (Matsuda et al., 2003).

Important features of conventional NMDARs are influenced by the presence of a GluN3 subunit and the formation of a GluN1/GluN2/GluN3-containing triheteromeric receptor complex (see 1.5.1 and 1.5.2). This is of special importance since the NMDAR-mediated mechanism of excitotoxicity through elevated calcium influx is related to various neurodegenerative diseases such as amyotrophic lateral sclerosis, multiple sclerosis, and Huntington’s disease (see chapter 1.4)(Tymianski et al., 1993; Chohan and Iqbal, 2006; Hardingham, 2009). When the expression of GluN3A is artificially prolonged in genetically modified mice, these mice show deficits in LTP, memory deficits and hypofunction of synaptic NMDARs (Roberts et al., 2009). Also, mice lacking GluN3A show strongly increased number of dendritic spines and an accelerated synapse maturation (Das et al., 1998). These mice show no direct phenotype, but exhibit strongly impaired olfaction; also, they show delayed recovery from stroke (Lee et al., 2015a,b). The lower Ca$^{2+}$ conductivity of GluN3-containing triheteromeric receptors might hinder overexcitation of the synapse (Nakanishi et al., 2009). This implies a neuroprotective role of GluN3A, and shows the importance of a correct tuning of GluN3A for development and cognitive function (Das et al., 1998; Henson et al., 2012).
GluN3B-containing receptors are strongly expressed in motorneurons, and their expression increases from birth till adulthood when at the same time the expression of GluN2A decreases (Fukaya et al., 2005). Accordingly, mice lacking GluN3B show motor deficits, although the animals are otherwise healthy (Niemann et al., 2007).

The existence of GluN3-containing excitatory glycine receptors has recently been proven in vivo: D-serine was able to elicit a response in optic nerve myelin (Piña-Crespo et al., 2010). This response could not be induced in optic nerve myelin from GluN3A knockout mice. Also, evidence for the existence of GluN1/GluN3-containing receptors in hippocampal neurons occurred recently: continuing LTP induction in hippocampal synapses lead to the incorporation of GluN1/GluN3-containing receptors and thus to a synapse weakening (Rozeboom et al., 2015).

1.6. Zinc and the Central Nervous System

Zinc is the second most abundant trace element in the human body, following iron. Most zinc (around 95%) is bound within enzymes and cofactors where it plays catalytic or structural roles. A well known example are zinc finger DNA-binding proteins, where the zinc ion plays a stabilizing structural role. An example for a catalytic role is the carbonic anhydrase, an enzyme that catalyzes the reaction of carbon dioxide and water to carbonic acid. Besides this pool of bound zinc, in the brain “free” zinc is mostly found in synaptic vesicles of glutamergic synapses (Pérez-Clausell and Danscher, 1985). In these vesicles zinc is accumulated via active pumping by the vesicular zinc transporter ZnT-3 (Palmiter et al., 1996). From these vesicles zinc can, upon excitation of the synapse, be released, possibly reaching a transient peak concentration of around 100 µM in the synaptic cleft (Vogt et al., 2000; Qian and Noebels, 2005). In contrast, the tonic zinc level in the CNS, measured in the cerebrospinal fluid (CSF), is around 19 nM (Frederickson et al., 2006). Although direct proof remains elusive, it seems likely that zinc and glutamate are coreleased from the presynaptic terminal (Assaf and Chung, 1984; Qian and Noebels, 2005; Frederickson et al., 2006).

The importance of zinc homeostasis, especially of synaptic zinc, can be understood when its role in Alzheimer’s disease is considered, for which strong evidence has been accumulated over the years. In Alzheimer’s disease, amyloid-β (A-β) is cleaved out of amyloid precursor protein (APP) and aggregates extracellularly. The A-β aggregation is promoted by zinc, as is its resistance towards proteolytic catabolism is increased by zinc (Bush et al., 1994a,b). Consequently, substances that can bind zinc show therapeutical potential. One example is 8-OH quinoline that can foster the dissociation of zinc from A-β (Ritchie et al., 2003; Adlard et al., 2008). In another example, ischemia, elevated zinc concentrations led to neurodegeneration. This effect could be prevented by a zinc chelator, calcium-EDTA (Koh et al., 1996).

Elevated concentrations of extracellular zinc (> 300 µM) are toxic for cells in vivo (Yokoyama et al., 1986). Dependent on the conditions, zinc can exhibit lethal effects already at far lower concentrations. For example, 20 µM were lethal to glucose-deprived cerebellar granule neurons (Isaev et al., 2012). In another study, 50% of cultured mouse cortical neurons died
1. Introduction

Zinc homeostasis
- Alzheimer’s disease
- ischemia
- cell death
- epilepsy
- depression
- memory deficits

- anticonvulsant
- LTP modulation

Figure 1.5: Physiological importance of correct zinc homeostasis. The basal cellular zinc level has anticonvulsant effects and is involved in mechanisms such as LTP. Increased as well as decreased cellular zinc levels take part in various diseases. Too high or low levels of zinc can lead to cell death via different mechanisms.

at 40 µM zinc (Bozym et al., 2010). The effects high intracellular zinc concentrations exhibit on neurons are mediated via various mechanisms. One prominent way is the activation of PKC, which then upregulates NADPH oxidase activity, thereby enhancing oxidative stress and promoting cell death (Noh et al., 1999; Kim and Koh, 2002). Raised zinc concentrations can also impaire the glycolytic metabolism via reducing the amount of NAD+, leading to a subsequent reduction in ATP amounts and cell death (Cai et al., 2006).

On the other hand, low zinc concentrations can also promote apoptotic cell death via caspase activation (Clegg et al. 2005, for an overview see Truong-Tran et al. 2001). Furthermore, zinc homeostasis has also implications in epilepsy, as mice lacking ZnT-3 are more prone to seizures than wild type mice, further supporting the suggested neuroprotective role of zinc (Cole et al., 2000). Also, zinc plays an important role in LTP: Endogenous zinc is required for the induction of LTP in mossy fiber and hippocampal synapses (Lu et al., 2000; Li et al., 2001; Takeda et al., 2009). Additionally, low zinc concentrations are linked to mechanisms of depression. The mechanistic implications involve a certain tonic inhibition of NMDARs at physiological concentrations (Maes et al., 1994; Nowak et al., 2003; Sowa-Kućma et al., 2008). This idea is supported by an altered composition of NMDARs in favor of GluN2A found in suicide victims, which decreases the potency of zinc on these receptors (Sowa-Kućma et al., 2013).

1.7. Objectives

The NMDARs play a crucial role in the formation of memory and the mechanism of learning. Zinc as an important and abundant trace element in the body is known to block the classical, GluN1/GluN2-containing NMDAR. However, information about the behavior of the third NMDAR subclass, GluN3, towards zinc remains sparse and only comparatively little research has been conducted with GluN3-containing receptors. At receptors contain-
ing GluN1 and GluN3A zinc shows diverging effects: At high concentrations zinc alone can act as an agonist, and it can potentiate the glycine-mediated currents at low glycine concentrations (Madry et al., 2008).

This interesting effect was only investigated for combinations of GluN1-a splice variants and GluN3A, and the exact mechanism of activation has not yet been analyzed. The main important other GluN1 splice variants, the “b” variants carrying the exon 5 that heavily alters receptor behavior in GluN1/GluN2-containing receptors (Traynelis et al., 1995) have been neglected. Concerning the GluN3 subunit, the transcription of GluN3A during development is downregulated after birth, while the expression of GluN3B elevates. The physiological role of GluN3B cannot – and should not – be underestimated. The NMDAR-mediated mechanism of excitotoxicity through elevated calcium influx is related to various neurodegenerative diseases (see above), and NMDARs containing the GluN3 subunit show reduced calcium permeability. Nevertheless, the behavior of GluN1/GluN3B-containing excitatory glycine receptors towards zinc remains elusive. Last, but not least, as the existence of diheteromeric, GluN1/GluN3-containing receptors in vivo has recently been proven (Piña-Crespo et al., 2010), the interest in this NMDAR receptor combination is not only academic. Furthermore, elevated concentrations of zinc are toxic for cells, and dysregulated zinc homeostasis plays important roles in various diseases such as Alzheimer’s disease, epilepsy, and depression.

Many questions remain unsolved: Does the excitatory glycine receptor formed by the GluN3B receptor show the same behavior towards zinc as has been reported for the GluN3A-containing receptor? Does the exon 5 play a role in the zinc modulation of the GluN3B-containing receptors? And how can zinc influence the receptor? The aim of this thesis is therefore to electrophysiologically investigate the mechanisms of zinc modulation of receptors containing GluN3B.

Zinc is a potent modulator of NMDARs, while the GluN3B subunit is an important member of the NMDAR family. A better understanding of the mechanisms underlying the zinc-mediated modulation will therefore provide a step forward in our knowledge of glutamate receptor function and hopefully will lead to further insights into their modulation.
2. Materials

2.1. Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Amplifier</td>
<td>Turbo Tec-10CX, npi electronic, Tamm</td>
</tr>
<tr>
<td>Autoclave</td>
<td>Varioklav Typ 500 E, H+P Labortechnik GmbH,</td>
</tr>
<tr>
<td></td>
<td>Oberschleißheim</td>
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<td>Binoculars</td>
<td>Stemi 2000, Carl Zeiss, Jena</td>
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<td></td>
<td>Stemi DRC, Carl Zeiss, Jena</td>
</tr>
<tr>
<td></td>
<td>Axiovert 25, Carl Zeiss, Jena</td>
</tr>
<tr>
<td>Binoculars – light source</td>
<td>KL1500 LCD, Schott, Mainz</td>
</tr>
<tr>
<td></td>
<td>KL1500 electronic, Schott, Mainz</td>
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<td></td>
<td>Biofuge pico, Heraeus, Hanau</td>
</tr>
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<td></td>
<td>Biofuge stratos, Heraeus, Hanau</td>
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<td></td>
<td>Centrifuge 5415 C, Eppendorf, Hamburg</td>
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<td></td>
<td>Centrifuge 5415 R, Eppendorf, Hamburg</td>
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<td></td>
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<td></td>
<td>Minispin, Eppendorf, Hamburg</td>
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<tr>
<td></td>
<td>RC 5B, Sorvall, Newtown, CT, USA</td>
</tr>
<tr>
<td></td>
<td>RC 28S, Sorvall, Newtown, CT, USA</td>
</tr>
<tr>
<td></td>
<td>Tischzentrifuge, Qualitron Karachi, PK</td>
</tr>
<tr>
<td>Computer (electrophysiology)</td>
<td>PowerMac G4, Apple, Cupertino, CA, USA</td>
</tr>
<tr>
<td>Electrode puller</td>
<td>P-97, Sutter Instruments, Novato, CA, USA</td>
</tr>
<tr>
<td></td>
<td>Pipette Puller PIP5, HEKA, Bellmore, NY, USA</td>
</tr>
<tr>
<td></td>
<td>DMZ Universal Puller, Zeitz Instruments, Martinsried</td>
</tr>
<tr>
<td>Elektrophoresis Power Supply</td>
<td>E844, Consort, Turnhout, B</td>
</tr>
<tr>
<td></td>
<td>ECPS 3000/150, Pharmacia, Uppsala, S</td>
</tr>
<tr>
<td></td>
<td>PowerPac 3000, Bio-Rad, Hercules, CA, USA</td>
</tr>
<tr>
<td>Gel documentation system</td>
<td>INTAS, Göttingen</td>
</tr>
<tr>
<td>Electrophorese Cchambers</td>
<td>MGU-200T (7 × 10 cm), C.B.S., Del Mar, CA, USA</td>
</tr>
<tr>
<td>Hoods</td>
<td>BH NG, Gelman Sciences, Ann Arbor, MI, USA</td>
</tr>
<tr>
<td>Ice machine</td>
<td>Scotsman AF-30</td>
</tr>
</tbody>
</table>
2. Materials

Injector
Nanoliter Injector, WPI, Sarasota, FL, USA

Micromanipulators
Märzhäuser, Wetzlar

Photometer
Nanophotometer, Implen, München

Pipettes
Pipetman P2, P10, P20, P100, P200, P1000, P5000, Gilson, Middleton, WI, USA
Comfort DVC2 / DVC10, Abimed, Langenfeld
Reference 10 µl, Research 20µl/200 µl, Eppendorf, Hamburg

pH-Meter
Labor-pH-Meter 766, Knick, Berlin

Scales
2205 MC1, Sartorius, Göttingen
BP211D, Sartorius, Göttingen

Thermocycler
PTC-200, MJ Research, Watertown, MA, USA

Ultra pure water system
Synergy UV, Millipore, Billerica, MA, USA

Vibration isolation table
Kinetic Systems Vibraplane, Boston, NY, USA
Physic Instruments, Auburn, MA, USA

Vacuum pumps
MZ 2CE, Vacuubrand, Wertheim
ME 2, Vacuubrand, Wertheim

Valves
Hamilton, Reno, NV, USA

2.2. Consumables

0.2- and 0.5 ml-PCR reaction tubes
Starlab, Ahrensburg
Sarstedt, Nümbrecht

1.5- and 2.0 ml reaction tubes
Starlab, Ahrensburg

1.5 ml reaction tubes, sterile
Biozym, Hess. Oldendorf
Sarstedt, Nümbrecht

1.5 ml screw cap reaction tubes
Starlab, Ahrensburg

14 ml tubes
Greiner, Frickenhausen

50 ml tubes
Sarstedt, Nümbrecht

Borosilicate glass capillaries
Art.-Nr. 1404501, Hilgenberg, Malsfeld
Art.-Nr. GB150ETF-10, Science Products, Hofheim am Taunus

Filter pipette tips
Starlab, Ahrensburg

Gloves
VWR, Radnor, PA, USA

Injection capillaries
No 4878, WPI, Sarasota, FL, USA

Petri dishes
Becton Dickinson, Franklin Lakes, NJ, USA
Greiner, Frickenhausen

Pipette tips
Starlab, Ahrensburg
2.3. Chemicals

All chemicals were ordered from the following companies in a purity of at least analytical grade:

- AppliChem, Darmstadt
- Biozym, Hess. Oldendorf
- Fluka, Buchs, CH
- Merck, Darmstadt
- Pierce, Rockford, IL, USA
- Roche, Basel, CH
- Sigma, Taufkirchen

Biomol Feinchemikalien, Hamburg
Bio-Rad, Richmond, CA, USA
J.T. Baker, Deventer, NL
Perkin Elmer, Waltham, MA, USA
Riedel-de-Häën, Hannover
Roth, Karlsruhe

MDL-29951 was at the beginning of this thesis not commercially available any more and was synthesized (spectroscopically pure, determined via ¹H-NMR) by Dr. Christian Wagner, Department of Organic Chemistry I, Workgroup Natural Product Chemistry, Prof. emerit. Dr. M. Feigel, according to Salituro et al. (1990).

2.4. Enzymes

All enzymes were ordered from the following suppliers:

- Fermentas, St. Leon-Rot (now Thermo Scientific, Waltham, MA, USA)
- Finnzymes, Espoo, FI (now Thermo Scientific, Waltham, MA, USA)
- New England Biolabs (NEB), Frankfurt am Main
- Promega, Madison, WI, USA

2.5. Oligonucleotides

All oligonucleotides were ordered either from Microsynth, Balgach, CH, or from Sigma, Taufkirchen.

2.6. Kits

The following kits were used:

- JetStar 2.0 Plasmid Purification Kit, Genomed, Löhne
- mMESSAGE mMACHINE Kit T7, Ambion, Austin, TX, USA
- my-Budget Double Pure Kit, Bio-Budget, Krefeld
- RNA Clean & Concentrator Kit, Zymo Research, Irvine, CA, USA
2. Materials

2.7. Organisms

2.7.1. Bacteria

For all cloning within this thesis the *Escherichia coli* (E. coli) K12 strains ER2925 and XL1-Blue were used. The strain ER2925 (NEB, Frankfurt) is resistant to chloramphenicol and streptomycin. This strain is DNA adenine methylase (Dam)- and DNA cytosine methylase (Dcm)-negative and can therefore also be cut by restriction enzymes sensitive to Dam or Dcm methylation.

**Genotype:** ara-14 leuB6 fhuA31 lacY1 tsx78 glmV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2

The strain XL1-Blue (Stratagene, La Jolla, CA, USA) is resistant to tetracycline. Contrary to the ER2925 it is Dam- and Dcm-positive, which has to be taken into account when choosing restriction enzymes, as some enzymes are not capable of cutting methylated DNA.

**Genotype:** recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacZDAM15 Tn10 (Tet’)]

2.7.2. Animals

Oocytes for electrophysiological experiments were obtained from female South African clawed frog *Xenopus laevis* (*X. laevis*, Nasco, Fort Atkinson, WI, USA).

2.8. DNA Constructs

The all-round vector carrying all receptor constructs was the pSGEM vector, derived from pGEMHE (Liman et al., 1992). The vector’s MCS is flanked by both the 5’ and 3’ UTRs of the *Xenopus β*-globin gene. Both vectors also contain the β-lactamase gene required for ampicillin resistance and an ori. The vector pSGEM-eGFP also contains the cds of eGFP.

![Figure 2.1.: Used vectors. Both pSGEM and the pSGEM-eGFP contain a T7 and an SP6 RNA polymerase promoter. The MCS is flanked by the 5’ and 3’ UTRs of the *Xenopus β*-globin gene. Both vectors also contain the β-lactamase gene required for ampicillin resistance and an ori. The vector pSGEM-eGFP also contains the cds of eGFP.](image-url)
of the *Xenopus* β-globin gene to enhance the expression efficiency in oocytes. For the *in vitro* transcription of deoxyribonucleic acid (DNA), the promoters of the T7 (sense) and SP6 (antisense) RNA polymerases are available. For efficient amplification of DNA an origin of replication (ori) and, to allow the selection of positive clones in transformed bacteria, also an ampicillin resistance via the β-lactamase gene are present in the vector. Besides the standard pGEMHE linearization sites, the vector pSGEM also contains two additional linearization sites (PciI and SfiI). The MCS is derived from the vector pBlue-script (Stratagene). The vector pSGEM-eGFP, generated by Dr. Daniel Tapken (Tapken, 2008), is a modified version of the pSGEM vector, where the coding sequence (cds) of the enhanced green fluorescent protein (eGFP) is inserted in the MCS. This allows to generate receptor constructs where eGFP is covalently and in frame linked to the receptor.

2.9. Used cDNAs

2.9.1. Wild type Receptors

Within this thesis, the following receptor constructs were used:

**GluN1**:  
GluN1-3a (GenBank: U08265)  
GluN1-3b (GenBank: U08266)

**GluN2**:  
GluN2A (GenBank: AF001423.1)

**GluN3**:  
GluN3A (GenBank: NM_001198583.1)  
GluN3B (GenBank: NM_130455)

2.9.2. Mutant Receptors

All receptor mutants and chimeras used within this thesis are listed below. Besides indicated exceptions, that were generated during a bachelor thesis supervised as a part of the teaching requirements, all constructs were generated by the author.

- GluN1-3a/pSGEM-eGFP  
- GluN1-3a(F466A)/pSGEM  
- GluN1-3a(F466Y)/pSGEM  
- GluN1-3a(N616Q)/pSGEM  
- GluN1-3a(D669N)/pSGEM  
- GluN1-3a(F466A/D669N)/pSGEM  
- GluN1-3a(F466A/N616Q)/pSGEM  
- GluN1-3a(R505A)/pSGEM (Jonas Ahlers)  
- GluN1-3a(D714A)/pSGEM (Jonas Ahlers)  
- GluN1-3b/pSGEM-eGFP
2. Materials

GluN1-3b(F487A)/pSGEM
GluN1-3b(F487Y)/pSGEM
GluN3A(ΔUTR)/pSGEM
GluN3B/pSGEM-eGFP
GluN3B(Y482A)/pSGEM
GluN3B(Y482F)/pSGEM
GluN3B(D722A)/pSGEM (Jonas Ahlers)
GluN3B(R515A)/pSGEM (Jonas Ahlers)
GluN3B(ΔATD)/pSGEM
GluN3B(ΔATDs)/pSGEM
GluN3B(ATD 1-a)/pSGEM
GluN3B(ATD 1-b)/pSGEM
3. Methods

3.1. Molecular Biology

3.1.1. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an *in vitro* technique to amplify DNA, using deoxy nucleotides, oligonucleotides (primers) flanking the region of interest and a DNA polymerase. The usage of modified primers allows the generation of specific mutations in the amplified DNA fragment. A typical PCR protocol consists of three repeated steps: denaturation, annealing and elongation.

During denaturation, the DNA double strand and possible primer dimers are separated. The denaturation temperature depends on the polymerase used. For the polymerase used here, the Phusion™ polymerase (Finnzymes, now Thermo Scientific), 98°C are required. The denaturation time was kept as short as possible to avoid the risk of damaging the DNA. The initial denaturation was performed for 60 s.

During the annealing period primers bind to the DNA strand. The temperature of this step is dependent on the melting temperature of the primers. This temperature is also dependent on the ionic strength of the PCR solution, the pH value and on the used polymerase. For the Phusion™ polymerase primers > 20 nt should be annealed at a temperature +3°C above the lowest melting temperature of the primers, whereas for primers < 20 nt the annealing temperature should be equal to the lowest melting temperature of the primers. The melting temperature was calculated with the nearest neighbour method. The annealing time can be varied between 10 and 30 s.

During the elongation the DNA polymerase adds nucleotides to the 3' end of the primer, generating a new DNA strand. The temperature required for this step is dependent of the polymerase; the Phusion™ polymerase used here requires a temperature of 72°C. In general, the extension time is 15 s per kb, but can be varied for different cases. The final elongation step was 5 min long. A typical PCR program is depicted in Table 3.1.

For a PCR protocol polymerases are necessary that do not denature at the high temperatures required for DNA denaturation. Such polymerases can be found in thermophilic bacteria living in hot springs. Polymerases differ from each other in various regards, for example the processivity and the proofreading capability. Two common thermostable polymerases are the Taq (originating from *Thermus aquaticus*) and Phusion (based on *Pyrococcus furiosus*) polymerases. The Taq polymerase has the disadvantage that it lacks a proofreading capability, a feature the Phusion polymerase has. Through a 3’-5’ exonuclease activity it can cleave false nucleotides and therefore correct mistakes in the synthesized strand. A disadvantage of this feature is that primers can be truncated. To avoid this, the reactions were assembled whilst keeping the reaction tubes on ice and by adding the
3. Methods

Table 3.1.: Example of a standard PCR program

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>98 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>30</td>
<td>Denaturation</td>
<td>98 °C</td>
<td>15 s</td>
</tr>
<tr>
<td></td>
<td>Hybridization</td>
<td>variable</td>
<td>30 s</td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
<td>72 °C</td>
<td>15 s/kb</td>
</tr>
<tr>
<td>1</td>
<td>Final elongation</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

polymerase as the last compound. The Phusion polymerase is dependent on Mg$^{2+}$. For most reactions the Mg$^{2+}$ concentration achieved through the reaction buffer is sufficient, but in some cases it is necessary to increase the amount of magnesium by adding MgCl$_2$. A typical PCR (see Table 3.2) was performed with a 50 µl sample in thin wall 0.2 ml reaction tubes.

Table 3.2.: Components of a PCR reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense primer</td>
<td>100 pM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Antisense primer</td>
<td>100 pM</td>
<td>1 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>10 mM each</td>
<td>2 µl</td>
</tr>
<tr>
<td>Phusion™ HF buffer</td>
<td>5×</td>
<td>20 µl</td>
</tr>
<tr>
<td>Phusion™ polymerase</td>
<td>1 U/µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td></td>
<td>up to 15 ng</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>ad 50 µl</td>
</tr>
</tbody>
</table>

3.1.2. Overlap Extension PCR

The overlap extension PCR is a modified version of a standard PCR and can be used to introduce point mutations into plasmids or to exchange domains between two different constructs. To perform a mutagenesis, primers carrying a mismatch for the desired mutation are used. In the first step, one standard primer and one mutagenesis primer are used together in a standard PCR reaction. In parallel, a second PCR is performed, where the corresponding antisense mutagenesis primer is used together with a second standard primer. In the second step a third PCR is performed where in the first cycles the two PCR products anneal without primers at their overlapping part and the DNA strand is elongated. Here, a prolonged elongation time up to five minutes is used and the annealing temperature is set to the melting temperature of the overlapping ends. Afterwards the outer standard primers are added and a standard PCR protocol to amplify the new product that carries the desired mutation is performed. A typical overlap extension PCR protocol is shown in Table 3.3.
3.1. Molecular Biology

Table 3.3.: Example of an overlap extension PCR programme

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>98 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Adding Phusion® polymerase

<table>
<thead>
<tr>
<th></th>
<th>Denaturation</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
<td>98 °C</td>
<td>15 s</td>
</tr>
<tr>
<td></td>
<td>Hybridization</td>
<td>variable</td>
<td>30 s</td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
<td>72 °C</td>
<td>15 s/kb</td>
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</table>

Adding outer primers

<table>
<thead>
<tr>
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<th>Denaturation</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td></td>
<td>98 °C</td>
<td>15 s</td>
</tr>
<tr>
<td></td>
<td>Hybridization</td>
<td>variable</td>
<td>30 s</td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
<td>72 °C</td>
<td>15 s/kb</td>
</tr>
<tr>
<td>1</td>
<td>Final elongation</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Using primers containing a tail not corresponding to the template (tail primer) makes it possible to exchange domains between different coding sequences. Three reactions are performed in parallel. The first reaction contains the acceptor DNA and two primers: one standard primer and the first tail primer with a tail complementary to a part of the donor DNA. The second reaction contains the donor DNA and two standard primers. The third reaction again consists of the acceptor DNA and two primers, one standard primer and another tail primer with a tail complementary to a part of the donor DNA. The amplified DNA fragments are then purified and used for a fourth PCR. In this reaction all three DNAs are used in one reaction with a programme as described for the overlap PCR: the first cycles are run with a prolonged elongation time and without primers. Afterwards, the two outer standard primers also used in the first and third PCR are added and the reaction is continued.

3.1.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis separates nucleic acid fragments according to their length, taking advantage of their negatively charged phosphate groups, as in an electric field the nucleic acids migrate to the anode. Linear DNA fragments migrate through such a gel with a speed inversely proportional to the logarithm of their molecular weight. DNA can be visualized by adding ethidium bromide (EtBr), a dye that intercalates in DNA double strands and fluoresces under ultraviolet (UV) light. Agarose is a polysaccharide isolated from algae and can be used in different concentrations. Higher concentrations promote the separation of smaller molecules but hinder the separation of larger molecules. Here, gels with a concentration between 0.8 and 1 % were used. An appropriate amount of agarose was dissolved in 1 x Tris-borat-EDTA (TBE) buffer by briefly boiling the mixture. Ethidium bromide was added to a final concentration of 1 µg/ml. The solution was poured into a plastic gel tray provided with a slot-forming comb that was removed once the gel had

25
solidified. Half a volume of 1× loading buffer (containing bromophenol blue and xylene cyanol to follow the migration progress) was added to the DNA samples and pipetted into the gel slots. An appropriate DNA marker (GeneRuler, 100 bp or 1 kb DNA Ladder) supplied with 6× loading buffer was also pipetted in one slot to determine the length of the DNA fragments. The electrophoresis was performed at 80–120 V, depending on the size of the fragments. Afterwards, the gel was put on a UV table and photographed for further analysis.

Table 3.4.: Contents of TBE and MOPS running buffer

<table>
<thead>
<tr>
<th>1× TBE running buffer</th>
<th>10× MOPS buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 mM Tris-Borat pH 8.0</td>
<td>200 mM MOPS</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>50 mM Sodium acetate</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>50 mM Sodium acetate</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>with NaOH</td>
</tr>
</tbody>
</table>

For ribonucleic acid (RNA) gel electrophoresis a slightly different protocol was used. The gel tray and the comb were cleaned well with ribonuclease (RNase) free water, and instead of using TBE buffer 3-(N-morpholino)propanesulfonic acid (MOPS) buffer was used. The agarose concentrations were in the same range as for DNA. Instead of ethidium bromide, the GelStar™ nucleic acid gel stain (Lonza, Basel, CH) was used. As this gel stain is sensitive to light, the electrophoresis itself was performed in the dark as far as possible, and, to assemble the gel, plasticware was used, as the stain adheres to glassware. The 2× loading dye provided with the mMESSAGE mMACHINE T7 Kit (Ambion, now Life Technologies, Darmstadt, see also 3.1.12) was used. The chosen RNA marker was the RiboRuler High range RNA ladder (Thermo Scientific, Waltham, MA, USA).

3.1.4. DNA Extraction from Agarose Gels

Following an agarose gel electrophoresis, DNA can be isolated for further usage. Therefore, the agarose fragment containing the DNA was excised from the gel under UV light, avoiding excess agarose. The DNA was extracted from the agarose using the BioBudget Double Pure kit. This kit uses the principle of anion exchange. The agarose is dissolved and the sample loaded on a silica column under high salt conditions. Subsequent washing steps remove proteins and residual agarose, afterwards the DNA can be eluted with a low salt buffer.

3.1.5. Restriction Digest

Restriction enzymes are used to cut double-stranded DNA at specific sites. Originally they are part of the protection mechanisms of bacteria against alien DNA, restricting, e.g. unmethylated viral DNA, whereas the own, methylated DNA cannot be restricted. Cleavage of the DNA can lead to blunt ends when the enzyme cuts both strands at the same position, or, when it cuts the two strands at different positions, generate “sticky” ends with a 5’ or a 3’ overhang. The 5’ end usually contains a phosphate group, whereas
the 3’ end contains a hydroxyl group. Contrary to blunt ends, sticky ends can be ligated much easier and, when using different enzymes, can also be used to avoid an incorrectly oriented insert, and were therefore always the first choice. A successful restriction strongly depends on the reaction conditions. Salt, DNA and enzyme concentrations as well as the reaction temperature play a role. Reactions were set up using the appropriate reaction buffer and incubated at the best working temperature for the enzyme. Also, bovine serum albumin (BSA) was added to the reaction. The amount of enzyme necessary for sufficient restriction was calculated using formula 3.1. 1 U is the amount of enzyme necessary to digest 1 µg of a reference DNA in 1 h.

\[
\text{Units} \frac{\mu g \cdot h}{n} = \frac{[n] \text{ Restriction sites substrate DNA}}{[n] \text{ Restriction sites reference DNA}} \cdot \frac{\text{Length reference DNA [bp]}}{\text{Length substrate DNA [bp]}}
\] (3.1)

It is important to use the proper amount of enzyme, as too much enzyme can lead to unspecific cutting (“star activity”) and too little enzyme can leave uncut template DNA. A typical restriction digest is depicted below.

• 2 µl 10× Restriction buffer
• 2 µl 10× BSA
• x U Restriction enzyme
• 2 µl DNA
• ad 20 µl Water

For use in in vitro transcriptions (see 3.1.12), linear DNA is inevitable to avoid concatemeric RNA. Therefore a modified version of the protocol was performed in a total volume of 50 µl and incubated overnight, using a single restriction site in the vector.

### 3.1.6. Dephosphorylation of DNA

Prior to its usage in a ligation, the 5’ phosphate groups of restricted vector DNA have to be removed. This is necessary to prevent the vector DNA from religation. Therefore 1 µl (= 5 U) Antarctic phosphatase and 2.3 µl of its reaction buffer were added directly after the restriction digest and the mixture was incubated at 37°C for 30 min. A typical dephosphorylation reaction is depicted below.

• 20 µl of the restriction digest
• 1 µl (= 5 U) Antarctic phosphatase
• 2.3 µl 10× Antarctic phosphatase buffer

Afterwards the phosphatase was deactivated by a 10 min incubation at 65°C.

### 3.1.7. Phosphorylation

To use PCR products, which lack the 5’ phosphate group, in a ligation it is necessary to phosphorylate them, as otherwise the 5’ and 3’ ends cannot be linked together. In this thesis the T4 polynucleotide kinase (PNK) and ligase buffer as an adenosine 5’-triphosphate (ATP) source were used. A typical phosphorylation reaction is shown below.
3. Methods

- 8 µl PCR product
- 1 µl (= 5 U) T4 PNK
- 1 µl 10× Ligase reaction buffer

The reaction was incubated at 37 °C for 1 h and the kinase subsequently heat-inactivated for 30 min at 65 °C.

3.1.8. Ligation

Ligases are enzymes capable of covalently linking the 5′ phosphate and the 3′ hydroxyl group of DNA fragments. Here, the T4 DNA ligase was used. The required energy is provided by ATP included in the ligase buffer. Both blunt and sticky ends can be ligated, where sticky ends must share the same sequence, e.g. through digestion with the same restriction enzyme. The linkage of the DNA fragments by the ligase is a purely stochastical process. It can be enhanced by the addition of polyethylene glycol (PEG) to the reaction to increase the DNA fragment concentration by crowding out the water space. The molar ratio of insert and vector DNA is another important factor. Usually, a ratio of 3:1 insert:vector was used, resulting in a threefold excess of the insert DNA. The appropriate amount of DNA was calculated using formula 3.2.

\[
\text{mass insert [ng]} = \frac{\text{mass vector [ng]} \cdot \text{length insert [bp]}}{\text{length vector [bp]}} \cdot \text{ratio insert/vector} \quad (3.2)
\]

A typical ligation reaction was assembled as seen below, mixed, and incubated at 17 °C overnight.

- 2 µl 10× Ligase reaction buffer
- 1 µl PEG 4000
- 1 µl T4 DNA Ligase
- x µl Vector DNA
- x µl Insert DNA
- ad 20 µl Water

3.1.9. Transformation in Competent E. coli Bacteria

During a transformation alien DNA is incorporated in bacteria. These bacteria are made ready (“competent”) for uptaking DNA via treatment with calcium chloride. In this thesis, the heat shock method was used. 100 µl bacteria that were previously stored at -80 °C were briefly thawed. From a standard 10 µl ligation 5 µl were added to the bacteria, gently mixed and incubated on ice for 30 min. During this time the DNA adheres to the bacteria. The heat shock was performed for 45 s at 42 °C, when the adherent DNA is able to permeate the cell membrane. The suspension was briefly placed on ice. After incubation at room temperature for 2 min the mixture was filled up to 1 ml with 2× yeast-tryptone growing medium (YT) and incubated with shaking at 37 °C for 45 min. The bacteria were pelleted by centrifugation at 3000 g for 3 min. Afterwards, the supernatant was discarded and the
bacteria were plated onto Luria-Bertani growing medium (LB) agar plates containing an antibiotic suitable for the selection of the transformed bacteria. For example ampicillin-containing LB plates allow to select for positive clones successfully transformed with a pSGEM DNA construct, as the pSGEM vector contains an ampicillin resistance (see 2.8). The LB plates were incubated at 37°C overnight and then single colonies were picked for use in DNA preparations.

Table 3.5.: Contents of LB agar plates, YT and LB media

<table>
<thead>
<tr>
<th>LB agar plates</th>
<th>LB media</th>
<th>2× YT media</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g/l Tryptone</td>
<td>10 g/l Tryptone</td>
<td>16 g/l Tryptone</td>
</tr>
<tr>
<td>5 g/l Yeast extract</td>
<td>5 g/l Yeast extract</td>
<td>10 g/l Yeast extract</td>
</tr>
<tr>
<td>5 g/l NaCl</td>
<td>5 g/l NaCl</td>
<td>5 g/l NaCl</td>
</tr>
<tr>
<td>14 g/l Bacto agar pH 7.2 with NaOH</td>
<td>2× YT media</td>
<td>pH 7.4 with NaOH</td>
</tr>
<tr>
<td>pH 7.2 with NaOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.1.10. Preparation of Plasmid DNA

Small amounts of DNA (10 – 20 µg) were extracted from transformed bacteria by alkaline lysis. With this method the cell membrane is disrupted under alkaline conditions using sodium dodecyl sulfate (SDS)/sodium hydroxide (NaOH). Proteins, the chromosomal and the plasmid DNA are also denatured, but the plasmid DNA can renature later on. Afterwards the mixture is neutralised by adding potassium acetate. Denatured proteins, remains of the cell wall and chromosomal DNA form an insoluble precipitate that can be removed by centrifugation, whereas the plasmid DNA stays in the supernatant. After precipitating the DNA by adding isopropanol, it is washed with ethanol and dissolved in TE buffer.

For a typical minipreparation, single colonies of transformed bacteria were taken from the corresponding LB plate and incubated in 2 ml YT medium set up with an appropriate antibiotic at least for 12 hours, usually overnight, at 37°C in a shaker. When a DNA preparation was set up of existing glycerol stocks, a sample of the stock was directly transferred from the frozen stock into the medium. The next day 1.5 ml of the culture were passed to a reaction tube and the bacteria pelleted by centrifugation at 13,000 g for 1 min. The remaining 0.5 ml were stored at 4°C for further usage, e.g. to set up midipreparations. The supernatant was discarded, the pellet resuspended in 0.2 ml ice-cold buffer P1 and the mixture incubated on ice for 5 min. Afterwards, 0.4 ml of buffer P2 were added. The samples were mixed and again incubated on ice for 5 min. 0.3 ml ice-cold buffer P3 were added and, after gently mixing the samples, incubated on ice for 5 min before pelleting the cell debris and genomic DNA by centrifugation at 13,000 g for 5 min. The supernatant, containing the plasmid DNA, was transferred to a new reaction tube and the plasmid DNA was precipitated by adding 0.7 ml isopropanol and incubating the mixture for 5 min at room temperature. After centrifugation at 16,000 g for 5 min, the supernatant was removed, the pellet washed by adding 1 ml 70% ethanol and again centrifuged at 16,000 g for 5 min. The
3. Methods

supernatant was discarded, the pelleted DNA briefly dried and resuspended in 30 µl TE buffer. After determining the concentration, roughly 1 µg DNA was used for an analytical restriction digest to identify potentially positive clones (see 3.1.5).

Table 3.6.: Contents of the minipreparation buffers and the TE buffer

<table>
<thead>
<tr>
<th>Buffer P1</th>
<th>Buffer P2</th>
<th>Buffer P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris-HCl pH 8.0</td>
<td>200 mM NaOH</td>
<td>3 M Potassium acetate</td>
</tr>
<tr>
<td>20 mM Glucose</td>
<td>1 % (w/v) SDS</td>
<td>2 M Acetic acid</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To obtain larger amounts (100 – 200 µg) DNA midipreparations with 50 ml bacteria-containing medium were used. To this end, appropriate antibiotics and 10 µl culture of a positive clone retrieved from a minipreparation were added to 50 ml LB medium. After incubation at 37 °C in a shaker for at least 12 h, usually over night, the bacteria were pelleted by centrifugation at 4 °C for 15 min. The DNA was isolated using the JetStar 2.0 Plasmid kit (Genomed) which takes advantage of anion exchange columns, where plasmid DNA can be bound under low salt conditions and, after removing the cell debris, can be eluted under high salt conditions in, e.g. TE buffer. The DNA extractions were performed according to the manual provided with the kit. Briefly, the pelleted bacteria were resuspended in 4 ml buffer E1, and after adding 4 ml buffer E2 and mixing by gentle inversion the mixture was incubated at room temperature for 5 min. Then, 4 ml neutralisation buffer E3 were added and the samples were gently mixed by inversion until the mixture was homogeneous. The samples were centrifuged at 16,000 g for 10 min at room temperature to pellet cell debris and genomic DNA. Lipids, possibly forming a thin layer at the top of the mixture, could easily be removed with a pipette tip. The supernatant was added to columns (previously equilibrated with 10 ml E4) and drained by gravity. Then, the columns were washed twice with 10 ml wash buffer E5 by gravity flow.

DNA was eluted in a sterile 15 ml tube by adding 5 ml elution buffer E6 to the column. To precipitate the DNA, 3.5 ml isopropanol were added, the tube mixed and centrifuged at 15,000 g, 4 °C for 30 min. The supernatant was removed and the pelleted DNA washed with 1 ml 70 % ethanol before centrifugation as above for 15 min. The supernatant was discarded and the pelleted DNA, after brief vacuum-drying, dissolved in 70 µl TE buffer. Afterwards, the concentration was determined photometrically, the correctness of the plasmid checked by an analytical restriction (see 3.1.5) and, if the restriction digest was positive, the construct was sequenced (see 3.1.11).
3.1.11. Sequencing

The correctness of cloned constructs and mutations was verified by sequencing. The sequencing was performed by chain-termination PCR using standard primers but a nucleotide mixture that contains standard bases and also four dideoxynucleoside triphosphates (ddNTPs), each connected to a different fluorophor. When one of this ddNTPs is incorporated into the newly synthesized DNA, the chain elongation stops. This stochastic incorporation therefore produces all possible termination fragments with different lengths, that can later be separated by electrophoresis with simultaneous recording of the fluorophors. This allows the reconstruction of the original DNA sequence. All sequencing was performed by Annette Tolle, Sabine Laerbusch or Björn Peters on the Applied Biosystems 3130 x1 Genetic Analyzer owned by the departments of Biochemistry I and II at the Ruhr University Bochum.

3.1.12. In vitro Transcription

With an in vitro transcription, RNA is synthesized from a DNA template in vitro by RNA polymerases. RNA polymerases, e.g. from T7 or SP6 bacteriophages, bind to specific promoter sequences on a DNA template. To avoid concatemeric RNA constructs, the DNA has to be linearized prior to its usage in an in vitro transcription (see 3.1.5).

The in vitro transcription reactions were performed with the mMESSAGE mMACHINE T7 Kit (Ambion, now Life Technologies, Darmstadt). A typical transcription reaction is depicted below. The NTP/Cap mix contains the NTPs for the transcription reaction and forms a 5’-cap structure that increases the stability of the RNA against RNases. After assembling the reaction, the samples were incubated at 37°C for 2h. The reaction was then stopped by adding 1 µl DNase (provided with the kit) to the reaction and further incubation at 37°C for 15 min. The RNA was then purified as described in 3.1.13.

3.1.13. RNA Purification

The RNA generated in the in vitro transcription (see 3.1.12) was purified with the RNA Clean & Concentrator Kit (Zymo Research). The purification was done according to the manual provided with the kit. Briefly, each sample of RNA solution obtained from the in vitro transcription was filled up to a total volume of 50 µl with DEPC-treated water.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearized DNA template</td>
<td>x µl (~ 1 µg)</td>
<td></td>
</tr>
<tr>
<td>2× Nucleotide/Cap Mix</td>
<td>2×</td>
<td>10 µl</td>
</tr>
<tr>
<td>10× Reaction buffer</td>
<td>10×</td>
<td>2 µl</td>
</tr>
<tr>
<td>T7 Enzyme mix</td>
<td></td>
<td>2 µl</td>
</tr>
<tr>
<td>DEPC water</td>
<td></td>
<td>ad 20 µl</td>
</tr>
</tbody>
</table>
3. Methods

Afterwards, 100 µl RNA binding buffer and 150 µl ethanol were added. The mixture was transferred into a spin column, the column placed in an empty receiver tube and centrifuged for 1 min at 12,000 g. The flow-through was discarded, 400 µl RNA preparation buffer were added and centrifuged at 12,000 g for 30 s. After discarding the flow-through, two subsequent wash steps with 800 µl and 400 µl RNA wash buffer were performed, each followed by a centrifugation at 12,000 g for 30 s. The flow-through was discarded and the column was centrifuged for 2 min at 12,000 g in an emptied receiver tube to remove residual ethanol. Afterwards, the spin column was placed in an RNase-free reaction tube and the RNA was eluted with 25 µl DEPC-treated water by centrifugation for 30 s at 10,000 g.

The obtained RNA was analyzed by agarose gel electrophoresis (see 3.1.3), the concentration determined photometrically and subsequently adjusted to 0.2 or 0.4 µg/µl. The RNA was then stored at -80°C or directly used.

3.2. Confocal Microscopy

A major disadvantage of conventional microscopy is the fact that, besides the image of the focal plane, also the areas in front and behind of the focal plane disturb the view. The confocal microscopy is a way to circumvent this problem: areas outside the focal plane are not pictured. Via a lens the sample is illuminated by a laser and the light is, through a beam splitter and a pinhole, guided to the detector. By adjusting the pinhole to the sample it is then possible to picture only the light from the focal plane. As only point signals are detected the sample is then scanned in the x and y axis to generate a complete picture. Moving the sample in the z axis permits the creation of 3D images.

To visualize samples with the confocal microscopy, the samples have to reflect light or to fluoresce. This can be done, for example for cell compartments, with different dyes or expression of fluorescent proteins or, for single proteins, by tagging them with fluorescent proteins. It is also possible to use antibodies coupled to a fluorescent dye.

For fusion proteins the use of green fluorescent protein (GFP) is most common: GFP is a fluorescent protein from the jellyfish *Aequorea victoria*, consisting of 238 amino acids that form a barrel-like structure of β-sheets surrounding an α-helix. This helix contains a tripeptide of serine, tyrosine and glycine that forms the chromophore. Whereas in the jellyfish the energy for the fluorescence is transferred from the Ca²⁺-binding protein aequorin, GFP can also be excited directly by light as it contains two absorption maxima, one at 395 and one at 470 nm. The resulting light emission has a maximum at 509 nm. By introducing point mutations it was possible to alter the properties of GFP regarding quantum efficiency and fluorescence, e.g. the “enhanced” eGFP. Also, variations fluorescing at different wavelengths were generated, e.g. enhanced yellow fluorescent protein (eYFP) or enhanced cyan fluorescent protein (eCFP).

Within this thesis, fusion proteins of the different receptor subunits with eGFP were generated.
3.3. Electrophysiology

3.3.1. The *Xenopus laevis* Oocyte Expression System

The oocytes of the South African clawed frog *Xenopus laevis* contain all components important for protein biosynthesis. As the oocytes contain endogenous RNA, RNases are effectively downregulated, therefore injected exogenous RNA will not be degraded. Proteins are posttranslationally processed and transported to their final destination. As these features also come with a relatively large size (around 1 mm), which makes the handling of the cells quite easy both when injecting RNA and performing electrophysiological measurements, the *X. laevis* oocyte expression system is a long-established system to characterize ion channels.

However, there are also disadvantages. The size of the oocytes makes it impossible to measure fast kinetics, e.g. current deactivation or current desensitization, as an applied agonist never reaches all receptors at the same time. The measured current always represents the steady state current of all non-desensitized receptors. Another problem are endogenous ion channels. Most importantly, the oocytes express an endogenous GluN2 subunit, *Xen*GluN2B (Schmidt and Hollmann, 2008). The expression level of this subunit has to be checked via single expression of a GluN1 subunit that can form a functional heteromeric ion channel with *Xen*GluN2B. This is especially important when very small current responses have to be measured as was often the case in this study (see results, chapter 4.1.1). Oocyte batches showing too large background currents had to be discarded, because an interpretation is not possible, e.g. when the current responses of the subunit combination is in the range of the background. Endogenous Ca$^{2+}$ channels, which reveal themselves through short peak currents at the beginning of a measurement, can be handled via calcium-free Ringer’s solutions. For example calcium can be substituted with barium.

3.3.2. *X. laevis* Oocyte Preparation

The surgery of *X. laevis* frogs was performed by Björn “Earnie” Peters and Stephanie Nolte, as was the maintenance of the frog facility. Briefly, a female *X. laevis* was anesthetized with tricaine and placed on ice. Through a small cut in the abdominal wall parts of the ovary were removed, cut into little pieces and placed in Barth’s solution without Ca$^{2+}$. The cut was closed, sewed and disinfected. The parts of the ovary were subsequently digested with collagenase (type 2) in Barth’s medium without Ca$^{2+}$ at 18°C for 1 – 2 h to remove the follicle cells and extracellular matrix and liberate the oocytes. The treatment was stopped by washing the solution with increasing concentrations of Ca$^{2+}$-containing Barth’s medium and the supernatant was removed. Oocytes of stage V or VI (Dascal, 1987; Dumont, 1972) were selected for the injection of RNA (see 3.1.12) at the same or, at the latest, the following day. The oocytes were incubated at 17°C.

3.3.3. RNA Injection into Oocytes

RNA was injected into previously selected oocytes (see 3.3.2) via small glass capillaries filled with non-toxic mineral oil. These glass capillaries were prepared with an electrode/pipette
3. Methods

### Table 3.8: Barth’s solution

<table>
<thead>
<tr>
<th>with Ca(^{2+})</th>
<th>without Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>88 mM NaCl</td>
<td>88 mM NaCl</td>
</tr>
<tr>
<td>1.1 mM KCl</td>
<td>1.1 mM KCl</td>
</tr>
<tr>
<td>2.4 mM NaHCO(_3)</td>
<td>2.4 mM NaHCO(_3)</td>
</tr>
<tr>
<td>0.8 mM MgSO(_4)</td>
<td>0.8 mM MgSO(_4)</td>
</tr>
<tr>
<td>15 mM HEPES-NaOH pH 7.6</td>
<td>15 mM HEPES-NaOH pH 7.6</td>
</tr>
<tr>
<td>63 µg/ml Penicillin G sodium salt</td>
<td>63 µg/ml Penicillin G sodium salt</td>
</tr>
<tr>
<td>40 µg/ml Streptomycin sulfate</td>
<td>40 µg/ml Streptomycin sulfate</td>
</tr>
<tr>
<td>0.3 mM Ca(NO(_3))(_2)</td>
<td>0.3 mM Ca(NO(_3))(_2)</td>
</tr>
<tr>
<td>0.4 mM CaCl(_2)</td>
<td>0.4 mM CaCl(_2)</td>
</tr>
<tr>
<td>100 µg/ml Gentamicin</td>
<td>100 µg/ml Gentamicin</td>
</tr>
</tbody>
</table>

puller and afterwards broken back until the inner diameter of the tip was ca. 20 µm. One capillary was then mounted on a micro-injector.

The RNA was thawed at 4 °C on ice and briefly spun down in a microcentrifuge. The desired amount of RNA was pipetted into the lid of a sterile reaction tube. Usually, 8 fmol RNA per subunit were injected, 16 fmol for coexpression of two receptor subunits.

#### 3.3.4. Two-Electrode Voltage Clamp Recording

The two-electrode voltage clamp (TEVC) system allows the recording of an ion flux across a membrane. One electrode, the potential electrode, constantly measures the intracellular potential compared to a reference electrode in the bath solution. This value (typically -70 mV) can be set by the operator. To “clamp” this value, a current is applied via the current electrode until the resting potential is reached. When ions are flowing through ion channels in the membrane, this change in charge leads to a compensating current to keep the resting potential constant. This applied current has the same amplitude as the current flowing through the ion channels but the opposite direction and is the actual measured signal. This signal, via an analog/digital (A/D) converter, is put out to a computer with the Pulse recording software.

Oocytes were placed in a recording chamber with an influx hole for the recording solutions and an outflux drain connected to a vacuum pump. The flow of the agonist solutions is regulated by an 8-way Hamilton valve connected to 50 µl syringes. For recordings, an oocyte was placed in the recording chamber filled with normal frog Ringer’s (NFR) solution. Glass capillaries were pulled similarly to the injection capillaries without braking them back and had a tip opening of 1 – 2 µm. They were filled with 3 M KCl and mounted on silver wire electrodes with an Ag/AgCl coating. The potential and the current electrode were immersed in the NFR solution and capillary resistances were checked. Capillaries with a resistance above or below 0.2 – 1.5 MΩ were replaced. The offset of both electrodes was set to zero and the potential electrode inserted into the oocyte. When a stable resting potential was reached, the current electrode was inserted into the oocyte, the NFR flow was started.
and the recording mode switched to voltage clamp. When the leak current of the oocyte had reached a stable level, the recording could be started. In the first 10 s of every measurement only NFR was applied to have a baseline for data analysis. After this, NFR supplied with the desired agonist was applied by switching the valve to the respective syringe until a stable steady state was achieved. Subsequently, the agonist solution was washed out by switching back to NFR. The recording was then analyzed with the Pulse software.

**Table 3.9.:** Components of NFR. For experiments with altered pH values (see 4.4.3), the pH was adjusted accordingly to pH 6.8 with NaOH.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>115 mM</td>
<td>NaCl</td>
<td>2.5 mM</td>
<td>KCl</td>
</tr>
<tr>
<td>1.8 mM</td>
<td>CaCl₃</td>
<td>10 mM</td>
<td>HEPES</td>
</tr>
<tr>
<td></td>
<td>pH 7.2</td>
<td></td>
<td>with NaOH</td>
</tr>
</tbody>
</table>
4. Results

The heavy metal zinc is a long-known inhibitor of the conventional, GluN1- and GluN2-containing NMDA receptor, but little is known about its effect on the unconventional, GluN1/GluN3-containing excitatory glycine receptors. First light was shed on these combinations with interesting results, as it was found that zinc could potentiate, instead of block, the glycine-mediated currents of GluN3A-containing receptors. Zinc also showed interesting additional effects, namely a “supralinear potentiation”, when coapplied with MDL-29951 (Madry et al., 2007b). This is especially interesting as MDL-29951 was originally found to be an antagonist of conventional, GluN1- and GluN2-containing NMDA receptors, binding to the GluN1 glycine binding site. Originally, it was developed as an anticonvulsant (Baron et al., 1992; Salituro et al., 1990).

However, until now it remains elusive what – if any – effects zinc has on the second possible combination of an unconventional excitatory glycine receptor, combining GluN1 and GluN3B-subunits. GluN3B is of special interest, not only, but also due to its expression increasing first post-natally at a time when the expression of GluN3A decreases. Also, the GluN3-containing excitatory glycine receptors have been proven to exist in vivo in optic nerve myelin (Piña-Crespo et al., 2010).

The goal of this thesis therefore was to explore the characteristics of diheteromeric receptors containing GluN1 and GluN3B regarding the influence of zinc. Therefore, in the first project the focus was laid on the effects of zinc and glycine alone on GluN3B-containing receptors. The second objective was the potential effect the NMDA receptor blocker MDL-29951 on GluN3B-containing NMDA receptors and whether the “supralinear potentiation” seen in GluN3A-containing receptors also shows up in the GluN3B-containing variant. In a third step, the underlying mechanisms were investigated. Last but not least, the membrane expression of all receptor constructs generated in the context of this project was verified, showing that eGFP-tagged receptor subunits are a proper tool to study NMDA receptor expression.

Also, to take a glance at the comparability of GluN3A and GluN3B-containing receptors, GluN3A-containing receptors were tested at exactly the same conditions as the GluN3B-containing receptors concerning the concentrations of zinc and MDL-29951, alone or in combination. A quick look was taken at the pH sensitivity of GluN3A-containing receptors, as a side project.
4. Results

4.1. Activation of GluN3B-Containing Receptors by Zinc and Glycine

To test for the interactions of zinc when coapplied with glycine, first of all a set of experiments was carried out in order to nail down the exact effects of zinc and glycine alone. Though the concentration-dependent effects of glycine on GluN3B-containing NMDA receptors, resulting in a bell-shaped activation curve, had previously been described (Awobuluyi et al., 2007; Madry et al., 2007a), it is especially important to check the background expression of endogenous Xen GluN2B that can assemble with GluN1 subunits, giving rise to a functional conventional NMDA receptor that might interfere with any measurements. Additionally, the behavior of receptors containing GluN3B and a GluN1-b subunit including the exon 5, as opposed to GluN1-a that lacks exon 5, remains elusive. As the exon 5 has modulatory potential, e.g. for the pH dependency and zinc block in conventional receptors, an alternative concentration-dependent behavior for GluN1-b-containing receptors regarding the glycine concentration-dependent activation might be expected. However, as the focus of the previously published research on GluN1/GluN3A-containing NMDA receptors was relatively unspecific and covered only some specific concentrations (Madry et al., 2008), a completely new, very systematic approach was chosen, screening many possible combinations of glycine and zinc to reveal where interesting interactions may be located. For expression in Xenopus oocytes receptors containing GluN1-3a or GluN1-3b together with GluN3B, each subcloned into the vector pSGEM cRNAs of the two subunits were injected in a molar ratio of 1:1 GluN1 to GluN3. Measurements were conducted via the two-electrode voltage clamp (TEVC) method. All recorded current responses represent the steady state currents.

4.1.1. Glycine-Mediated Effects

Figure 4.1 summarizes the results of the application of 1 – 10,000 µM (10 mM) glycine to receptors containing either GluN1-3a and GluN3B or GluN1-3b and GluN3B. Both combinations show a robust current response already at 1 µM glycine. For GluN1-3a-containing receptors, the current response at 1 µM glycine was 40.5 ± 2.5 nA. Increasing the glycine concentration to 10 µM led to 961 ± 48 nA, a 24-fold increased current response and the highest observable current in this experiment. Further increasing the glycine concentration to 100 µM led to a current response of 186 ± 14 nA, comprising a 4.6-fold increase. Current responses elicited by 1,000 µM (68.0 ± 7.4 nA) and 10,000 µM (50.7 ± 8.2 nA) glycine showed only a marginal increase in agonist-induced currents comparable to that elicited by 1 µM glycine (1.6-fold for 1,000 µM and 1.2-fold for 10,000 µM glycine). This bell-shaped response towards agonist application is a very uncommon feature, as usually ionotropic receptors reach a plateau response at a saturating concentration, where the current amplitude is not further altered. However, this decrease seen in GluN1/GluN3-containing NMDA receptors is believed to be an effect of the desensitization of the receptor complex by the GluN1 subunit (Awobuluyi et al., 2007; Madry et al., 2007a; Kvist et al., 2013).
4.1. Activation of GluN3B-Containing Receptors by Zinc and Glycine

Receptors containing GluN1-3b and GluN3B showed a very similar picture regarding the current pattern. The current response for 10 µM glycine gave maximal currents and, at 1787 ± 297 nA was 31-fold higher than the current response yielded by 1 µM glycine (56.6 ± 8.8 nA). 100 µM glycine (370 ± 96 nA) still showed a 6.5-fold increase in current compared to 1 µM glycine, but already had a decreased current response compared to that yielded by 10 µM glycine. 1,000 µM (162 ± 33 nA) and 10,000 µM (136 ± 32 nA) glycine led to a 2.8-fold (1,000 µM) and 2.4-fold (10,000 µM glycine) higher current response compared to the application of 1 µM glycine. Again, the same bell-shaped dose-response behavior was observed instead of reaching a plateau. As for the GluN1-3a-containing receptors, this effect can be attributed to the desensitizing effect of glycine on the GluN1 subunit.

Note that the here depicted current amplitudes for GluN1-3a/GluN3B- and GluN1-3b/GluN3B-containing receptors are not directly comparable as they originate from different batches of oocytes.

The endogenously expressed Xen GluN2B (Schmidt and Hollmann, 2008), which can form a functional receptor together with single GluN1 subunits, is an important issue. It can lead to a significant background current, therefore additional experiments were carried out in order to take this into account. Oocytes that were injected only with cRNA of the corresponding GluN1-3 subunit were measured with glycine concentrations ranging from 1 – 10,000 µM (10 mM). Any effect mediated by this “hybrid” receptor under those conditions would than reveal itself by showing a current response that could be taken into account. These control experiments were conducted in the same batch of oocytes, so that

Figure 4.1.: The concentration-dependent effect of glycine on GluN3B-containing receptors. Diagrams A and B: Current responses in nA elicited by 1 – 10,000 µM glycine (logarithmic scale) on receptors containing either GluN1-3a and GluN3B (A) or GluN1-3b and GluN3B (B). Data shown here represent the mean values ± SEM, n = 5 – 10. C and D: Representative glycine-induced current responses, equivalent to the glycine concentration depicted above in A or, respectively, B. Red bars indicate the period of agonist application, blue bars indicate the scales of the current traces.
4. Results

Figure 4.2.: Direct comparison of glycine-induced currents in oocytes injected with only a GluN1-3 subunit (-) or GluN1-3 together with GluN3B (+). Green bars show the current responses in nA (mean values ± SEM) of the depicted (1 – 10,000 µM) glycine concentrations, grey bars the current response on oocytes injected only with GluN1-3a in (A) or in GluN1-3b (B). For the sake of simplicity the scales of current response were fit to the maximal current response of the respective agonist concentration. A: Oocytes injected with GluN1-3a alone (-) or together with (+) GluN3B in the same experiment as shown in 4.1. 1 µM glycine showed no current response on the individually expressed GluN1-3a subunit. (** = p < 0.005; nd = not determined; n = 5 – 10). B: Oocytes injected with GluN1-3b alone (-) or together with (+) GluN3B in the same experiment as shown in 4.1. Here, neither 1 nor 10 µM glycine could elicit a detectable current response. (** = p < 0.01; nd = not determined; n = 5).

the data shown for GluN1-3a coexpressed with GluN3B or GluN1-3a expressed alone (see above and Figure 4.1) allow a direct comparison. Alike, data shown for GluN1-3b expressed alone or together with GluN3B were gained from one batch. The results are shown in Figure 4.2, where each agonist concentration is directly compared between oocytes injected with GluN1-3a or GluN1-3b alone (-) or together with GluN3B (+).
4.1. Activation of GluN3B-Containing Receptors by Zinc and Glycine

Referring to the individual expression of GluN1-3a, 1 µM glycine was not able to elicit any current response at all. At a glycine concentration of 10 µM a first response was visible, amounting to only 1.1 ± 0.04 nA. The current response received through application of 100 µM glycine, 2.2 ± 0.1 nA was only slightly increased. Upon application of 1,000 µM glycine a current response of 2.8 ± 48.0 nA was seen, which is a small, but further increase. 10,000 µM glycine, with a current response of 3.3 ± 0.1 nA, yielded the highest current response regarding this control. Individually expressed GluN1-3a, for all measured glycine concentrations, compared to receptors combining both GluN1-3a and GluN3B, showed a highly statistically significant (p < 0.005; Student’s t-test) difference.

Oocytes expressing only GluN1-3b do respond to neither 1 µM nor 10 µM glycine. Upon the application of 100 µM glycine a small current response of 1.7 ± 0.09 nA was visible. Increasing the glycine concentration to 1,000 µM also increased the current response to 3.1 ± 0.32 nA. The highest used concentration of glycine, 10,000 µM, was able to elicit a current of 9.6 ± 0.61 nA, which again was an increase. Compared to oocytes expressing both GluN1-3b and GluN3B these marginal current responses are always significantly smaller (p < 0.01; Student’s t-test).

These results show unequivocally that the Xen GluN2B-mediated background is no major problem in these experiments. In general, for all experiments the Xen GluN2B-mediated background was controlled in this way by injecting the individual GluN1-3 subunit and recording its current responses to exclude false positives within the results.

Summary: Both the GluN1-3a/GluN3B- and GluN1-3b/GluN3B-containing receptors showed a biphasic concentration-dependent pattern upon glycine-mediated activation. The maximal current was induced by 10 µM glycine. Oocytes injected only with the GluN1-3a or GluN1-3b subunits were used to check background expression of Xen GluN2B, which was negligible.

4.1.2. Zinc-Mediated Effects

In previous studies it had been shown that zinc alone, without any other agonist, had agonistic effects on GluN1/GluN3A-containing excitatory glycine receptors (Madry et al., 2007b). Again, there is no evidence in the literature on the effects of zinc alone on GluN3B-containing excitatory glycine receptors. To see whether the described zinc effect occurs also in GluN3B-containing receptors, 1 – 10,000 µM (10 mM) zinc, just as for the concentrations of glycine, were used to search for possible effects of zinc applied alone.

Both 1 µM and 10 µM zinc showed no effect on receptors combining GluN1-3a and GluN3B. Whereas upon the application of 100 µM zinc a robust current response of 21.1 ± 2.6 nA could be seen, an increase of the zinc concentration to 1,000 µM zinc led only to a lesser current response with a mean amplitude of 7.5 ± 2.0 nA. The effects of 10,000 µM zinc were not determinable as the oocytes under those conditions became unstable and showed a strong rundown upon agonist application.

For receptors containing the GluN1-3b variant, the application of neither 1, 10 nor 100 µM zinc yielded any detectable current response. Applying 1,000 µM zinc led to an overall current response of 2.0 ± 0.2 nA. Again, the effect of 10,000 µM zinc was not determinable.
4. Results

Figure 4.3.: Zinc-mediated effects when zinc was used as the sole agonist on GluN3B-containing receptors. Diagrams A and B: Current responses in nA elicited by 1 – 10,000 µM zinc (logarithmic scale) on receptors containing either GluN1-3a and GluN3B (A) or GluN1-3b and GluN3B (B). Data shown here represent the mean values ± SEM in nA, nd = not determined, n = 7 – 13. C and D: Representative zinc-induced current responses, equivalent to the glycine concentration depicted above. Red bars indicate the period of agonist application, blue bars the scale of the elicited current responses.

Summary: GluN1-3a/GluN3B-containing receptors were activated by zinc at 100 and 1000 µM. GluN1-3b/GluN3B-containing receptors showed virtually no current responses.

4.1.3. Buffering the Zinc Concentration with Tricine

Contaminating zinc in buffer solutions and labware is an important issue, possibly falsifying any results that involve zinc. This was indicated by the observation that agonist-induced currents of conventional NMDARs, composed of GluN1-a or GluN1-b and GluN2A which can be blocked by zinc, could be potentiated by using a zinc chelator in the recording solutions (Paoletti et al., 1997). Additionally, there are reports suggesting that zinc can reach levels up to 8 nM, sufficient to also influence the classical, inhibitory GlyR (Cornelison and Mihic, 2014; Harvey et al., 1999; Suwa et al., 2001). In conclusion, the existence and – if any – effect of contaminating zinc in the labware and buffer solutions had to be examined.

Tricine, a zinc-chelating buffer reagent, was used to determine these possible effects of zinc. Therefore, tricine was added in a concentration of 10 mM to the NFR that was later on used to prepare agonist solutions and directly compared to NFR and agonist solutions without tricine. For this purpose, a receptor composed of GluN1-3a and GluN3B and both a saturating concentration of glycine (10 µM) and a non-saturating concentration (1 µM) were used. This combination was chosen as a concentration of 1 µM zinc was already sufficient to significantly reduce the glycine-mediated current response in this receptor combination (see chapter 4.1.4). Hence, any effect of chelating contaminating zinc traces...
4.1. Activation of GluN3B-Containing Receptors by Zinc and Glycine

4.1.4. Interplay between Glycine and Zinc

The influence of glycine and zinc – and their potential interplay – on excitatory glycine receptors containing GluN3B was investigated by coexpressing the respective subunits, GluN1-3a or GluN1-3b together with GluN3B. To screen for effects occurring when combining both zinc and glycine, various combinations of concentrations of glycine together with zinc were measured and normalized to the current response given by glycine alone. The concentration of zinc was chosen to be still within the physiological range, which meant it to be maximally 100 µM (Vogt et al., 2000; Qian and Noebels, 2005). However, after the first experiments had been done it was eventually adjusted to even higher concentrations. The results are depicted in Figure 4.5 and Table 4.1.
4. Results

Figure 4.5.: Influence of zinc on glycine-induced currents. A: GluN1-3a + GluN3B; B: GluN1-3b + GluN3B. Indicated concentrations of glycine (µM, y-axis) were combined individually with several concentrations of zinc (µM, x-axis). Black bars represent the normalized current response in % (mean values ± SEM) seen upon the application of glycine alone (= 100%). Red bars indicate a statistically significant block, green bars a significant potentiation of glycine-induced currents by zinc (at least p < 0.05, Dunnett’s one-way ANOVA), whereas grey bars display combinations where no statistically significant effect was seen. All zinc effects are calculated relative to the application of glycine alone. Thin black lines protruding from the top of the bars represent the SEM, n = 6 – 36.

At different glycine concentrations, the addition of zinc to receptors containing GluN1-3a or GluN1-3b and GluN3B led to a varying pattern of responses, shown in Figure 4.5 and Table 4.1. The overall picture shows a kind of “tent”-like structure. Both subunit combinations show a “summit” where a certain zinc concentration powerfully potentiates the current response mediated by glycine. The “edges”, areas where zinc does not or only minimally alter the glycine-induced currents, are in direct proximity to the concentration where zinc can positively modulate. The “rim” is then formed by concentrations where zinc blocks the currents induced by glycine, or where the glycine concentration is too low to elicit any current response. The maximum differs only slightly between GluN1-3a- and GluN1-3b-containing receptors. For GluN1-3a-containing combinations, at 1 µM glycine 100 µM zinc show the highest potentiation, whereas for GluN1-3b-containing receptors the maximum is also at 1 µM glycine, but seen upon the coapplication of 300 µM zinc.

The first subunit combination investigated was GluN1-3a and GluN3B. Whereas the addition of zinc to 1 µM glycine significantly reduced the glycine-induced current response to 43.9 ± 7.6% (1 µM zinc) and 53.1 ± 8.0% (10 µM zinc), the addition of 30 µM zinc already showed a different picture: it potentiated the glycine-induced current slightly, about 1.6-fold to 163 ± 19.3 %, yet not significantly (Dunnett’s one-way ANOVA). 100 µM zinc potentiated the current response about 4-fold (419 ± 50.0 %). The addition of 300 µM zinc potentiated the currents induced by 1 µM glycine by a lower degree with a factor of roughly 3-fold (329 ± 46.4 %). When zinc was coapplied to 3 µM glycine, the current responses fluctuated...
4.1. Activation of GluN3B-Containing Receptors by Zinc and Glycine

Table 4.1.: Matrix displaying all actual currents (in %, ± SEM) recorded for the different combinations of concentrations of glycine and zinc as depicted in Figure 4.5. The upper table shows the values for GluN1-3a and GluN3B, the lower table the values measured for GluN1-3b and GluN3B. Dark grey areas depict relative responses measured without the presence of zinc (= control values). All effects seen upon the addition of zinc were calculated referring to this level (100%). Light grey areas indicate values that did not significantly differ from the reference without zinc, whereas red areas show a significant block and green areas represent a significant potentiation by the addition of zinc (at least \( p < 0.05 \), Dunnett’s one-way ANOVA). For combinations indicated with “–” no current response was detectable, n.d. = not determined.

<table>
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<th>GluN1-3a + GluN3B</th>
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<th>10</th>
<th>3</th>
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<th>0.3</th>
</tr>
</thead>
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<td>µM Zinc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100 ± 5.0</td>
<td>100 ± 5.5</td>
<td>100 ± 7.4</td>
<td>100 ± 7.6</td>
<td>100 ± 9.8</td>
</tr>
<tr>
<td>1</td>
<td>70.0 ± 7.6</td>
<td>67.4 ± 6.9</td>
<td>90.4 ± 8.7</td>
<td>43.9 ± 7.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>60.9 ± 6.1</td>
<td>45.4 ± 5.0</td>
<td>91.4 ± 12.9</td>
<td>53.1 ± 8.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>30</td>
<td>73.9 ± 3.6</td>
<td>46.8 ± 4.5</td>
<td>93.8 ± 13.4</td>
<td>163 ± 19.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>100</td>
<td>67.9 ± 6.3</td>
<td>23.5 ± 2.0</td>
<td>115 ± 12.4</td>
<td>419 ± 50.0</td>
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<tr>
<td>300</td>
<td>53.9 ± 6.7</td>
<td>27.9 ± 3.4</td>
<td>84.8 ± 15.0</td>
<td>329 ± 46.4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<table>
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<th>10</th>
<th>3</th>
<th>1</th>
<th>0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM Zinc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100 ± 5.5</td>
<td>100 ± 6.2</td>
<td>100 ± 7.3</td>
<td>100 ± 6.3</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>94.6 ± 6.2</td>
<td>72.1 ± 8.1</td>
<td>79.0 ± 12.7</td>
<td>88.7 ± 15.5</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>67.1 ± 7.6</td>
<td>58.9 ± 4.5</td>
<td>75.2 ± 13.9</td>
<td>93.8 ± 12.4</td>
<td>–</td>
</tr>
<tr>
<td>30</td>
<td>63.4 ± 2.2</td>
<td>40.7 ± 1.5</td>
<td>86.8 ± 16.5</td>
<td>137 ± 11.7</td>
<td>–</td>
</tr>
<tr>
<td>100</td>
<td>46.5 ± 3.7</td>
<td>26.5 ± 1.8</td>
<td>122 ± 11.1</td>
<td>343 ± 39.2</td>
<td>–</td>
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<tr>
<td>300</td>
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<td>24.5 ± 1.3</td>
<td>106 ± 20.4</td>
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<td>–</td>
</tr>
<tr>
<td>1000</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>250 ± 32.8</td>
<td>–</td>
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</tbody>
</table>

around the values measured for glycine without the addition of zinc. 1 µM (90.4 ± 8.7%), 10 µM (91.4 ± 12.9%) and 30 µM (93.8 ± 13.4%) showed a very slight, yet not significant reduction of the glycine-induced current response. The addition of 100 µM zinc led to a slight increase of the current response (115 ± 12.4%), and the addition of 300 µM zinc again to a slight reduction (84.8 ± 15.0%), both effects not being significant. At 10 µM glycine, the coapplication of zinc led to a significant block at all used zinc concentrations: 1 µM zinc block the glycine-induced current response to 67.4 ± 6.9%, 10 µM further down to 45.4 ± 5.0% and 30 µM down to 46.8 ± 4.5%. 100 µM (23.5 ± 2.0%) and 300 µM (27.9 ± 3.4%) also showed a block of the glycine-induced currents. The same pattern was seen with 100 µM glycine: the coapplication of 1 µM zinc led to a reduction down to 70.0 ± 7.6%, with 10 µM zinc to 60.9 ± 6.1% and with 30 µM zinc to 73.9 ± 3.6%. Again, 100 µM (67.9 ± 6.3%) and 300 µM (53.9 ± 6.7%) zinc also showed a block. For 0.3 µM glycine only very small current responses could be measured, and the addition of zinc lead to no current response at all.

A similar pattern can be seen for GluN1-3b- and GluN3B-containing receptors. The addition of 1 µM or 10 µM zinc to 1 µM glycine had no statistically significant effect on the glycine-induced current response (88.7 ± 15.5% when adding 1 µM zinc and respectively 93.8 ± 12.4% with 10 µM zinc). The addition of 30 µM zinc was slightly, but not
4. Results

significantly, increasing the glycine-induced current response to $137 \pm 11.7\%$. Nevertheless, as well as with the GluN1-3a-containing receptor, the addition of $100 \mu M$ zinc led to a quite pronounced increase of the current induced by $1 \mu M$ glycine, by factors of 3.4-fold to $343 \pm 39.2\%$. Further increasing the zinc concentration to $300 \mu M$ also increased the potentiation by a factor of 6 to the maximal value of $616 \pm 69.5\%$. $1,000 \mu M$ (1 mM) zinc then showed a reduced potentiation by a factor of 2.5 ($250 \pm 32.8\%$). When zinc was coapplied with $3 \mu M$ glycine, none of the effects were statistically significant. For $1 \mu M$ ($79.0 \pm 12.7\%$), $10 \mu M$ ($75.2 \pm 13.9\%$), and $30 \mu M$ ($86.8 \pm 16.5\%$) zinc, a small decrease of the glycine-induced current response was observed. The addition of $100 \mu M$ ($122 \pm 11.1\%$) and $300 \mu M$ ($106 \pm 20.4\%$) zinc led to a slight increase of current responses. For all combinations of zinc combined with $10 \mu M$ glycine, a block was detected. The addition of $1 \mu M$ zinc reduced the current response to $72.1 \pm 8.1\%$. Adding $10 \mu M$ zinc led to a reduction down to $58.9 \pm 4.5\%$, adding $30 \mu M$ decreased it to $40.7 \pm 1.5\%$, and $100 \mu M$ zinc further reduced it to $26.5 \pm 1.8\%$. $300 \mu M$ zinc then reduced the current response induced by $10 \mu M$ glycine to $24 \pm 1.3\%$. For the GluN1-3b and GluN3B-containing combination, the addition of $1 \mu M$, $10 \mu M$, $30 \mu M$, $100 \mu M$, or $300 \mu M$ zinc to $100 \mu M$ glycine led to a reduction of current responses to $94.6 \pm 6.2\%$ ($1 \mu M$ zinc), $67.1 \pm 7.6\%$ ($10 \mu M$ zinc), $63.4 \pm 2.2\%$ ($30 \mu M$ zinc), $46.5 \pm 3.7\%$ ($100 \mu M$ zinc), and $33.1 \pm 1.9\%$ with $300 \mu M$ zinc. Here, all effects except the effect of the addition of $1 \mu M$ zinc, which was not significant, were statistically significant reductions of the current responses (Dunnett’s one-way ANOVA). When $0.3 \mu M$ glycine was used as an agonist, no current response at all was detectable.

Summary: Zinc had a concentration-dependent potential to, dependent on the glycine concentration used, either block or potentiate the glycine-mediated current responses. In general, high glycine concentrations (up to $100 \mu M$) were blocked by low and high zinc concentrations (up to $300 \mu M$). Low glycine concentrations (up to $3 \mu M$) were not influenced by low zinc concentrations (up to $30 \mu M$). Yet, the current responses induced by $1 \mu M$ glycine were potentiated by high zinc concentrations ($30 – 1000 \mu M$).

4.2. Coactivation of GluN3B-Containing Receptors by a Glycine Binding Site Antagonist

Given the scope of this thesis, the use of a potent and specific GluN1 glycine binding site antagonist was inevitable. As it was the goal to investigate the influence of the GluN3B subunit within the diheteromeric excitatory glycine receptor, the effects of the GluN1 subunit on the receptor characteristics had to be eliminated to isolate – and thereby uncover – the pure effect of GluN3B. This was especially difficult as both GluN1 and GluN3 contain a glycine binding site: therefore, it was essential to be sure that only the GluN1 glycine binding site was affected by this antagonist. The antagonist finally chosen for this study was MDL-29951, a long known glycine site antagonist originally developed to have therapeutic potential in epilepsy (Baron et al., 1992). This antagonist is also the same that was previously used by others for the study of zinc- and antagonist-mediated effects on GluN3A-containing receptors (Madry et al., 2008). For simplicity, MDL-29951 will henceforth be abbreviated as MDL.
4.2. Coactivation of GluN3B-Containing Receptors by a Glycine Binding Site Antagonist

Firstly, the IC$_{50}$ of MDL on a conventional receptor combining GluN1-3a or GluN1-3b with GluN2A was determined. Baron et al. (1992) had shown that MDL was capable to strongly reduce the efficacy of glycine to enhance NMDAR-induced Ca$^{2+}$ influx in primary cell culture of cerebellar granule cells, but they had not determined exact values for specific NMDA receptor compositions. Secondly, MDL’s concentration-dependent effects were tested on GluN1-3a- and GluN1-3b-containing receptors assembled with GluN3B. The glycine affinity of GluN3A is, relative to the glycine affinity of GluN1, about 600-fold higher, the glycine affinity of GluN3B even 1600-fold higher (Yao and Mayer, 2006; Yao et al., 2008). Literature data indicating an enhancement of glycine-mediated current responses in GluN3B-containing receptors by antagonism of the glycine binding site of GluN1 suggest that the desensitization of the receptor is mediated by the GluN1 subunit (Madry et al., 2008). To determine the efficacy of MDL on GluN1/GluN3B-containing receptors, where it acts as an apparent agonist when coapplied with glycine, and to compare it with its efficacy as an antagonist of conventional, GluN1/GluN2A-containing receptors the EC$_{50}$ of MDL on GluN1/GluN3B-containing receptors was determined.

4.2.1. Influence of MDL-29951 on GluN1- and GluN2-Containing Conventional NMDA Receptors

Currents were elicited in conventional NMDA receptors by saturating agonist concentrations (150 µM glutamate and 10 µM glycine). Without the addition of MDL, the current response for GluN1-3a/GluN2A-containing receptors was about 8 µA, and for GluN1-3b/GluN2A-containing receptors was about 10 µA. Figure 4.6.: Effect of MDL on glutamate- and glycine-evoked currents in conventional GluN1/GluN2-containing NMDA receptors. A: Representative current traces of the concentration-dependent MDL effect on glutamate/glycine-induced currents in GluN1-3a/GluN2A or GluN1-3b/GluN2A-containing receptors. Red bars indicate the period of agonist application, blue bars scale the elicited current responses. B: IC$_{50}$ values of MDL for conventional receptor combinations at saturating (150 µM glutamate and 10 µM glycine) agonist concentrations. Blue line/filled circles indicate GluN1-3a/GluN2A-containing, red line/open circles GluN1-3b/GluN2A-containing receptors. Results shown represent the mean values ± SEM normalized to the current response without coapplication of MDL, n = 3 – 10. Included in B) is the structural formula of MDL according to Baron et al. (1992).
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3b/GluN2A-containing receptors 13 µA. To examine the IC\textsubscript{50} of MDL, eight concentrations ranging from 0.01 µM to 30 µM MDL were used and coapplied with glutamate and glycine. The results are shown in Figure 4.6. To calculate the effect of MDL, the elicited current responses were normalized to the maximal current induced by glutamate/glycine alone to calculate the relative MDL-induced reduction of the current response.

A concentration of only 0.03 µM MDL already strongly reduced the current response of GluN1-3a-containing receptors to 0.85 ± 0.01-fold of the current response without MDL. For GluN1-3b-containing receptors a slightly lesser reduction to 0.94 ± 0.01-fold was seen. The same pattern – a more distinct block for GluN1-3a-containing receptors – was also seen at higher concentrations: 10 µM MDL blocked GluN1-3a-containing receptors to 0.03 ± 0.01-fold of the maximal current response, whereas GluN1-3b-containing receptors were merely blocked to 0.14 ± 0.03-fold. Yet, 30 µM MDL blocked 99% of the glutamate/glycine-induced current response in both receptor combinations: with respect to the maximal current response the factors were 0.009 ± 0.003-fold for GluN1-3a/GluN2A-containing receptors and 0.014 ± 0.001-fold for GluN1-3b/GluN2A-containing receptors. This finally resulted, for GluN1-3a/GluN2A-containing receptors, in an IC\textsubscript{50} for MDL of 0.61 µM. The respective IC\textsubscript{50} value for GluN1-3b/GluN2A-containing receptors was 0.82 µM.

**Summary:** The GluN1 glycine binding site antagonist MDL-29951 is a potent blocker of GluN1/GluN2-containing receptors, independently of the GluN1 N-terminal splice variant.

4.2.2. Effects of MDL-29951 on GluN1- and GluN3B-Containing NMDA Receptors

The further characterization of MDL included its effect on GluN3B-containing receptors, tested for heteromeric combinations with GluN1-3a and GluN1-3b. To determine the EC\textsubscript{50} of MDL on GluN3B-containing receptors, 1 µM glycine was used. The MDL concentrations of choice ranged from 0.0001 µM (0.1 nM) to 3 µM. All current responses were normalized to the current seen upon the application of 1 µM glycine alone and are represented as x-fold potentiation (mean value ± SEM). The results are summarized in Figure 4.7.

Adding MDL in a concentration of 0.0001 µM (0.1 nM) showed no effect on the glycine-induced current in GluN1-3a/GluN3B-containing receptors (1.05 ± 0.02-fold potentiation). At a concentration of 0.003 µM, the first potentiating effect was visible, leading to a potentiation by a factor of 1.44 ± 0.13-fold. The use of 0.3 µM MDL potentiated the current already in the saturating range, with a potentiation of about 3.4 ± 0.2-fold. For the GluN1-3a-containing receptors, the determined EC\textsubscript{50} was 8 nM. For GluN1-3b-containing receptors, the determined EC\textsubscript{50} was 7 nM, very close to the GluN1-3a-containing receptors. The first relative potentiation was seen upon the coapplication of 0.003 µM MDL, which led to currents enhanced by 1.23 ± 0.09-fold. A concentration of 0.01 µM MDL was saturating (1.78 ± 0.14-fold potentiation).

Interestingly, the MDL-mediated potentiation of the glycine-induced current seemed to be higher in receptors containing GluN1-3a compared to the ones with GluN1-3b. However, these experiments were performed in two different batches of oocytes, and the level of potentiation can be batch-dependent, as the maximal potentiation also seemed to depend on the maximal current response (general observation). Also as generally GluN1-3a-containing
4.2. Coactivation of GluN3B-Containing Receptors by a Glycine Binding Site Antagonist

Figure 4.7.: Effect of MDL on glycine-evoked currents on GluN1/GluN3B-containing NMDA receptors. A: Representative current traces showing the concentration-dependent MDL effect on currents induced by 1 µM glycine for GluN1-3a/GluN3B- and GluN1-3b/GluN3B-containing receptors. Red bars indicate the period of agonist application, blue bars scale the elicited current responses. B: EC50 values of MDL on GluN3B-containing receptor combinations. Results shown represent the mean values ± SEM normalized to the current response without coapplication of MDL, n = 6. Green line/filled squares indicate GluN1-3a/GluN3B-containing, purple line/open squares indicate GluN1-3b/GluN3B-containing receptors.

receptors yielded higher currents when directly (in one batch of oocytes) compared to GluN1-3b-containing receptors, this effect might be attributed to the presence of exon 5 in the latter combination (see Cavara 2009). Independently of the actual factor, the strong efficacy of MDL in the nanomolar range to potentiate glycine-induced current responses in GluN3B-containing receptors was impressive and showed its potential as a pharmacological tool for NMDA receptor research.

Summary: At GluN1/GluN3B-containing receptors, MDL-29951 highly efficiently potentiated the glycine-mediated current responses. This effect was independent of the N-terminal splice variant.

4.2.3. Potentiation of GluN1/GluN3B-Containing Receptors by Zinc and MDL-29951

As described in chapter 4.1.4, zinc was able to potentiate the glycine-induced currents at certain concentrations, at receptors coexpressing GluN3B with either GluN1-3a or GluN1-3b. Of the different concentrations of interest, the combination 1 µM glycine and 100 µM zinc was chosen for further investigation. This is the combination that showed the maximal potentiating effect in GluN1-3a/GluN3B-containing receptors (419 ± 50.0 %). Though this is not the zinc concentration with the highest potentiating effect, which would be 300 µM zinc in receptors assembled from GluN1-3b and GluN3B (616 ± 69.5 %), it was chosen as it displays the highest, widely accepted physiological value of zinc (Vogt et al., 2000). Concerning MDL a concentration of 0.2 µM was used. This concentration was saturating at GluN3B-containing receptors. Also, the application of 0.2 µM MDL alone to GluN1/GluN3B-containing receptors showed no effect.
4. Results

The results of the recordings for both GluN1-3a/GluN3B and GluN1-3b/GluN3B-containing receptors are shown in Figure 4.8.

Regarding GluN1-3a/GluN3B-containing receptors, the glycine-induced currents were strongly positively modulated (10.6-fold) upon the addition of zinc. The modulation seen upon coapplication of MDL was similar, as a potentiation of 12.9-fold could be seen. This slightly higher potentiation of MDL compared to zinc was not significant. Upon the addition of both zinc and MDL to glycine the extent of positive modulation was 11.2-fold. Again, the differences between zinc/MDL and either zinc or MDL alone were not significant and showed no tendency towards an additive or stronger than additive potentiation. In contrast, the differences in the potentiating effect of MDL, zinc, and MDL/zinc combined seemed to show the tendency that the coapplication of zinc/MDL yields a slightly lower potentiation than that yielded upon the coapplication of MDL alone. This lower potentiation seemed to be rather equal to that of zinc alone than the coapplication of MDL alone.

The same general pattern, just with smaller potentiation factors, was seen at GluN1-3b/GluN3B-containing receptors, on which both the coapplication of glycine, MDL, and zinc alone also showed a potentiating effect. The positive modulation mediated by the addition of zinc led to 4.1-fold higher currents. By contrast, MDL enhanced the glycine-
induced currents by a factor of 7.4, significantly higher than the potentiation seen upon the addition of zinc. Interestingly, when zinc and MDL were combined and coapplied with glycine, the 4.3-fold apparent potentiation of the glycine-induced current reached pretty much the same level as the potentiation seen upon the addition of zinc alone. Also, this effect differs, just as the effect of zinc alone, significantly from the potentiation mediated by MDL alone. This effect of MDL alone, seen as a small tendency in GluN1-3a-containing receptors, is here significant for GluN1-3b-containing receptors. This suggests a competitive component of the interaction between zinc and MDL (addressed later in chapter 4.3.5).

**Summary:** Glycine-induced current responses in GluN1-3a/GluN3B- and GluN1-3b/ GluN3B-containing receptors were strongly potentiated by zinc, MDL, or zinc plus MDL combined. Whereas in GluN1-3a/GluN3B-containing receptors zinc, MDL, and zinc/MDL potentiated by roughly the same factor, in GluN1-3b/GluN3B-containing receptors the coapplication of MDL resulted in a higher potentiation than seen upon the application of zinc or zinc/MDL combined.

### 4.3. Investigation of the Underlying Mechanism

#### 4.3.1. Influence of the Ligand Binding Domain

To gain insight into the mechanism underlying the potentiating effect of zinc, point mutations of the receptor subunits involved were generated. As for the GluN3B subunit and its glycine-binding LBD, no selective antagonist is available so far, the isolation of the pharmacological properties of the GluN1 subunit by blocking the GluN3B-mediated effects was not possible. The generated point mutations were designed to selectively influence the LBD. Besides the GluN3B mutants, GluN1-3a and GluN1-3b receptors with altered ligand binding domains were constructed. To achieve this, the mutation of the phenylalanine in position 466, an aromatic residue crucial for glycine binding in the GluN1, was used. This mutation had been shown to strongly decrease agonist-induced currents (raising the EC\textsubscript{50} for glycine about 6500-fold, Kuryatov et al. 1994) in GluN1/GluN2-containing receptors and to abolish their function. In GluN3-containing receptors, this mutation strongly increased the glycine-mediated current response (Madry et al., 2007a). It has already been effectively utilized to screen GluN1/GluN3-containing receptors for new, GluN3-selective antagonists (Kvist et al., 2013). This phenylalanine stabilizes the binding of the agonist via a π electron interaction, thus preventing the agonist from leaving the closed LBD (Furukawa and Gouaux, 2003). The GluN3B subunit at the homologous site contains a functionally conserved tyrosine rather than the phenylalanine found in the GluN1 subunit. In addition, two other amino acids, R505 and D735 (in GluN1-3a; R515 and D722 in GluN3B, respectively), were identified as conserved via sequence alignments and crystal structures in both GluN1 and GluN3B subunits and therefore selected for mutation (compare Figure 4.9).

All mutations were tested for their functionality by expressing them in *Xenopus* oocytes in heteromeric GluN1/GluN3B combinations and measuring their electrophysiological properties via TEVC. The effect of each generated mutant was controlled via comparison to the corresponding wild type receptor. As agonist the saturating concentration of 10µM
4. Results

Figure 4.9.: Sequence alignments and analysis of mutants. A: Crystal structure of the LBD of GluN1-1a (1PB7) and B: GluN3B (2RCA). Tagged with amino acid numbers and marked in white are the mutated residues with the bound glycine shown in the middle. C: Sequence alignment of GluN1-3a, GluN1-3b, and GluN3B. Highlighted in dark grey are the mutated residues, shown in red are conserved residues. D: Effects of the mutations on the electrophysiological properties of the receptors. The mutants were in one experiment each compared to the wild type, current responses were normalized to the wild type. Data shown represent the mean values ± SEM. *** = p < 0.005; * = p < 0.05, Dunnett’s one-way ANOVA or Student’s t-test, n = 7 – 24. E: Representative current traces. The red bars indicate the duration of agonist application, the blue bars scale the current amplitude.
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Figure 4.10.: Receptor containing the mutated GluN3B ligand binding site. A: GluN1-3a + GluN3B(Y482A). 1 µM glycine was applied alone, together with 100 µM zinc, 0.2 µM MDL, or zinc/MDL combined. All currents were normalized to the sole application of 1 µM glycine. Data shown represent the mean values ± SEM. Statistical differences were calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test (** = p < 0.005), n = 14. B: Representative current traces for the different agonist applications. The red bars show the duration of the agonist application whereas the blue bars scale the current amplitude.

glycine was chosen (compare 4.1.1). The results for all mutants are shown in Figure 4.9. As is clearly evident, the F466A mutant in GluN1-3a and GluN1-3b showed a large effect on the glycine-mediated current response, potentiating it about 3-fold (GluN1-3a) and 1.5-fold (GluN1-3b). Separately shown are the R505A and D714A mutants. Despite their position in the LBD, they failed, when mutated to an alanine, to produce the potentiation the F466A mutant showed, but rather strongly reduced receptor function. The corresponding mutations (R515A and D722A) in the GluN3B LBD also failed to generate any current responses above background, controlled via the sole expression of a GluN1-3a subunit, and were not used for further investigations. Interestingly, the GluN3B(Y482A) mutant was able to form a functional receptor when combined with GluN1-3a, albeit with a strongly reduced current response. This receptor’s functionality could be proven to be above the background current mediated by receptors containing the endogenous XenGluN2B, and was therefore used in further experiments. The R505A and D735A mutants of GluN1-3a were, due to their lack of functionality, not generated for GluN1-3b, and only the F466A mutant of that subunit used for further investigations.

Shown in Figure 4.10 are the results for the modulation of the GluN3B(Y482A)-containing receptor. Despite the highly reduced current responses, robust amplitudes could be detected. It was clearly visible that both zinc and MDL were still able to strongly potentiate the current responses seen upon application of 1 µM glycine. Yet, the potentiation factors
were strongly increased: whereas the wild type receptor combination was potentiated about 10-fold by the coapplication of zinc, here the potentiation factor was 36.6-fold. Also, MDL, which was able to potentiate the wild type glycine-induced current about 13-fold, now potentiated the current response by a factor of 43.8. Applying both zinc and MDL together enhanced the current response of 1 µM glycine 34.0-fold (11-fold in the wild type).

Interestingly, the GluN1 subunit carries a phenylalanine at position 466 and the GluN3B subunit a tyrosine at the homologous position 482. These two amino acids share one common feature: both carry a benzene group. This group, forming a delocalized $\pi$ electron system, is positioned almost rectangularly to the glycine in the binding pocket. Its deletion strongly alters the receptor function, although this amino acid is not thought to directly interact with the glycine. Rather, it is chiefly needed to stabilize the agonist binding. The only difference between GluN1 and GluN3B is the additional hydroxyl group (precisely: creating a phenol residue rather than a benzene group) in the tyrosine in GluN3B. This might be a coincidence, as the function is conserved via the benzene; yet, the importance of this hydroxyl group was tested. In the GluN1-3a and GluN1-3b subunits, the hydroxyl group was added, generating the F466Y mutant. Vice versa, in the GluN3B subunit the hydroxyl group was removed by exchanging the tyrosine for a phenylalanine (GluN3B(Y482F)).

![Figure 4.11.](image)

**Figure 4.11:** Effects of exchanging phenylalanine and tyrosine in GluN3B and GluN1-3 subunits. **A:** GluN3B mutants, tested with 10 µM glycine. Statistical significances were calculated with respect to the receptor combination containing the GluN3B wild type. **B:** GluN1-3a mutants, tested with 150 µM glutamate/10 µM glycine. Statistical differences were calculated with respect to the GluN1-3a(F466A)-containing receptor combination. Shown is the mean current (nA/µA) ± SEM. Statistical differences were calculated using the Dunnett’s one-way ANOVA test (** *= p < 0.005), n = 5 – 10. **C** and **D:** Representative current traces for the different receptor combinations. Red bars indicate the duration of the agonist application, blue bars scale the current amplitude.
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Figure 4.12.: Effects of the mutated GluN1 ligand binding site on zinc/MDL modulation in GluN3B-containing receptors. A: GluN1-3a(F466A) + GluN3B; B: GluN1-3b(F487A) + GluN3B. 1 µM glycine was applied alone, together with 100 µM zinc, 0.2 µM MDL or zinc/MDL combined. All currents were normalized to the sole application of 1 µM glycine. Data shown represent the mean values ± SEM. Statistical differences were, if not indicated otherwise, calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test or Student’s t-test (** = p < 0.005), n = 11 – 26. C and D: Representative current traces for the different agonist applications. The red bars indicate the duration of the agonist application, the blue bar scale the current amplitude.

The GluN1-3a(F466Y) and GluN1-3a(F466A) mutant (the latter already described above) were compared to the wild type and here characterized in coexpression with GluN2A. GluN3B(Y482F) and GluN3B(Y482A) were characterized together with GluN1-3a and and also compared to the wild type GluN3B. The results are shown in Figure 4.11.

Referring to the GluN3B mutants, the GluN1-3a/GluN3B wild type-containing receptor showed a robust glycine-induced (10 µM) current response of 150.0 ± 24.1 nA. The GluN3B(Y482A) mutant strongly decreased the agonist-induced current to 2.3 ± 0.4 nA. Interestingly, the functionally conserved mutant GluN3B(Y482F) showed almost even less current responses (1.2 ± 0.1 nA). The GluN1-3a mutants presented a completely different picture. Here, the structurally conserved mutant GluN1-3a(F466Y) showed almost no alteration in its reaction to agonist application compared to the wild type: a receptor combining GluN1-3a(F466Y) and GluN2A produced a current response of roughly 10.7 ± 0.5 µA upon application of 150 µM glutamate and 10 µM glycine. The difference to the wild type, that showed an average current response of 9.9 ± 0.8 µA upon agonist application, was not significant. The GluN1-3a(F466A) mutant showed, as had been expected based on data from Kuryatov et al. (1994), a strongly reduced (0.11 ± 0.02 µA) current response upon agonist application. Depicted are only the results for receptors containing GluN1-3a subunits, as the results for the GluN1-3b-containing receptors show virtually the same results.
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The modulation of glycine-induced current responses by zinc, MDL, or zinc and MDL together at the GluN1(F466A) mutant is shown in Figure 4.12. All current responses were normalized to the current response elicited by 1 µM glycine. After all, zinc in a concentration of 100 µM was still able to potentiate the glycine-induced current responses. In the GluN1-3a(F466A)-containing combination, this potentiation was, showing a 9.8-fold increase, more pronounced than in the wild type receptor. On the other hand, the positive upregulation that was seen by 0.2 µM MDL in the wild type receptor was virtually gone in the mutated version. Only a very small modulation could be seen that showed a 1.3-fold enhanced current. This picture again changed when both zinc and MDL were coapplied with glycine: a strong increase in current responses, similar to that seen upon the addition of zinc alone, was detected. In numbers, the potentiation was 9.3-fold. For GluN1-3b(F466A)-containing receptors the same pattern showed up with somewhat reduced potentiation factors. Zinc was still capable of inducing a strong increase of current responses to 3.1-fold of the basal current. Contrary to GluN1-3a-containing receptors, MDL showed a slight, yet not significant block of current responses to 80% of glycine applied alone. Both MDL and zinc applied together still potentiated with a factor of 3.4 to a degree similar to zinc alone (3.1-fold). Interestingly, all these potentiation factors were in the same range as the factors that were seen in the wild type receptors, as illustrated in chapter 4.8.

Summary: Mutations within the GluN1 LBD, influencing the glycine binding, enhanced glycine-induced current responses in GluN3B-containing receptors, and abrogated the MDL-mediated potentiation of glycine-induced current responses. The potentiation by zinc remained unaffected. A corresponding mutation within the GluN3B LBD reduced the glycine-induced current responses, yet had no influence on the modulation by zinc and MDL.

4.3.2. Role of Exon 5

The only difference between the GluN1-splice variants GluN1-a and GluN1-b is the lack of exon 5 in the “a” variants. This exon has been in the focus of research for a long time in both conventional, GluN2-containing, and GluN3-containing receptors. It has been shown to completely alter the receptor behavior, e.g. the proton sensitivity in GluN2-containing receptors; the GluN1-b-containing combination is proton-insensitive, whereas the GluN1-a-containing combination is sensitive towards proton block (Traynelis et al., 1998; Cavara et al., 2009). Possible alterations in the receptor behavior might include an influence of the loop formed by the 21 amino acids encoded by exon 5. In GluN1-b variants this loop “shields” the proton binding site against unrequested access (Traynelis et al., 1998). The zinc modulation might be dependent on the presence or absence of exon 5, with exon 5 either as the site of interaction or, vice versa, the mediator of interaction. Therefore a look into the role of exon 5 is indeed necessary.

As the removal of exon 5 from the GluN1-b splice variant is obviously redundant as it would simply generate the GluN1-a variant, mimicking of the exon 5-mediated effects is a proper tool. Long-known receptor variants with certain point mutations (Kashiwagi et al., 1996; Low et al., 2000; Traynelis et al., 1998) have been used. Two of them, the most promising D669N and the N616Q mutants, were used here. Both have been shown
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to strongly decrease the proton sensitivity of the GluN1-a subunit down to the sensitivity of GluN1-b-containing receptors in both GluN2A- and GluN2B-containing receptors (Low et al., 2000). As this proton shield is also one of the zinc interaction site in conventional NMDARs, these mutations are a good tool to investigate – and mimick – the influence of exon 5 on GluN3B-containing receptors.

Both point-mutated GluN1-3a subunits showed robust, albeit significantly reduced current responses: the currents elicited by 10 µM glycine were, compared to the wild type, reduced to 7.1 ± 1.1% for the GluN1-3a(D669N) variant and to 23.4 ± 3.3% for GluN1-3a(N616Q)(data not shown).

Whereas it was no problem to determine the effects of zinc and MDL on the GluN1-3a(D669N) mutant, it was impossible to quantify the effect of zinc on the glycine-induced current response in the GluN1-3a(N616Q) mutant. As can be seen in Figure 4.13 C), under this condition a steady rundown of the current response was seen that reached no steady state even after minutes of agonist application. Additionally, it was impossible to further measure other agonist combinations in the oocyte after application of glycine and zinc, as oocytes did not recover from this rundown. Despite this rundown during coapplication of zinc, the effect of MDL was still there and quite pronounced as it potentiated the glycine-

![Figure 4.13: Characteristics of receptors containing putative proton- and zinc-insensitive GluN1-3a subunits. A: GluN1-3a(N616Q) + GluN3B; B: GluN1-3a(D669N) + GluN3B. 1 µM glycine was applied either alone or together with 100 µM zinc, 0.2 µM MDL, or zinc/MDL combined. All currents were normalized to the sole application of 1 µM glycine. Data shown represent the mean values ± SEM, n = 9 – 21. Statistical differences were calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test (*** = p < 0.005). C and D: Representative current traces for the different agonist applications in both receptor combinations. The red bars indicate the duration of the agonist application, the blue bars scale the current amplitude.](image-url)
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Induced current 28.8-fold. The coapplication of zinc and MDL with glycine also showed a pronounced potentiation with a slightly reduced effect (24.7-fold potentiation). Here, no rundown effect was seen.

The GluN1-3a(D669N) mutant showed no rundown effect. Zinc and MDL, or the combination of both, were able to enhance the glycine-induced current significantly. The coapplication of zinc with glycine led to a distinct potentiation by 2.7-fold of the current amplitude mediated by glycine alone. Coapplying MDL with glycine showed, with 2.7 the same potentiation factor as that of zinc. Both MDL and zinc together with glycine resulted in an increase of current responses relative to glycine applied alone by a factor of 2.9. Small differences between zinc, MDL, or MDL and zinc together were not significant.

Although the exon 5 does not directly influence the LBD, the mutations that mimic the effect of exon 5 might do so. To see whether both, mimicking the exon 5 and mutating the LBD, leads to alterations in the electrophysiological properties of the receptor, the double mutant, containing also the (F466A) glycine binding site mutation was of interest. As the GluN1-3a(N616Q) mutant proved itself to be not useful for analysis, only the GluN1-3a(F466A/D669N) double mutant was generated. The results for the receptor containing GluN1-3a(F466A/D669N) are shown in Figure 4.14.

![Figure 4.14](image_url)

Figure 4.14.: Effects of GluN1-3a(F466A/D669N), a putative proton-insensitive receptor subunit with an impaired glycine binding site at the GluN1 subunit, on receptor behavior. **A:** GluN1-3a(F466A/D669N) + GluN3B. 1 µM glycine was applied alone, together with 100 µM zinc, 0.2 µM MDL, or zinc/MDL combined. All currents were normalized to the sole application of 1 µM glycine. Data shown represent the mean values ± SEM. Statistical differences were, if not indicated otherwise, calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test or the Student’s t-test (*** = p < 0.005), n = 20. **B:** Representative current traces for the agonist applications. The red bars indicate the duration of the agonist application, whereas the blue bars scale the current amplitude.
As the data clearly show, mutating both the ligand binding site (F466A) and mimicking the effect of exon 5 (D669N) did not alter the receptor behavior. The current response induced by 1 µM glycine was potentiated by a factor of 16.3 ± 2.8 through adding 100 µM zinc to the recording solution. The same potentiation factor (16.6 ± 2.8) could be seen upon the addition of both zinc and 0.2 µM MDL. Yet, as for the GluN1-3a(F466A) mutant, the coapplication of 0.2 µM MDL led to no effect (1.2 ± 0.1-fold potentiation). Interestingly, these potentiation factors were higher than those seen in the equivalent single mutant receptor: the GluN1-3a(F466A)/GluN3B-containing receptor was potentiated by zinc/MDL by a factor of 9.3, the GluN1-3a(D669N)/GluN3B-containing receptors by a factor of 2.9 (compare chapters 4.3.1 and 4.3.2).

Summary: Point mutations, known to mimic the effect of exon 5 to render a receptor containing GluN1-b and GluN2 proton- and zinc-insensitive, were introduced into GluN1-3a. These mutations did not alter the behavior of the GluN1-3a/GluN3B-containing receptor towards zinc, MDL, and zinc/MDL combined.

4.3.3. Removal of the ATD

The approach followed so far focussed on the role the LBDs of the different receptors play within the differential modulation by zinc and MDL. But, besides the LBD, the ATD of the receptor subunits also plays an important role for receptor function. Forming a bilobed structure similar to the “clam shell” structure of the LBD, it is associated with modulatory functions in the tetrameric receptor, e.g. in binding zinc or ifenprodil in the conventional receptor combinations containing GluN1 and GluN2. Mesic et al. (2016) have shown that neither the GluN1 ATD nor the GluN3A ATD was essential for functionality in a receptor containing GluN1 and GluN3A. The receptor with a deleted ATD, in either one subunit or both subunits, was still functional and could be activated by glycine. To test for a similar role of the GluN3B ATD, two ATD deletion mutants were constructed. The first mutant, GluN3B(Δ23-409) had the complete sequence between the signal peptide (SP) and the S1 domain removed, and will be referred to as GluN3B(ΔATD). For the second mutant, a short “linker” was left between the SP and the S1. This mutant, GluN3B(Δ31-396), will be referred to as GluN3B(ΔATDs)(s for “short”). Amino acids are counted with the initial methionine as 1.

Contrary to data in the literature (Mesic et al., 2016), a receptor containing the GluN3B-(ΔATD) mutant coexpressed with a GluN1-a subunit (here GluN1-3a) showed no noticable current responses upon application of glycine (neither 10 µM nor 1 µM). Oocytes injected with the GluN1-3b subunit in coexpression with the GluN3B(ΔATD) also lacked any glycine-inducible currents. The first possibility here is a current response below the detection threshold of the TEVC system, given that many of the receptor constructs investigated within this thesis showed only relatively low agonist-induced current responses. Yet, the control of the membrane expression of all receptor subunits conducted later indicated that this particular subunit (GluN3B(ΔATD)) is not exported to the membrane (see chapter 4.5.2): the coexpression of GluN3B(ΔATD) with either the eGFP-tagged GluN1-3a or GluN1-3b subunits in oocytes and subsequent confocal microscopy showed no fluorescence that was above the fluorescence level of the uninjected control oocytes. By contrast, the
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Figure 4.15.: A receptor containing GluN3B ATD-exchange mutants. A: GluN1-3a + GluN3B-(ATD 1-b), B: GluN1-3b + GluN3B(ATD 1-b), E: GluN1-3a + GluN3B(ATD 1-a), F: GluN1-3b + GluN3B(ATD 1-a). 1 µM glycine was applied alone or together with 100 µM zinc, 0.2 µM MDL, or zinc/MDL combined. All currents were normalized to the sole application of 1 µM glycine. Data shown represent the mean values ± SEM, n = 13 – 35. Statistical differences were, if not indicated otherwise, calculated with respect to the application of glycine alone, using the Dunnell’s one-way ANOVA test or Student’s t-test (** = p < 0.005; *** = p < 0.01; * = p < 0.05). C, D, G and H: Representative current traces for the different agonist applications in the receptor combinations. The red bars indicate the duration of agonist application, the blue bars scale the current amplitude.
construct GluN3B(ΔATDs) could, together with GluN1-3a or GluN1-3b, form a receptor complex for that membrane expression could be proven. Yet upon electrophysiological characterization, this receptor combination also showed no detectable current responses upon the application of glycine. As the membrane expression could be proven it might simply be that the current responses in receptors containing GluN3B(ΔATDs) were below the detection threshold of the TEVC system.

Summary: In order to investigate the influence of the GluN3B ATD on zinc modulation, the ATD was removed either completely or with a short linker of 15 AS left between the signal peptide and the S1 domain. Both mutants showed, when coexpressed with either GluN1-3a or GluN1-3b, no agonist-inducible current response. Whereas the membrane expression of the GluN3B mutant with the ATD completely removed was later disproven, the second mutant with a short linker was shown to be plasma membrane-expressed.

4.3.4. Substitution of the ATD

As removing the ATD from the GluN3B subunit produced no constructs that yielded detectable current responses, the ATD of the GluN3B subunit was replaced with the corresponding ATD of the GluN1-a or GluN1-b subunits. If the ATD of the GluN3B subunit plays an essential role for the zinc modulation of the receptor, a change in electrophysiological properties would be detectable. Receptors composed of a GluN1 subunit and the GluN3B(ΔTD 1-a) or GluN3B(ΔTD 1-b) variant were fully functional and showed robust current responses upon application of glycine. The results of the electrophysiological measurements of chimeric GluN3B receptors with exchanged ATDs are shown in Figure 4.15.

Interestingly, the receptor containing GluN1-3a and GluN3B(ATD 1-b) showed the same pattern of zinc/MDL modulation as the GluN1-3b/GluN3B wild type. Coapplying 100 µM zinc resulted in a 6.2 ± 1.0-fold potentiation of the current response elicited by 1 µM glycine. The potentiation resulting from the coapplication of 0.2 µM MDL was about twice the value compared to that of zinc: 11.0 ± 1.7-fold. Adding both zinc and MDL produced a 7.0 ± 1.0-fold potentiation. The general potentiation factors were approximately twice as high compared to that in the wild type (see chapter 4.8).

Just as in the wild type, the pattern in the GluN1-3b/GluN3B(ΔTD 1-b)-containing receptor remained constant. Only the extent of the potentiation was reduced by a factor of approximately 2: where 100 µM zinc could potentiate the glycine-induced current in the wild type around 4-fold, in the GluN3B(ΔTD 1-b)-containing receptor, this potentiation was 2.1 ± 0.2-fold. MDL produced a potentiation factor of 4.8 ± 0.4 compared to the application of glycine alone. Combining MDL, zinc, and glycine resulted in a potentiation like that of zinc alone, 2.7 ± 0.2-fold.

Composing a receptor with four GluN1-a-ATDs led to a receptor that showed the same potentiation pattern as the wild type receptor that contained GluN1-3a and GluN3B. Both zinc and MDL potentiated the glycine-induced current response. Potentiation factors for the coapplication of zinc were 2.7 ± 0.3-fold and for MDL 3.5 ± 0.4-fold. Applying both MDL and zinc led to a relative potentiation of 3.0 ± 0.3-fold. The alteration in current amplitudes and potentiation factors between zinc, MDL, and zinc/MDL together were not significant for any combination.
4. Results

The combination of the GluN1-3b subunit with a GluN3B subunit that carries the ATD of the GluN1-a subunit also showed current potentiation for the coapplication of zinc and MDL. Adding 100 µM zinc to the recording solution potentiated the current response, compared to the application of glycine alone, by a factor of 3.0 ± 0.2. When 0.2 µM MDL were added, the potentiation was increased to 4.4 ± 0.4. The coapplication of both zinc and MDL with glycine led to a potentiation just as with zinc alone, 3.0 ± 0.4-fold compared to 1 µM glycine alone. Here, the potentiation mediated by MDL was significantly larger than the potentiation induced by zinc, or by zinc and MDL alone.

**Summary:** The GluN3B ATD does not play a role in zinc modulation of the GluN1-3a/GluN3B- or GluN1-3b/GluN3B-containing receptors. A receptor composed of GluN3B, containing the transplanted GluN1-b ATD plus GluN1-3a showed glycine-induced currents that were modulated by zinc and MDL with a pattern as in the wild type, where MDL potentiates stronger than zinc or MDL plus zinc.

4.3.5. Competition Between Zinc and MDL

As described in chapter 4.8, a receptor combining GluN1-3b and GluN3B showed an unexpected pattern. The ability to potentiate the glycine-induced current was higher for MDL alone as for the coapplication of MDL and zinc. The latter combination instead reached the same current level as zinc alone did, indicating a competitive behavior. To test this, the GluN1-3b-subunit was coexpressed with the GluN3B subunit and measured with zinc and MDL, where the zinc concentration remained at 100 µM, but the MDL concentration was increased 1000-fold to 200 µM, resulting in a two-fold excess over zinc.

Interestingly, increasing the MDL concentration did indeed effect the potentiation of the current response, as shown in Figure 4.16. 100 µM zinc potentiated the glycine-induced current as usual for the GluN1-3b-containing receptor combination in the range of 2-fold (here 1.8 ± 0.1-fold). The coapplication of 200 µM MDL and glycine led to 3.2 ± 0.1-fold enhanced current response. When glycine was coapplied with zinc and 200 µM MDL, the current response was also potentiated 3.2-fold (± 0.2) compared to the current response elicited by 1 µM glycine alone. This contrasted with the use of 0.2 µM MDL, where the potentiation by the coapplication of zinc and MDL led to a potentiation in the range of zinc alone (see chapter 4.2.3, Figure 4.8).

**Summary:** In a GluN1-3b/GluN3B-containing receptor, MDL was shown to be able to compete with zinc.

4.4. Comparison with GluN3A

Considering GluN3A-containing receptors, it is known that they can be potentiated by the coapplication of zinc (Madry et al., 2007a). Yet, it so far remained unclear how GluN3B behaves in direct comparison to GluN3A. Considering the differential developmental expression profiles of GluN3A and GluN3B, the role the different subunits play during developmental processes cannot be underestimated. In order to gain insight into any specific differences, the same concentrations of glycine, zinc, and MDL as for GluN3B-containing
4.4. Comparison with GluN3A

Figure 4.16.: Competition between zinc and MDL. A: GluN1-3b + GluN3B. 1 µM glycine was applied either alone or together with 100 µM zinc, 200 µM MDL, or zinc/MDL combined. All currents were normalized to the sole application of 1 µM glycine. Data shown represent the mean values ± SEM, n = 12 – 13. Statistical differences were calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test (*** = p < 0.005). B: Representative current traces for the different agonist applications in both receptor combinations. Red bars indicate the duration of agonist application, blue bars scale the current amplitude.

combinations were applied to GluN3A. Additionally, a GluN3A subunit lacking the 5’ UTR was constructed to see what, if any effect, the UTR has on translation efficacy. This might be a useful tool to gain enhanced current responses.

In addition, the proton-sensitivity of GluN3A-containing receptors was tested. The conventional GluN2-containing NMDA receptor is blocked by protons when containing a GluN1-a subunit. When a GluN1-b subunit is coexpressed, the receptor is rendered proton-insensitive, as the 21 AS loop formed by the exon 5 shields the proton binding site (Traynelis et al., 1998). By contrast, GluN3B-containing excitatory glycine receptors had been found to be proton-sensitive regardless of the GluN1 splice variant (Cavara et al., 2009).

4.4.1. Effect of MDL and Zinc on GluN3A-Containing Receptors

The effect of zinc and MDL on GluN3A-containing excitatory glycine receptors was determined via the coexpression of GluN1-3a and GluN3A. To allow the direct comparison between GluN3A- and GluN3B-containing receptors, the same agonist concentrations that were used for GluN3B-containing combinations were employed. 1 µM glycine was used and coapplied with 100 µM zinc, 0.2 µM MDL, or zinc/MDL together. The results are summarized in Figure 4.17.
4. Results

Figure 4.17.: Effect of zinc and MDL on GluN3A-containing receptors. A: GluN1-3a + GluN3A. 1 µM glycine was applied alone, together with 100 µM zinc, 200 µM MDL, or zinc/MDL combined. All currents were normalized to the application of 1 µM glycine alone. Data shown represent the mean values ± SEM, n = 5. Statistical differences were, if not indicated otherwise, calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test or the Student’s t-test (*** = p < 0.005, ** = p < 0.01). B: Representative current traces for the different agonist applications in both receptor combinations. The red bars indicates the duration of agonist application, blue bars scale the current amplitude.

In GluN3B-containing receptors, the coapplication of glycine and zinc led to a pronounced potentiation of the glycine-induced currents. Similarly, GluN3A-containing receptors were significantly potentiated by the coapplication of 100 µM zinc. The relative potentiation of the glycine-induced current (compared to the application of glycine alone) was 2.9 ± 0.4-fold. Regarding the use of 1 µM glycine together with 0.2 µM MDL, GluN3B-containing receptors were potentiated in the same range as by the coapplication of zinc. Interestingly, the coapplication of MDL and glycine to a receptor containing GluN3A showed no potentiation at all (1.1 ± 0.1-fold compared to glycine alone). Comparing GluN3A- and GluN3B-containing receptors, this was a completely different behavior.

Regarding the coapplication of MDL and zinc, a GluN3A-containing receptor behaved just like the GluN3B-containing combination and showed a potentiation by the factor of 3.1 ± 0.4. As in the GluN3B-containing receptor, the level of potentiation between the coapplication of 100 µM zinc alone and the coapplication of 100 µM zinc and 0.2 µM MDL was not significant.

Summary: The concentration of zinc used for GluN3B-containing receptors, 100 µM, potentiated the current response induced by 1 µM glycine in GluN1-3a/GluN3A-containing receptors, whereas 0.2 µM MDL failed to potentiate the current responses. The potentiation of MDL and zinc together reached the same level as the potentiation by zinc alone.
4.4. Comparison with GluN3A

4.4.2. Removal of the GluN3A 5' UTR

The 5' UTR of the GluN3A receptor subunit consists of 473 base pairs, whereas the 5' UTR of the GluN3B subunit is composed of only 177 base pairs. To identify a possible influence of the 5' UTR, as a UTR can substantially influence protein translation, a GluN3A subunit lacking the 5' UTR was generated, heterologously expressed together with GluN1-3a and electrophysiologically characterized. As with the wild type GluN3A receptor (see previous chapter), the same agonist concentrations that were used for GluN3B-containing receptors were utilized to allow comparison between the subunits. The data of the electrophysiological recordings are depicted in Figure 4.18.

![Figure 4.18](image_url)

**Figure 4.18:** Zinc and MDL modulation of a receptor containing a GluN3A subunit lacking the 5' UTR. **A:** GluN1-3a + GluN3A(∆UTR). 1 µM glycine was applied alone, with 100 µM zinc, 200 µM MDL, or zinc/MDL combined. All currents were normalized to the application of 1 µM glycine alone. Data shown represent the mean values ± SEM, n = 4. Statistical differences were calculated with respect to the application of glycine alone, using the Dunnett’s one-way ANOVA test (** = p < 0.005). **B:** Representative current traces for the different agonist applications in both receptor combinations. The red bars indicate the duration of agonist application, blue bars scale the current amplitude.

As for the GluN3A wild type, the coapplication of 100 µM zinc strongly potentiated the glycine-induced current. The actual potentiation factor, 4.2 ± 1.2, was larger than for the wild type. However, this fact could also be due to batch-dependent differences. 0.2 µM MDL were again not capable of potentiating the current response mediated by 1 µM glycine. An interesting effect was seen for the coapplication of both zinc and MDL with glycine: combining the two agonists showed a potentiation much stronger than the potentiation mediated by the coapplication of zinc or MDL alone. The actual potentiation factor was 14.0 ± 3.2, almost three-fold larger than the potentiation seen upon the application of zinc alone. Thus, by simply removing the 5' UTR the potentiating effects of MDL and zinc...
could be changed enormously. It has to be noted that, though the potentiating effect of zinc alone is quite distinct, it is not significant when compared to the application of glycine alone. This is an effect of the statistical analysis.

Summary: Upon removal of the 5' UTR of the GluN3A subunit, the GluN1-3a/GluN3A-containing receptor was still potentiated by zinc and showed no effect upon MDL application. Surprisingly, the coapplication of zinc and MDL led to a potentiation significantly higher than the potentiation by zinc alone.

4.4.3. PH Dependency of Agonist-Induced Currents

The strong pH dependency of conventional, GluN2-containing NMDA receptors is influenced by the presence or absence of exon 5 (Traynelis et al., 1998). Whereas receptors containing a GluN1-a subunit are proton-sensitive, GluN1-b-containing receptors are insensitive towards protons. For GluN3B-containing receptors, however, a different picture had emerged: such receptors are proton-sensitive regardless of the GluN1 variant (Cavara et al., 2009). Until recently (Cummings and Popescu, 2016) it remained unclear how GluN3A-containing receptors behave towards protons and what – if any – influence the different GluN1 splice variants might have. In order to identify possible effects, GluN1-3a or GluN1-3b were coexpressed together with GluN3A and electrophysiologically characterized using NFR adjusted to pH 6.8 and standard NFR pH 7.2. To exclude any concentration-dependent effects, glycine was used in concentrations of 1, 10, and 100 µM. The results are shown in Figure 4.19.

A receptor composed of GluN1-3a and GluN3A showed no sensitivity towards protons: increasing the pH from 6.8 to 7.2 had no significant effect on the current response elicited by 1, 10, or 100 µM glycine. For GluN1-3b/GluN3A-containing receptors the same picture

![Figure 4.19: Proton sensitivity of GluN3A-containing receptors. A: GluN1-3a + GluN3A. B: GluN1-3b + GluN3A. The pH dependency of glycine-induced current responses was determined with NFR pH 6.8 compared to NFR with a pH of 7.2 at 1, 10, or 100 µM glycine. Current responses were normalized to pH 6.8 (= 1). Data shown represent the mean values ± SEM, n = 6 – 16. None of the differences were significant.](image-url)
4.5. eGFP-Tagged NMDA Receptor Subunits

In general, the GluN1-1 and GluN1-3 subunits carry an ER retention signal that prevents individual receptor subunits from being exported to the plasma membrane, a motif that is not present in the GluN1-2 and GluN1-4 subunits. However, a PDZ binding motif in the GluN1-3 subunit is thought to partially be able to compensate for this effect and, to a certain degree, allow for some plasma membrane export (Standley et al., 2000). In order to form membrane-expressed receptors, this ER retention signal is normally masked by another NMDA receptor subunit, e.g., a GluN2 or GluN3 subunit. Additionally, the endogenous \textit{Xen}GluN2B is able to form a functional receptor complex with exogenously expressed GluN1 subunits in the \textit{Xenopus} oocyte expression system (Schmidt and Hollmann, 2008).

Tagging receptor subunits with eGFP is a long-known method used, e.g., to investigate expression profiles. Also, the use of split eGFP molecules attached to different proteins, a method called bimolecular fluorescence complementation (BiFC), is a useful tool to search for potential protein interactions. For glutamate receptors it is often used for a variety of auxiliary subunits such as the transmembrane AMPA receptor regulatory protein (TARP) family. To track down spatial expression profiles, photobleaching of fluorescent probes, e.g., receptors tagged with eGFP, and regain of fluorescence in the bleached area can be used.

To test whether eGFP-tagging of NMDA receptors also provides access to this multitude of methods and their applications, different NMDA receptor subunits were covalently tagged with eGFP. This was done via the vector pSGEM-eGFP. The receptor subunits were then heterologously expressed in \textit{Xenopus} oocytes. Subsequent confocal microscopy then was used to verify the expression of the tagged subunits. The eGFP-tagged receptor subunits generated for this purpose were GluN1-3a-eGFP, GluN1-3b-eGFP, and GluN3B-eGFP. Of special interest was the degree of plasma membrane export of the tagged GluN1-3 subunit, as this subunit should be able to be exported to the plasma membrane to a certain degree, without a second subunit masking the ER retention signal (Standley et al., 2000).

4.5.1. Individual Expression of eGFP-Tagged Receptor Subunits

Although the GluN1-3 splice variant possesses an ER retention signal, this signal that can be overridden by a PDZ binding motif, leading to forward trafficking and plasma membrane expression of the subunit even when it is expressed alone (Xia et al., 2001; Standley et al., 2000; Scott et al., 2001). As already mentioned, endogenously expressed \textit{Xen}GluN2B is a problem as it is able to form functional, membrane-expressed NMDA receptor heteromers with any exogenously expressed GluN1 subunit. To quantify the expression level
4. Results

![Image of two micrographs: A: 1-3a-eGFP, 1-3a-eGFP + 3B. B: 1-3a-eGFP.](image)

**Figure 4.20.: Expression of GluN1-3a-eGFP alone or together with GluN3B.** A: A batch with very low fluorescence for the individually expressed GluN1-3a-eGFP subunit, indicating weak expression of endogenous *Xen* GluN2B, compared to the positive control. B: Batch showing substantial fluorescence for the GluN1-3a-eGFP subunit expressed alone, with fluorescence being in the same range as the positive control.

The expression of GluN1-3a-eGFP was assessed to understand the strength of its interaction with the GluN3B subunit. The expression of GluN1-3a-eGFP alone was compared to a positive control, which was coexpressing GluN1-3a-eGFP with GluN3B, resulting in a functional, membrane-expressed and fluorescence-tagged receptor complex. The GluN1-3a-eGFP subunit expressed alone could reach the membrane to a certain extent due to a PDZ binding motif (see above) and depending on the expression level of the endogenous *Xen* GluN2B subunit. As shown in Figure 4.20, the background expression of the *Xen* GluN2B in the oocyte was strongly batch-dependent. In most batches the background expression was negligible (Figure 4.20 A).

However, in some batches, the background was a real problem, showing fluorescence at almost the same level as the positive control (Figure 4.20 B). Thus, special attention had to be paid to the batch-specific background expression when it came to expression analysis.

Another issue was the orientation of the oocyte during confocal microscopy. Each oocyte has a melanin-pigmented black, animal pole, and a white, unpigmented, vegetal pole. As in confocal microscopy experiments a picture of only a certain section of the oocytes was taken, the orientation of the oocytes in the dish was very important. By taking several pictures that were subsequently merged into one, the analysis of the spatial expression differences with a picture of high resolution and details was possible. For this experiment oocytes injected with GluN1-3a-eGFP in addition to positive controls coexpressing GluN3B were used. Figure 4.21 shows the results. One oocyte injected only with GluN1-3a-eGFP was placed next to an oocyte injected with the positive control (GluN1-3a-eGFP/GluN3B), the white vegetal pole facing each other (see inset detailing the orientation in Figure 4.21).

The positive control showed a distinct fluorescence with only weak alteration all over the oocyte, whereas the negative control, GluN1-3a-eGFP alone, showed moderate fluorescence only on the vegetal, white pole. Almost no fluorescence was seen in the animal pole. In general, all oocytes injected with a tagged GluN1-3 subunit showed a more or less pronounced fluorescence in the vegetal pole. As the positive control (or any other fluorescent
Figure 4.21.: Region-dependent expression of tagged receptors in oocytes. A: Comparison of an uninjected oocyte to the negative and the positive control. At the top the negative control (GluN1-3b-eGFP alone) is placed. Below: On the right side the positive control (GluN1-3b/GluN3B) and on the left side an uninjected oocyte is arranged. B shows a control oocyte injected only with GluN1-3a-eGFP (left) compared to a positive control (GluN1-3a-eGFP and GluN3B, right). The orientation (vegetal/animal pole) is schematically depicted besides the corresponding image.

Sample) should show fluorescence all over the oocyte, this problem could be circumvented by positioning the oocytes with the dark, animal poles facing each other.

Oocytes injected only with a tagged GluN1-3 subunit as a negative control and oocytes injected with GluN1-3-eGFP and GluN3B as a positive control were in addition compared to uninjected oocytes that can show intrinsic plasma membrane fluorescence as well. In Figure 4.21 an exemplary picture is shown. The tagged GluN1-3b subunit was used. The GluN1-3b-eGFP/XenGluN2B-mediated background fluorescence was minimal in this batch, while the positive control showed bright fluorescence. The uninjected oocyte showed no fluorescence at all. Thus, the fluorescence of the positive control and even the negative control clearly predominated over any intrinsic fluorescence of an uninjected oocyte at standard amplification settings used at the confocal microscope.

Summary: Expression of GluN1-3/GluN3B receptors containing an eGFP-tagged subunit led to strong membrane fluorescence independently of the N-terminal splice variant. Upon single expression of an eGFP-tagged GluN1-3 subunit as background control, only weak fluorescence was seen in the vegetal pole of the oocytes. However, some batches of oocytes showed pronounced background fluorescence. This is supposedly mediated by the XenGluN2B, that is able to form a membrane-exported receptor with GluN1 subunits.

4.5.2. Artificial NMDA Receptor Constructs

The here generated three eGFP-tagged subunits, namely GluN3B-eGFP, GluN1-3a-eGFP, and GluN1-3b-eGFP, were used to test the plasma membrane expression of the used receptor constructs. To answer the question whether, e.g., the GluN3B(ATD GluN1-a) is membrane-located, the coexpression of this subunit with a tagged GluN1-3 subunit was
4. Results

sufficient. Under the confocal microscope, fluorescence would be visible in the case of a plasma membrane expression. If the receptor complex is not transported to the plasma membrane, no fluorescence would be visible. To assure the exclusion of false positives through *Xen*GluN2B-mediated membrane export the sole GluN1-3 subunit was used as a control (see chapter 4.5.1).

All these factors that were found to influence the fluorescence were taken into account. The electrophysiological properties of receptors containing eGFP-tagged subunits had been tested previously and were unaltered compared to untagged receptors. The expression of the wild type GluN3B subunit together with either the eGFP-tagged GluN1-3a or GluN1-3b subunit showed bright fluorescence. Compared to this, the background fluorescence seen in oocytes injected with a tagged GluN1 subunit alone was negligible. Uninjected oocytes showed no detectable fluorescence at all. Vice versa, the expression of wild type GluN1-3 subunits together with eGFP-tagged GluN3B also showed a high fluorescence intensity, whereas there was almost no background fluorescence seen in oocytes injected with only GluN3B-eGFP. The uninjected oocytes also showed no fluorescence.

Table 4.2.: Table showing all receptor subunit mutants tested for membrane fluorescence upon coexpression with eGFP-tagged partner subunits. Each mutated receptor subunit was expressed with a subunit tagged with eGFP. The mutated GluN1-3 subunits were coexpressed with the tagged GluN3B-eGFP, the GluN3B variants in independent experiments were expressed with the tagged GluN1-3a-eGFP and GluN1-3b-eGFP subunits. For most of the combinations the expression of wildtype and mutant receptors could be proven. Exceptions with a negative outcome for the test of membrane expression are marked with an asterisk.

<table>
<thead>
<tr>
<th>Mutant Subunit</th>
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<tr>
<td>1-3a</td>
<td>3B-eGFP</td>
<td>3B</td>
<td>1-3a-eGFP</td>
</tr>
<tr>
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<td>3B(Y482A)</td>
<td>1-3a-eGFP</td>
</tr>
<tr>
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<td>3B(Y482F)</td>
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<tr>
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<td>3B(R515A)</td>
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<tr>
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<td>3B(D722A)</td>
<td>1-3a-eGFP</td>
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<tr>
<td>1-3a(N616Q)</td>
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<td>3B(ΔATD)</td>
<td>1-3a-eGFP*</td>
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<tr>
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<td>3B-eGFP</td>
<td>3B(ΔATDs)</td>
<td>1-3a-eGFP</td>
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<tr>
<td>1-3a(F466A/N616Q)</td>
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<td>3B(ΔATD 1-a)</td>
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</tr>
<tr>
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<td>3B(ATD 1-b)</td>
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By means of this method the membrane expression of all but one altered receptor constructs could successfully be proven. The results are depicted in Table 4.2 (for example pictures see Appendix, Figures A.1 – A.4). The wild type and mutant GluN1 constructs were tested via coexpression of the GluN3B subunit and showed bright fluorescence. The GluN3B subunits were tested with both GluN1-3a-eGFP and GluN1-3b-eGFP and were also – with one exception – found to be membrane-expressed.
The one combination for that no plasma membrane expression was seen was the GluN3B-(\(\Delta\)ATD) mutant. This was found to be independent of the coexpressed eGFP-tagged GluN1-3 subunit. This fits to the electrophysiological data, where this mutant failed to show any glycine-induced current responses. The results for this combination are separately shown in Figure 4.22. The comparison of an uninjected oocyte and the positive control showed no abnormality. The comparison of the positive control, the negative control and a receptor containing the GluN3B(\(\Delta\)ATD) mutant showed fluorescence only for the positive control. The fluorescence mediated by the negative control – GluN1-3b-eGFP expressed alone – is at the same level as that seen in the oocyte injected with GluN1-3b-eGFP and GluN3B(\(\Delta\)ATD). After adjusting the confocal microscope by pushing the amplification to the limit to get the background fluorescence to saturating levels, it became clear that there was indeed no difference between the fluorescence intensity of the negative control and the GluN3B(\(\Delta\)ATD)-containing receptor.

**Summary:** Via eGFP-tagging of the receptor subunits, the membrane export of almost all subunits used could successfully be proven. One exception is the GluN3B subunit where the complete ATD was removed. For this subunit no membrane fluorescence could be seen.
5. Discussion

5.1. Summary

To reveal the behavior of GluN1 and GluN3B-containing NMDA receptors towards zinc, firstly the general behavior of receptors containing GluN1-3a and GluN3B as well as receptors containing GluN1-3b and GluN3B was electrophysiologically characterized, using various combinations of glycine and zinc concentrations. Interestingly, a dichotomic behavior occurred: zinc could block the currents induced by high glycine concentrations (10/100 µM), whereas high zinc concentrations (100 µM) potentiated the currents induced by low glycine concentrations (1 µM). This pattern was independent of the GluN1 splice variant analyzed, i.e., the presence or absence of exon 5.

The additional increase of the zinc-mediated potentiation by an antagonist, MDL-29951, that was seen in GluN1-1a/GluN3A-containing receptors (Madry et al., 2008), does not occur in GluN3B-containing receptors.

Further analysis then revealed that the ligand binding site of the GluN1 subunit plays no role in the zinc potentiation, as the abrogation of glycine binding did not alter zinc sensitivity. Yet competition experiments between the antagonist MDL and zinc showed that at least some structural elements of the ligand binding site are involved in the potentiating effects mediated by zinc, albeit these elements may not include residues crucial for glycine binding. Furthermore, the GluN3B ligand binding site proved itself to be completely irrelevant for zinc modulation. The GluN1 ATD, however, does play a role. When the exon 5 (in the GluN1-b variants) was included in the receptor, the zinc potentiation was reduced compared to the receptor combination without exon 5 (GluN1-a variants). When the known proton and zinc binding sites of the GluN1 receptor that are present in the GluN1/GluN2-containing classical NMDA receptor were mimicked, the zinc potentiation was still present. Together with the fact that the reduced zinc potentiation occurred regardless of the position of exon 5, as found out by ATD exchange mutants, this suggests a second component of the zinc modulation, probably mediated by elements at the ATD dimer interface of the two subunits.

Analysis of GluN3A-containing receptors showed that the concentrations of zinc, glycine, and MDL that show potentiating effects in the GluN3B-containing receptors are ineffective. However, when the 5’ UTR of the GluN3A receptor was removed, this potentiation showed up and even the “supralinear” potentiation reported in the literature to be triggered by coapplication of zinc and MDL with glycine was seen. The principle that underlies this effect remains elusive. The GluN3A-containing receptor was also found to be not sensitive to protons.
Expression analysis carried out with eGFP-tagged NMDA receptor subunits showed that the forward-trafficking of the GluN1-3 subunit, when expressed alone, is not as pronounced as previously thought. This is illustrated by the fact that the fluorescence of positive control cells with a coexpressed GluN3B subunit forming a functional, diheteromeric receptor, was much stronger compared to that where the tagged GluN1-3 was expressed alone. However, in some experiments a strong background, reaching the level of the positive control, was seen. This can be attributed to high levels of endogenous *Xen* GluN2B that is able to form functional, membrane-exported diheteromeric conventional NMDA receptors.

5.2. Zinc-Modulation of GluN3B-Containing NMDA Receptors

Concerning the behavior of GluN3A-containing excitatory glycine receptors towards zinc some published data exists. However, this data is limited to a few apparently randomly picked concentrations of glycine and zinc. To systematically gather information about the modulation of GluN3B-containing receptors by zinc, a large matrix of zinc and glycine concentrations was tested in electrophysiological measurements.

Additionally, the possible differences between GluN1 splice variants have been totally neglected and were addressed by using variants of the GluN1 subunit that do and do not incorporate exon 5, the main functional element influenced by alternative splicing. In particular, the GluN1-3a and GluN1-3b subunits were used, as they generate, in combination with GluN3B, the largest currents of all eight GluN1 variants. The other splice variants GluN1-1, GluN1-2, and GluN1-4 not tested here do not show electrophysiological properties that differ from GluN1-3 (Cavara et al., 2009).

5.2.1. Zinc has only marginal Agonistic Potential

The well-known, biphasic concentration-dependent glycine activation of receptors containing GluN1-1a and GluN3A occurs also at both GluN1-3a/GluN3B and GluN1-3b/GluN3B-containing receptors. Just as in the GluN3A-containing receptors (Awobuluyi et al., 2007; Madry et al., 2007a) assuming a different behavior of the two receptor subunits can explain the observed modulation: binding of glycine to the GluN3B subunit gates the receptor, whereas glycine binding to the GluN1 subunit induces a reduction of steady-state currents by rapid desensitization. Although the current response at elevated glycine concentrations is decreased, it is at all concentrations far above the current response observed in controls where only the GluN1 subunit was expressed in the oocyte. Thus, the endogenous *Xen* GluN2B subunit that can form functional receptors together with an exogenous GluN1 subunit cannot be efficiently activated without the presence of glutamate, the mandatory primary agonist for gating of a GluN2-containing receptor.

One of the first experiments within this PhD project was the test whether the agonistic effects zinc (with an EC$_{50}$ of 178 µM) can have on GluN3A-containing excitatory glycine receptors (Madry et al., 2008) also exist in GluN3B-containing receptors. Zinc alone shows no agonistic effects on GluN1-3a/GluN3B-containing receptors when used in concentrations...
of 1 or 10 μM. However, a concentration of 100 μM is able to elicit a small but robust current response. For the GluN1-3b/GluN3B-containing receptor, zinc only at a concentration of 1000 μM is able to elicit a minimal current response.

Between these two latter receptor subunit combinations only one difference exists, which is the presence of exon 5 in the GluN1-3b/GluN3B variant. In conventional, GluN1/GluN2-containing receptors, the presence of exon 5 renders the receptor insensitive towards modulation by protons and zinc, most likely via shielding the proton binding site (Traynelis et al., 1998). Though zinc blocks the conventional receptor, it can evidently activate the GluN3B-containing variant, at least at relatively high concentrations. The observation that this agonistic behavior of zinc preferentially occurs at the GluN1-3a-containing receptor, strongly suggests that this effect is mediated via the GluN1 subunit and includes structural elements of the GluN1 subunit that can be shielded by the loop formed by exon 5 in the GluN1-b variants. As this effect of zinc presents itself only at relatively high concentrations, with 100 μM zinc the maximum that to date is accepted to be physiological, it does most likely play no role in vivo.

A check for contaminating zinc in the used glassware and chemicals showed that there are no tricine-chelatable zinc traces levels left within the recording solutions.

5.2.2. The Dichotomic Effect of Zinc

The search for modulating effects of zinc on the glycine-mediated current response was quite challenging. In the end the multitude of experiments showed a very interesting pattern. For both combinations of receptors, GluN1-3a/GluN3B and GluN1-3b/GluN3B, the latter incorporating exon 5, the addition of zinc led to a pronounced block of most of the currents induced by glycine application. Some zinc concentrations, though, when combined with glycine, led to a pronounced potentiation of the agonist-induced current response.

![Figure 5.1.](image)

Figure 5.1.: Schematic representation of zinc-modulated glycine-induced currents in GluN1-3a/GluN3B and GluN1-3b/GluN3B-containing receptors. A: GluN1-3a/GluN3B-containing receptors. B: Receptors combining GluN1-3b and GluN3B. Green indicates an upregulation of the glycine-induced current response, red a block, and grey no or insignificant modulation.

Why does zinc itself only potentiate current responses when present in a relatively high concentration? Here, it is tempting to speculate that at a small concentration, zinc is not yet able to efficiently bind to the modulating site in the GluN1 ligand binding domain. At higher concentration it can bind and hinders the receptor from desensitizing through binding of glycine to the GluN1 subunit.

But why is zinc then not able to also potentiate the higher glycine concentrations? The widely accepted explanation for the biphasic behavior of GluN3-containing receptors to-
wards glycine is that agonist binding to the GluN3 subunit is sufficient to open the receptor, whereas the binding of glycine to the GluN1 subunit induces rapid current reduction by desensitization of the receptor. With an increasing glycine concentration, this desensitizing effect dominates the receptor and the current response decreases proportionally to the glycine concentration. Assuming that zinc has only a limited binding affinity, it would then not be able to bind to the already – via the GluN1 subunit – desensitized receptor, with glycine still bound to the GluN1 subunit.

Only at an accurately balanced ratio of glycine and zinc ions their interplay leads to this potentiating effect. The existence of a clear maximum potentiation at a defined concentration, when all other concentrations show, relative to this maximum, decreasing effects, supports this idea.

5.2.3. MDL-29951 Differentially Influences NMDA and Excitatory Glycine Receptors

The potent NMDA receptor antagonist MDL-29951, that is selective for the GluN1 subunit, binds to the glycine binding site of the GluN1 subunit. MDL is known to efficiently block conventional, GluN2-containing receptors through this binding to GluN1. On GluN3-containing excitatory glycine receptors, however, MDL displays a phenomenologically quite different behavior: it potentiates the glycine-induced current responses. Via its binding to the GluN1 glycine binding site it competitively prevents glycine from binding and, as the receptor desensitizes via the glycine-activated GluN1 subunit (Awobuluyi et al., 2007; Madry et al., 2007a; Kvist et al., 2013), inhibits also the desensitization, thus enhancing the glycine-induced current response. Receptor gating can, according to this model, still occur via the GluN3 subunit.

On the first hand, the determination of the IC\textsubscript{50}/EC\textsubscript{50} values of MDL revealed its real potential. The IC\textsubscript{50} for the conventional receptors lies clearly higher than the EC\textsubscript{50} of MDL for GluN3B-containing excitatory glycine receptors. Both values do not significantly overlap. MDL thus proves itself to be an important tool for NMDA receptor research and to be much more than just a blocker of conventional NMDA receptors: the use of low concentrations of MDL in combination with glycine potentiates the currents induced by glycine. The small current responses usually seen with the GluN3-containing receptors, which might escape the attention due to high detection thresholds and methodological problems, can be potentiated and thereby rendered detectable. Without influencing the conventional NMDA receptors, as for their activation glycine and glutamate are necessary and MDL would potentially block them, the currents of GluN3-containing excitatory glycine receptors can thus be isolated.

Especially in primary neurons or brain slices this pharmacological scheme can be a good strategy to search for the existence of GluN1/GluN3-containing excitatory glycine receptors. Indeed, recently evidence of the existence of GluN1/GluN3-containing NMDA receptors in hippocampal neurons emerged (Rozeboom et al., 2015). As so far only one study could provide evidence for the existence of GluN3-containing receptors \textit{in vivo} (Piña-Crespo et al., 2010), MDL could be used to search for further evidence of the \textit{in vivo} expression of these unconventional receptors.
5.2.4. Potentiation of GluN3B-Containing Excitatory Glycine Receptors by MDL-29951 and Zinc

The condition where for GluN1-3a- and GluN3B-containing receptors the highest potentiation factor was seen (1 µM glycine and 100 µM zinc) was chosen to investigate the modulatory effects of zinc and MDL. Whereas one might argue that the maximal potentiation was seen in GluN1-3b- and GluN3B-containing receptors, in that case with 1 µM glycine and 300 µM zinc, 100 µM zinc represents the currently accepted maximal concentration that can be released into the synaptic cleft and is therefore more physiologically relevant (Vogt et al., 2000; Qian and Noebels, 2005). Hence, 100 µM zinc was used for this part of the study.

The major question to be clarified here was if the “supralinear potentiation” by zinc and MDL seen in GluN3A-containing GluN1/GluN3 receptors is also preserved in GluN3B-containing GluN1/GluN3 receptors. The first receptor subunit combination that was tested for the effects of zinc, MDL, and the coapplication of both consisted of GluN1-3a and GluN3B. The 11-fold potentiation of glycine-induced currents that is seen upon the coapplication of 100 µM zinc is not significantly exceeded by the potentiation by 0.2 µM MDL (roughly 13-fold). When zinc and MDL were coapplied, the potentiation was 11-fold and thus not supralinear, nor even additive: it was simply at the same level that each compound induced by itself. When the receptor consisting of GluN1-3b and GluN3B was measured, zinc showed a potentiation of only roughly 4-fold. Contrary to the GluN1-3a-containing receptor, MDL potentiated the current response to a greater extent, about 7-fold. The coapplication of zinc and MDL to glycine then, surprisingly, showed again only a potentiation by a factor of 4, which is the level of the potentiation of zinc alone.

Taken together, in GluN1-3a/GluN3B-containing excitatory glycine receptors each compound potentiates the glycine-induced current response by the same factor. However, in GluN1-3b/GluN3B-containing excitatory glycine receptors, MDL potentiated the current response to a greater extent, about 7-fold. The coapplication of zinc and MDL to glycine then, surprisingly, showed again only a potentiation by a factor of 4, which is the level of the potentiation of zinc alone.

This is a remarkable difference between the two receptor combinations that leads to the following conclusions:

1. The only structural difference between a GluN1-3a/GluN3B- and a GluN1-3b/GluN3B-containing excitatory glycine receptor is the presence of the exon 5 of GluN1 in the latter receptor combination. If exon 5 can reduce zinc modulation compared to MDL modulation in the GluN1-3b/GluN3B-containing combination, exon 5 evidently has an influence on the zinc modulation: thus, one structural element influencing zinc modulation resides within or nearby the GluN1 ATD, where the exon 5 is inserted. The MDL binding is assumed to be not altered, as the ligand binding sites in GluN1-3a and GluN1-3b have the same structure.

2. A second structural element, elsewhere in the receptor, influencing zinc modulation exists: the zinc potentiation is reduced, but not abolished by the presence of exon 5 in the GluN1-3b/GluN3B-containing receptor. In that subunit combination, MDL alone potentiates the glycine-induced currents by a higher level than zinc and MDL com-
bined. This suggest a competitive component between zinc and MDL potentiation. As MDL binds at the GluN1 glycine binding site and zinc evidently shows competitive behavior, the second structural element has to involve the glycine binding site in the GluN1 LBD.

3. The potentiation of the glycine-induced current response at GluN1-3b/GluN3B-containing excitatory glycine receptors by zinc is smaller than at GluN1-3a/GluN3B receptors. Thus, the second structural element involving the GluN1 LBD has a lower potency: its effect is in the GluN1-3a/GluN3B-containing receptor most likely “hidden” within the stronger effect mediated by the first structural element that involves the GluN1 ATD.

The GluN3B-containing receptors towards zinc show a different reaction when compared to the GluN3A-containing receptors. Both zinc and MDL potentiate the glycine-induced current responses of GluN3B-containing receptors, but there is no “supralinear” potentiation when zinc and MDL are applied together, a feature that was seen in the GluN3A-containing combination (see also 5.3)(Madry et al., 2008).

5.2.5. Involvement of Structural Elements in the Zinc Modulation

5.2.5.1. Important Structural Elements within the Ligand Binding Domain

Ligand binding in the GluN1 subunit was abolished by mutagenesis. Different amino acids were chosen of which the phenylalanine perpendicular to the glycine in the ligand binding site was the most promising. Thought to shield the agonist from leaving the binding pocket after binding, this phenylalanine forms a π electron interaction with the agonist. Removal of this phenylalanine potentiates the current induced by glycine, most likely because the receptor cannot be desensitized through GluN1 any more, as there is no agonist bound to GluN1 – the receptor is gated only via the GluN3 subunit (Kuryatov et al., 1994). The binding of MDL, which itself is an aromatic ring system, is abolished in the phenylalanine mutants, abolishing also the potentiating effect of MDL. The mechanism by which MDL appears to potentiate currents – stabilizing the closed cleft conformation and thus preventing receptor desensitization – is not possible in this mutant.

The GluN1-3a(F466A) and the GluN1-3b(F487A) mutants do not show any altered behavior towards the agonists. Zinc still potentiates, with the same potentiation factors of the wild type, the only difference being the complete lack of MDL potentiation. This shows that the actual potentiation by zinc is not mediated by the ligand binding site itself. At first glance this contradicts the second conclusion – a structural element involving the GluN1 glycine binding site – yet it supports the idea of an involvement of structural elements within the LBD rather than the actual ligand binding site. Also, if the GluN1-3b(F466A) mutant would destroy the zinc binding in the GluN1-3b/GluN3B-containing receptor, no zinc modulation would be seen at all: the second zinc-modulating element

1In the work conducted by Madry et al. (2008) the supralinear potentiation occurred when 100 µM zinc and 0.2 µM MDL were applied prior to the application of 10 mM glycine. The preapplication of the agonists – MDL, zinc, MDL/zinc – was tested here, too, but showed no different effects than the coapplication.
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negatively influences zinc modulation via exon 5. If both elements were abolished, no zinc potentiation would be existent any more.

All in all, these results stand in clear contrast to the GluN3A-containing receptors, where the GluN1(F466A) mutant abrogated zinc potentiation (Madry et al., 2008). Such a contrast between the behavior of the two GluN3 subunits is impressive, yet not completely unexpected, as both subunits share only 47% sequence identity (Chatterton et al., 2002).

The aromatic residue equivalent to GluN1-3a(F466) and GluN1-3b(F487) in the GluN3B subunit, Y482, had no effect on the potency of either zinc or MDL. The ligand binding domain of GluN3B accordingly has neither influence on zinc potentiation nor MDL potentiation. The latter is not surprising, as MDL is known to bind only to the GluN1 ligand binding site, yet was an important proof of principle for the applicability of MDL.

The GluN1 ligand binding site mutants, carrying the aromatic phenylalanine in position 466 were also tested in coexpression with GluN2A. As expected, the exchange of the phenylalanine for an alanine strongly reduced the glutamate/glycine-induced current responses. However, when this phenylalanine was instead exchanged for a tyrosine the current responses were only marginally altered. Thus, the additional hydroxyl group plays no role for ligand binding.

The effect of the exchange of the corresponding aromatic residue in the GluN3B subunit, a tyrosine in position 482 for an alanine, was tested by coexpression with GluN1-3a. As expected, this mutation strongly reduced the glycine-induced current response. A very interesting effect occurred when the tyrosine was exchanged for a phenylalanine: the agonist-induced current response was even more reduced, virtually destroying receptor function. Thus, whereas the addition of a hydroxyl group in the GluN1 subunit (F → Y) has no effect, the subtraction of a hydroxyl group in the GluN3B subunit (Y → F) destroys receptor function. This is a very interesting effect, as it is intuitive to think that in such a restricted area as the ligand binding pocket the addition of any size-increasing element would rather disturb the ligand binding by steric hindrance. Yet, no such effect was observed. On the other hand, the lack of the hydroxyl group destroys ligand binding in the GluN3B ligand binding site. A possible explanation for this effect is that the hydroxyl group of the tyrosine in the GluN3B ligand binding site may be needed to form hydrogen bonds to hydrophilic side chains of the ligand, and that the GluN1 ligand binding site is considerably less sensitive to such alterations.

5.2.5.2. Important Structural Elements within the Amino Terminal Domain

The most important difference in the ATD of the GluN1 receptor subunit is the presence of exon 5 in the “b” subunits, which renders GluN1-b/GluN2-containing receptors proton-insensitive. However, this “classical” proton modulation site of GluN1 that exists in conventional, GluN1-b/GluN2-containing receptors, has no influence on the proton sensitivity of GluN1/GluN3B-containing excitatory glycine receptors: it was shown that the GluN3B-containing receptors are proton-sensitive independent of the GluN1 splice variant (Cavara et al., 2009). Here, the proton binding deficiency mediated by exon 5 (Traynelis et al., 1998) was mimicked via point mutations (GluN1-3a(N616Q) and GluN1-3a(D669N)) in order to investigate the influence of the classical proton modulation site of GluN1 in a
receptor complex with GluN3B. However, these mutations did not evoke the current behavior seen in GluN1-3b wild type-containing receptors which, relative to the zinc-mediated potentiation, has a higher MDL-mediated potentiation.

The influence of exon 5 in conventional NMDA receptors can be mimicked by these mutations, yet this does not necessarily mean that they mimic all exon 5-mediated effects also in the completely differently structured GluN3B-containing receptors. Although the proton/zinc binding site that modulates conventional NMDA receptors is abrogated, exon 5 can evidently influence GluN3B-containing excitatory glycine receptors via other, so far unknown mechanisms outside of the “classical” modulator site which might be not accessible in conventional, GluN1/GluN2-containing receptors.

As the exon 5 resides in the ATD, the consequential next step was the removal of the ATD to see which part of the zinc-mediated effect might be affected. Two mutants, GluN3B(Δ23-409) and GluN3B(Δ31-396) were generated. The removal of the complete GluN3B ATD (GluN3B(Δ23-409), here named GluN3B(ΔATD)) upon coexpression with GluN1 led to a receptor that showed no current responses in the electrophysiological experiments and was proven to be not membrane-exported (see chapter 5.4.3). The second ATD deletion construct, where a few amino acids remain both at the start and at the end of the N-terminal domain (GluN3B(Δ31-396), here named GluN3B(ΔATDs)), was coexpressed with GluN1 and found to be exported to the plasma membrane, but showed no current responses. It is very likely that this receptor subunit is indeed functional, yet the agonist-induced current responses are below the detection threshold. This is interesting, as Mesic et al. (2016) and Madry et al. (2008) stated that a receptor with a deleted ATD is perfectly functional. It appears possible that the use of a different expression vector might lead to a stronger membrane expression and could push the current responses above the detection threshold.

Beside the removal of the GluN3B ATD, which due to the expression problems described failed to be useful for the investigation of mechanisms underlying the potentation mechanisms, the ATD of the GluN3B subunit was exchanged for the ATD of the GluN1-a or GluN1-b subunits. The domains were exchanged in all possible combinations: if exon 5 has an influence on the mechanism of zinc-potentiation, it might be possible to produce this effect independently of the subunit which it sits on, either GluN1 or GluN3B. Of special interest was the combination of GluN1-3a, the subunit without exon 5, and GluN3B(ΔTD b), with the transferred ATD containing exon 5. Accordingly, the ATD of the GluN3B was exchanged by that of either a GluN1-a or GluN1-b variant. All possible combinations of the mutants were then tested: GluN1-3a with GluN3B(ΔTD 1-a) and GluN3B(ΔTD 1-b), and GluN1-3b with GluN3B(ΔTD 1-a) and GluN3B(ΔTD 1-b). All chimeric receptors formed by these mutants proved to be fully functional and showed robust glycine-induced current responses.

The combination of the receptor subunits GluN1-3a and GluN3B(ΔTD 1-a) does not possess exon 5, yet the ATD of GluN3B is substituted. If the ATD of the GluN3B subunit would have an influence on zinc-potentiation, it should become visible via alterations of the effect of zinc on the glycine-induced current responses. Interestingly, no such effect was observed. This combination behaved, regarding the pattern of zinc and MDL potenti-
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The only difference noted were in potentiation factors, which show a reduction compared to the wild type. These results also showed that the GluN3B ATD does not participate significantly in zinc potentiation.

Building a receptor incorporating exon 5 only at the GluN3B subunit whilst using the GluN1-3a subunit that lacks exon 5 showed another interesting effect. Although this receptor is assembled with the GluN1-3a subunit, regarding the zinc and MDL potentiation it shows the same pattern as the wild type receptor formed by GluN1-3a and GluN3B. 0.2 µM MDL potentiate the current response induced by 1 µM glycine significantly larger than 100 µM zinc alone or zinc and MDL together.

The other receptor combinations, GluN1-3b/GluN3B(ATD 1-a) and GluN1-3b/GluN3B-(ATD 1-b) resembled the wild type GluN1-3b/GluN3B receptor combination. The current pattern of the different agonists was the same as in the wild type, albeit with somewhat reduced potentiation factors.

The results of the GluN1-3a/GluN3B(ATD 1-a) receptor, together with the results of the GluN1-3a/GluN3B(ATD 1-b) chimera, further support the idea of two independent zinc modulation sites. One of these sites is susceptible to inhibition by the loop formed by exon 5, regardless of the exact position. This is also a hint towards the idea that the zinc modulation in the excitatory GluN3B-containing glycine receptors occurs not at the zinc binding site in the GluN1 subunit of the conventional GluN2-containing receptors.

![Figure 5.2: Structural alignment of the zinc-bound GluN2B ATD (3JPY) with the GluN3B ATD (2RCA), and the sequence alignment of the GluN2B and GluN3B ATDs.](image)

To further support this results, the sequences of the GluN2B ATD and the GluN3B ATD were aligned in order to check for possibly existing similarities. Specifically, the GluN2B was used as a crystal structure for the zinc-bound GluN2B ATD exists (3JPY, published in 2009 by Karakas et al.). Additionally, a structural alignment of the GluN3B ATD (2RCA, Yao et al. 2006) with the zinc-bound GluN2B ATD (Karakas et al., 2009) was generated.
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using SWISS-MODEL (Arnold et al., 2006; Guex et al., 2009; Kiefer et al., 2009; Biasini et al., 2014). The sequence alignment showed that in the GluN3B subunit the amino acid residues that are in the GluN2B subunit the most important to coordinate zinc, H127 and E284, have no corresponding residues. The analysis of the structural alignment supported this, as there is no homologous amino acid in the corresponding part of the ATD of the GluN3B. The results are shown in Figure 5.2.

Also, the participation of the GluN3B ATD in the zinc modulation can be excluded, as its exchange did not alter the receptor activation or the modulation pattern. As the presence of exon 5 in the receptor, even when only present in the GluN3B subunit, always led to the zinc-mediated potentiation pattern seen in the wild type, this supports the idea of one element that lies within the ATD of the receptor. It is conceivable that the potentiation by zinc happens somewhere at the dimer interface between the GluN1 and GluN3 ATDs: the classical proton and zinc modulation site that can be blocked by exon 5 lies within the GluN1 ATD. In the GluN3B-containing receptors, this classical modulation site has no effect when its properties are mimicked in the GluN1-3a subunit. As the GluN3B ATD does not influence the behavior towards zinc, but the behavior can be modulated by exon 5 regardless of the subunit it sits on in the tetrameric receptor complex, the modulatory site must sit somewhere close to or between the ATDs to be in reach of the exon 5, even when it resides in the GluN3B subunit. If it would sit somewhere else at the GluN1 subunit it would be out of reach for the exon 5 when that is present in the chimeric GluN3B subunit (the GluN3B(ATD 1-b) subunit), yet the receptor composed of GluN1-3a/GluN3B(ATD 1-b) shows the same modulation pattern as the GluN1-3b/GluN3B wild type.

5.2.5.3. Competition between Zinc and MDL-29951

The experiments with the wild type receptors GluN1-3a/GluN3B and GluN1-3b/GluN3B suggested that there is competition between MDL and zinc. The potentiation by MDL at GluN1-3b-containing receptors reached a higher factor than that mediated by zinc; yet, the potentiation mediated by zinc and MDL together resulted in the same factor as for zinc alone. The use of 200 µM MDL, a concentration 1000-fold higher than the usually used concentration and 2-fold higher than the zinc concentration potentiated the glycine-induced current as expected. The glycine-induced currents were as expected potentiated by 200 µM MDL, as the usually used 0.2 µM are already saturating. When 200 µM MDL and zinc were coapplied, the potentiation seen had the same level as that reached by the application of MDL alone.

It is therefore evident that the zinc and MDL binding site share – and can compete for – similar structural elements, even though mutations of the ligand binding site affect glycine efficacy and MDL potentiation, but not zinc modulation.

5.2.5.4. Derived Structural Implication

Regarding zinc the GluN1/GluN3B-containing excitatory glycine receptor shows a dichotomous behavior towards the coapplication of zinc and glycine: zinc can both block the glycine-induced currents and potentiate them. This potentiation can be divided into two
5.3. Comparison Between GluN3B- and GluN3A-Containing Receptors

components, one influenced by, one independent of exon 5. A schematic representation of
the deduced zinc modulatory sites is displayed in Figure 5.3.

The more potent element, which can be influenced by exon 5, most likely resides some-
where at the ATD dimer interface of the GluN1 and GluN3B subunits. It is not one of the
classical proton and zinc modulation sites (Traynelis et al., 1995, 1998) as mutants that are
known to mimic the effects of exon 5 showed no effect. Also, this element is not present
in GluN1/GluN3A-containing excitatory glycine receptors, as in these combinations the
ATD had no influence on zinc modulation (Madry et al., 2008).

Figure 5.3.: Schematic representation of postulated zinc modulation sites in excitatory
glycine receptors containing GluN1-3a/GluN3B or GluN1-3b/GluN3B. A: GluN1-3a/GluN3B-
containing receptors. B: Receptors combining GluN1-3b and GluN3B.

The second element involves structural features within the ligand binding domain. How-
ever, it lies not directly at the ligand binding site: zinc shows competition with the GluN1
glycine binding site antagonist MDL, but abolishing the binding of the antagonist does
not prevent zinc from potentiating the current response. This is also a major difference
compared to GluN1/GluN3A-containing receptors, where the zinc-mediated effect could
be tied down to the glycine binding site (Madry et al., 2008).

5.3. Comparison Between GluN3B- and GluN3A-Containing
Receptors

Although the GluN3A-containing excitatory glycine receptor is known to be potentiated by
zinc and MDL, and that both MDL and zinc together lead to a potentiation that is higher
than just additive (Madry et al., 2008), it is important to know if there are differences
between the GluN3A- and the GluN3B-containing receptors with respect to modulation
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by zinc and MDL and to compare the concentration dependency of both modulators. This is especially important as Madry et al. (2008) analyzed very few and very different, non-intuitive concentrations: the current response induced by 1 mM glycine was potentiated by 50 µM zinc about 10-fold. The potentiating effect of MDL (24-fold compared to zinc), however, was measured at 10 mM glycine, not at 1 mM as for zinc. The 120-fold “supralinear” potentiation of 0.2 µM MDL was then measured not with 50 µM zinc, but with 100 µM and again at 10 mM glycine, not 1 mM as was used for the controls. As the effects of agonists and antagonists are strongly concentration-dependent and show different effects, the comparability of these different concentrations and the calculation of the potentiation factors is disputable.

Besides this, until recent findings by Cummings and Popescu nothing was known about the proton sensitivity of GluN3A-containing receptors. To counter this lack of information the pH dependency of glycine-mediated current responses in the GluN3A-containing receptor was tested.

5.3.1. The 5' UTR of GluN3A Influences its Electrophysiological Properties

GluN3A-containing receptors can be potentiated by both zinc and MDL (Madry et al., 2008) at certain concentrations. To see how the GluN3A-containing receptors behave when directly compared to the GluN3B-containing receptors, the very same concentrations used on the latter combination (1 µM glycine, 100 µM zinc and 0.2 µM MDL) were applied. GluN3A-containing receptors are not as susceptible towards the GluN1 glycine binding site antagonist MDL as GluN3B-containing variants: it was interesting to see that zinc was able to potentiate the glycine-induced currents by a factor of 2.9-fold, but 0.2 µM MDL alone showed no effect. Also, for this combination of concentrations the “supralinear” potentiation by zinc and MDL reported by Madry et al. (2008) did not occur.

As the current responses gained with the GluN3A-containing receptors were generally very low, the 5' UTR of the receptor was removed in an attempt to raise the agonist-induced currents, as the 5' UTRs of mRNAs can contain binding sites for other proteins sometimes negatively affecting translation. Removing the 5' UTR of the GluN3A receptor, however, did hardly affect the steady-state amplitude of the current. Zinc could potentiate the glycine-induced current as usual, with a slightly higher potentiation factor (4.2-fold) than the construct containing the 5' UTR (2.9-fold). This difference in potentiation factors potentially can also be a batch-dependent effect, as the actual potentiation factors between different batches do not always share the exact same value. MDL again was not capable of potentiating the current response. When zinc and MDL were coapplied with glycine, the potentiation surprisingly reached a much higher current response (14.0-fold) than for coapplication of zinc alone. Although MDL alone showed no effect, it can further enhance the zinc-induced current potentiation. Due to the huge effect of zinc and MDL, the effect by zinc alone, although recognizable as a tendency, appears to be not significant; a statistical fluke effect due to the one-way ANOVA test, which could be overcome by a higher n.

Why the potentiation occurred only after the removal of the 5' UTR is baffling and still awaits a convincing explanation. Although UTRs in general are known to contain binding
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sites for modulatory proteins affecting the translation, no such effect has been described for GluN3 subunits. The 5' UTR of the GluN3A subunit contains 473 bp. A daring hypothesis might be that within this region an unknown regulatory element or even protein is encoded. However, no open reading frame (orf) can be found within this area. The mechanism by which the absence of the 5' UTR generates the capability of the receptor to be modulated therefore remains elusive. However, it is rather a curiosity and will most likely play no role in vivo, as in vivo the UTRs are always present, and therefore this non-linear behavior of zinc together with a glycine binding site antagonist will most likely not occur.

5.3.2. GluN3A-Containing Receptors are Proton-Insensitive

Whereas it is known that the GluN3B-containing excitatory glycine receptors are proton-sensitive, until recently no such data existed for GluN3A-containing receptors. Only recently evidence was reported that protons affect GluN3A-containing receptors, potentiating their peak currents by reducing desensitization (Cummings and Popescu, 2016). These data were obtained in HEK293 cells.

The data shown here, however (see chapter 4.4.3) paint a different picture. Although there are minimal differences between the current responses recorded at different pH values (7.2 compared to 6.8), none of these differences are statistically significant. This effect is completely independent of the GluN1 splice variant analyzed.

Nevertheless, these results do not necessarily contradict each other. One of the limitations of the oocyte expression system is the lack of the possibility to measure single-channel kinetics, so that no statement about the peak currents or desensitization kinetics can be made, which might be enhanced. Still, as the claim is that the reduced desensitization is the working mechanism, the effect should be recognizable also in oocytes, which it does not. It is therefore most likely that there is some as of yet unidentified factor or protein that is not present in one of the two expression systems, and that has an influence on the electrophysiological properties. This is of course plausible, as the two expression systems are completely different, both having advantages and disadvantages: The HEK cell system is a human-derived cell system, whereas the oocyte expression system makes use of frog oocytes.

5.4. On the Use of eGFP-Tagged NMDA Receptor Subunits

The tagging of glutamate receptors with eGFP is a long-known approach to investigate their plasma membrane expression. Also, the interaction with, e.g. auxiliary subunits such as the TARP family can be investigated via fluorophore tagging, in particular the BiFC method: half the fluorophore is attached to the receptor, the second half to the potentially interacting protein of choice. If the protein interacts with the receptor it comes close enough to allow the two non-fluorescent halves of the fluorophor to form the intact, fluorescence-competent fluorophore. The fluorescence can than be detected for example by confocal microscopy.
5. Discussion

In an attempt to explore the usefulness of tagged receptor for expression control, GluN1-3a, GluN1-3b, and GluN3B were covalently tagged with eGFP. Although there are some drawbacks, it could be shown that tagging receptor subunits with eGFP is a proper tool to test NMDA receptor expression. As for the expression of a functional NMDA or excitatory glycine receptor always two subunits are necessary, and as one subunit alone (except, to a certain extent, the GluN1 subunit) is not membrane-exported, the use of one eGFP-coupled subunit is sufficient to prove the plasma membrane expression of the entire complex. When the tagged wild type receptors are available, the expression of any mutant receptor can easily be tested: the tagged wild type, e.g., a GluN2 subunit, is coexpressed with a mutant GluN1 subunit. As only an intact receptor is exported, membrane fluorescence means membrane expression, and any electrophysiological impairments thus can not be due to failed plasma membrane export.

However, the amount of protein expression cannot be reliably quantified with this method, as the fluorescence heavily depends on the regional expression in the oocyte membrane, and the exact positioning of the oocyte, which is required to allow for perfect comparability, cannot be guarantied at all times.

5.4.1. Weak Plasma Membrane Export of the GluN1-3 Subunit

The GluN1-3 splice variant contains an ER retention signal within the C1 cassette of exon 21, which can be overridden by the C2' cassette that carries a PDZ binding motif, leading to membrane export all the same (Sugihara et al., 1992; Hollmann et al., 1993; Standley et al., 2000). The GluN1-3 subunit was chosen for this investigation as it should be membrane-exported to a certain degree, yet the level of export was unclear.

If the forward-trafficking of the GluN1-3 subunit would be as pronounced as postulated, high background fluorescence would be seen in all batches expressing a tagged GluN1-3 subunit. However, in some batches a bright fluorescence occurred in the control oocytes which expressed only GluN1-3a-eGFP or GluN1-3b-eGFP, whereas most batches showed only little background fluorescence. The background fluorescence therefore appears to depend mainly on the expression of the oocyte-endogenous *Xen* GluN2B subunit that coassembles with the GluN1 subunit to a membrane-exported receptor (Schmidt and Hollmann, 2008). Oocyte batches with strong background fluorescence caused by endogenous *Xen* GluN2B were discarded and not used for analysis. When the normally low background fluorescence was compared to the positive controls, which incorporated GluN1-3a or GluN1-3b together with GluN3B, it was very low. Thus, the plasma membrane export of individually expressed GluN1-3 subunits is not as strong as thought, which in turn means that the PDZ binding motif in the C2' cassette is not as powerful as thought so far: if it were more powerful, the membrane fluorescence of the tagged GluN1-3 subunits expressed alone would reach the level of the positive controls in all cases, not only in a few batches and should not fluctuate that much between the batches.

None of the GluN1 subunits is capable of forming a functional, homomeric receptor; thus, the coexpression of at least one additional subunit (GluN2 or GluN3) is required (Monyer et al., 1992; Laube et al., 1998; Ulbrich and Isacoff, 2007). From a physiological point of view this makes sense: an inefficient membrane export of monomeric, non-functional receptors
5.4. On the Use of eGFP-Tagged NMDA Receptor Subunits

is a good way to prevent the protein synthesis machinery of a cell to produce protein that is not required – a feedback mechanism designed to save metabolic energy. While the GluN1-2 and GluN1-4 subunits carry no known ER retention signals and accordingly are transported to the plasma membrane, the lack of membrane export of the GluN1-1 subunit and the weak membrane export of the GluN1-3 subunit clearly show the consequences of ER retention signals (Standley et al., 2000).

5.4.2. Uneven Spatial Expression of eGFP-Tagged Receptors

The membrane expression of the tagged receptor subunits is not equally distributed across the oocyte plasma membrane. The oocytes injected only with a single GluN1-3a or GluN1-3b subunit tagged with eGFP that were used as negative/background controls showed in most cases a weak membrane fluorescence that occurred mainly in the vegetal (light-colored) pole of the oocyte. The positive controls, receptors with a tagged GluN1-3a or GluN1-3b subunit together with GluN3B, showed bright fluorescence all over the oocyte plasma membrane, independently of the animal or vegetal pole. In some batches, presumably those with a high expression of the Xen GluN2B, the fluorescence of the negative controls reached levels as high as those in the positive controls in both poles. Such batches had to be taken out: with high fluorescence also in the control oocytes, the actual sample signal might also be mediated by the background and would lead to false positives.

Altogether, a certain background fluorescence is always present and noticeable in the vegetal pole. Whether this background fluorescence is mediated by the assembly of the tagged GluN1-3 subunit with the endogenous Xen GluN2B or only via its own forward-trafficking can also be explained. It is most likely mediated via the plasma membrane export of the GluN1-3 subunit alone: the background fluorescence was always present, yet in most batches negligible. Only the strength of the signal varied between the batches. This variation in strength then can be explained by the assembly with the endogenous Xen GluN2B, as a high level pushes the background fluorescence, sometimes up to the level of the positive control, and the expression level of the Xen GluN2B is known to be highly batch-dependent (Schmidt and Hollmann, 2008). A promising approach to further elucidate this would be to perform electrophysiological measurements to quantify the Xen GluN2B-mediated background in the same oocytes that are used for control of plasma membrane expression. Unfortunately, due to time limitations, this experiment could not be carried out. The spatial differences in the fluorescence can result from the melanin present in the animal pole, as melanin can quench the light of the fluorescence with high efficacy.

The eGFP-tagged GluN3B subunit showed in single expression without GluN1-3 no abnormality and only in some experiments hardly noticeable background fluorescence. As the presence of an ER retention signal as well as the lack of a forward trafficking signal is known, this behavior fits the data published in previous studies (Matsuda et al., 2003).

5.4.3. Membrane Expression of Chimeric and Mutant Receptors

To exclude the possibility of the electrophysiological data, in particular the negative data, to be only artifacts of the expression system, the plasma membrane export of the receptor
has to be proven. The mentioned approach with eGFP-tagged NMDA receptor subunits, where the eGFP-tagged wild type of one of the two mandatory subunits is coexpressed with the mutant of interest as the second subunit was successful. The membrane export of all mutant receptors but one variant could be proven. This exception is the GluN3B(ΔATD) mutant, for which no fluorescence could be seen, neither when coexpressed with GluN1-3a nor with GluN1-3b. This was observed in various batches.

The second ATD-deleted subunit, GluN3B(ΔATDs), was membrane-exported, as the bright fluorescence indicated. The difference between both mutants is that in the “short” (s) variant 8 AS are left of the 5’ end of the ATD and 13 AS of the 3’ end. These short “linkers” are evidently of considerable importance for the membrane export. As directly ahead of the ATD and the start codon the SP is placed, it is possible that, without some short linkers, the SP is too close to the LBD. Thus, it might be that the SP can not be recognized by the signal recognition particle (SRP) or later, in the ER, by the signal peptidase, and further translocation is impaired. It is also conceivable that the stability of the LBD might be impaired and the receptor subunit is recognized as misfolded and degraded.

5.5. Open Questions

It is obvious from the data that the zinc modulation in the GluN3B-containing excitatory glycine receptor is mediated by two separate structural elements. One of those lies at the ATD of the GluN1 subunit and is only accessible upon coassembly with GluN3B, but not with GluN3A or a GluN2 subunit. It is most likely facing the interface of the GluN1/GluN3B dimer, as it can be influenced by exon 5. It remains elusive which exact amino acids within the GluN1 subunit are involved in zinc modulation. A mutational approach could scan for cysteines or histidines at the surface of the GluN1 ATD, as such residues are known to modulate zinc modulation behavior in conventional, GluN2-containing receptors (Choi and Lipton, 1999; Fayyazuddin et al., 2000; Paoletti et al., 2000; Choi et al., 2001; Karakas et al., 2009). One future experiment could also be to extend the chimeric approach and build GluN1(ATD 3B) receptors and to combine those with the GluN3B(ATD 1-a) and GluN3B(ATD 1-b) subunits.

Concerning the GluN1-3a/GluN3A-containing receptor investigated here, it is unexplainable why the “supralinear” effect of the potentiation of MDL and zinc combined with glycine occurs only when the 5’ UTR of the GluN3A receptor is removed. The results concerning the lack of proton-sensitivity of the GluN3A-containing receptors presented here stand in contrast to a recent publication showing a proton modulation of these receptors (Cummins and Popescu, 2016). This discrepancy might originate from the different expression systems, as here the Xenopus oocyte system was used and not, as in Cummings and Popescu, the HEK cell expression system. Most likely, some not yet identified factors or proteins that are existent only in one of the two expression systems have some additional modulatory influence. However, further research needs to be conducted to clarify this interesting divergence which might hold clues to the actual mechanism of proton modulation in GluN1/GluN3A-containing excitatory glycine receptors.
5.6. Conclusion

Another open question is how strong the surface expression of the other GluN1 subunits is. GluN1-2 and GluN1-4 subunits should be, when expressed alone, transported to the membrane at the same extent as when they are coexpressed with a GluN3 or GluN2 subunit. The GluN1-1 subunit should not be exported at all. Tagging with eGFP followed by confocal microscopy expression analysis of the GluN1-1, GluN1-2, and GluN1-4 subunits not investigated yet is the logical approach.

The proven existence of triheteromeric receptors containing GluN1, GluN2, and GluN3 (Perez-Otano et al., 2001; Schüler et al., 2008) has also to be taken into further consideration. As GluN3B lowers the Ca\(^{2+}\) permeability and the Mg\(^{2+}\) block of the conventional, GluN1/GluN2-containing receptors, it will be interesting to see what influence zinc has on a triheteromeric GluN1/GluN2/GluN3-containing receptor.

5.6. Conclusion

The GluN3B-containing excitatory glycine receptors have two zinc modulation sites that can act independently: one in proximity to – and sharing structural elements with – the ligand binding domain in GluN1 and a second one at the interface of the GluN1 and GluN3B ATD. This second element supposedly lies in the area of the proton modulation site of conventional NMDA receptors.

The specific activation pattern described here upon the application of glycine and zinc shows that zinc plays a very important role in regulating receptor activity. Concerning GluN1/GluN3B-containing excitatory glycine receptors, it seems that these receptors, just like the GluN1/GluN3A-containing receptors (Das et al., 1998; Henson et al., 2012) have a neuroprotective role: the activity of the receptor is lowered by zinc at almost all concentrations of glycine, preventing the receptor from overstimulation. If these receptors exist in synapses, they might attenuate the synaptic signal and prevent synaptic overexcitation. A weakening of synaptic signaling after induction of LTP by recruitment of GluN1/GluN3-containing receptors into hippocampal neurons has indeed recently been shown by Rozeboom et al. in 2015. As zinc is thought to be able to be co-released with glutamate and glycine into the synaptic cleft (Assaf and Chung, 1984; Frederickson et al., 2006), zinc is at the right place at the right time to play an important role in synaptic homeostasis.

Additionally, the coactivation by zinc, only seen upon neatly orchestrated concentrations of zinc and glycine, might be beneficial: in synaptic signalling, precise and strong signals are extremely important, especially for mechanisms as LTP and LTD that undergo fast changes, but alter the signalling on a long term. Indeed, the here shown matrix of glycine and zinc concentrations shows exactly that: the possibility to evoke a very strong signal by a very precise trigger. This would be the here described combination of a low glycine and a high zinc concentration, where the basal current response induced by 1 µM glycine could be enhanced up to 4-fold by simply adding – physiological existing and possible – 100 µM zinc. The existence of such a mechanism could thereby substantially enhance the possibility of fine-tuning the synaptic signal transduction if GluN1/GluN3B-containing receptors are present in synapses in vivo.
On the other hand, a prolonged coactivation of the GluN1/GluN3B-containing receptor by zinc could potentially also lead to overactivation. This perfectly fits the fact that zinc homeostasis is – and has to be – precisely tuned, as dysregulation is connected with various diseases such as Alzheimer’s disease (Bush et al., 1994a,b) or epilepsy (Cole et al., 2000). Overactivation might lead to zinc-related negative effects such as cell death. However, if in a disease state GluN1/GluN3B-containing excitatory glycine receptors are affected, it might be possible to counteract potentially negative effects, such as overexcitation mediated by these receptors, via the regulation of zinc homeostasis, i.e. via a downregulation of zinc release.

Also, conventional NMDA receptors are important for medical intervention. In some diseases, the downregulation of NMDA receptors is desirable to lower glutamergic excitability. One example is epilepsy, where overexcitability of receptors has to be attenuated. Yet, populations of excitatory glycine receptors containing GluN1 and GluN3 that might reside in the PSD could be potentiated by these antagonists, as exemplarily shown here for MDL-29951. When an attenuation is tried with NMDA receptor antagonists that act at the GluN1 ligand binding site, this might lead to side effects, as instead of an attenuation a potentiation might be induced. This would counteract the desired attenuation of the excitation and might be one of the reasons why only few clinical trials that took this approach had a positive outcome (Muir, 2006).

Further proof of the existence of excitatory combinations in other areas of the brain besides the optic nerve myelin is therefore urgently needed. Recent evidence of the existence of GluN1/GluN3-containing NMDA receptors in hippocampal neurons by Rozeboom et al. (2015) is therefore an important development and shows the potential importance of these receptor combinations.

Ultimately, the results presented in this work further show the importance of the rather neglected GluN3B subunit when combined with GluN1 into an excitatory glycine receptor: not only it this receptor differentially modulated by zinc, but it also can be activated instead of blocked by a classical antagonist of the GluN1 subunit.
6. Bibliography


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A. Appendix

A.1. Pictures of eGFP-Tagged NMDA Receptor Subunits

Figure A.1.: Tagged receptor fluorescence compared to uninjected oocytes and negative controls. A and B: Uninjected oocytes compared to negative controls injected only with a tagged GluN1-3 subunit and positive controls injected with tagged GluN1 and GluN3B. C and D: GluN3B-eGFP expressed alone compared to uninjected oocytes and positive controls. E and F: Expression test of the indicated GluN1 mutant by GluN3B-eGFP. Scale bar = 150 µM.
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Figure A.2.: Tagged receptor fluorescence compared to controls. A, B, and C: Indicated GluN1 mutants tested with the eGFP-tagged GluN3B subunit. D – H: Indicated GluN3B mutants tested with GluN1-3a-eGFP or GluN1-3b-eGFP. Scale bar = 150 µM.
Figure A.3.: Tagged receptor fluorescence compared to controls. **A:** Expression of GluN3B(ATD 1-a) tested with GluN1-3a-eGFP (left) and GluN1-3b-eGFP (right). **B:** Expression of GluN3B(ATD 1-b) tested with GluN1-3a-eGFP (left) and GluN1-3b-eGFP (right). Scale bar = 150 µM.

Figure A.4.: Receptor fluorescence of the GluN3B(ΔATD) mutants compared to negative controls and positive controls. **A:** GluN1-3b-eGFP injected alone as negative control (left) together with the positive control injected with GluN1-3b-eGFP and GluN3B (right) compared to GluN1-3b-eGFP coinjected with GluN3B(ΔATD)(below). **B:** As in A), with GluN1-3a-eGFP. Scale bar = 150 µM.
A. Appendix

A.2. Curriculum Vitae

Name: Kevin Lars Gottschling
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Education

10/2011 – present PhD Thesis at the Department of Receptor Biochemistry, Ruhr University Bochum
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A.3. List of Publications

Original Publications


Poster Presentations


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No quote this time.