Dissertation

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Regulation of AMPA receptors by type II-TARPs

The research was conducted at the Department of Biochemistry I - Receptor Biochemistry, Ruhr-University Bochum

Sandra Lemos

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“Live as if you were to die tomorrow. Learn as if you were to live forever.”

- Mahatma Gandhi
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<tr>
<td>A</td>
<td>alanine</td>
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<tr>
<td>aa</td>
<td>amino acid</td>
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<td>amp</td>
<td>ampicillin</td>
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<td>double destiled water</td>
</tr>
<tr>
<td>ddNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>desoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxothymidine triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>glutamate</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia Coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>F</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>G</td>
<td>glycine</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GK</td>
<td>guanylate kinase</td>
</tr>
<tr>
<td>glu</td>
<td>glutamate</td>
</tr>
<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>GRIP</td>
<td>glutamate receptor interacting protein</td>
</tr>
<tr>
<td>GSG11</td>
<td>germ cell-specific gene</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5′-triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>H</td>
<td>histidine</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>high fidelity</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>I</td>
<td>isoleucine</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic glutamate receptor</td>
</tr>
<tr>
<td>IRES</td>
<td>encephalomyocarditis virus internal ribosome entry site</td>
</tr>
<tr>
<td>I/V</td>
<td>current/voltage</td>
</tr>
<tr>
<td>K</td>
<td>lysine</td>
</tr>
<tr>
<td>KA</td>
<td>Kainite</td>
</tr>
<tr>
<td>kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>KAR</td>
<td>Kainite receptor</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>L</td>
<td>leucine</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>methionine</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>mEPSC</td>
<td>miniature excitatory postsynaptic current</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MgR</td>
<td>magnesium Ringer</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>asparagine</td>
</tr>
<tr>
<td>NETO</td>
<td>Neuropilin Tolloid like</td>
</tr>
<tr>
<td>NFR</td>
<td>normal frog Ringer</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside triphosphate</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ori</td>
<td>origin of replication</td>
</tr>
<tr>
<td>P</td>
<td>proline</td>
</tr>
<tr>
<td>PA</td>
<td>polyacrylamide</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PNK</td>
<td>polynucleotide kinase</td>
</tr>
<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
<tr>
<td>PSD-95</td>
<td>postsynaptic density protein 95</td>
</tr>
<tr>
<td>Q</td>
<td>glutamine</td>
</tr>
<tr>
<td>R</td>
<td>arginine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>S</td>
<td>serine</td>
</tr>
<tr>
<td>SAP</td>
<td>synapse-associated protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SOL</td>
<td>suppressor of lurcher</td>
</tr>
<tr>
<td>SV</td>
<td>simian virus</td>
</tr>
<tr>
<td>SynDIG</td>
<td>synapse differentiation-induced gene</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TARP</td>
<td>transmembrane AMPA receptor regulatory protein</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borat-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TG</td>
<td>tris-glycine</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>valine</td>
</tr>
<tr>
<td>W</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>YT</td>
<td>yeast extract and tryptone growing medium</td>
</tr>
</tbody>
</table>

Standard abbreviations were used for units
Abstract

Glutamate is the most common neurotransmitter in the CNS. At the synapse, glutamate binds to different receptors, which include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. AMPA receptors are in turn responsible for the vast majority of fast excitatory synaptic transmission in the brain and contribute to synaptic plasticity. They are therefore responsible for complex cognitive processes such as memory and learning. Abnormal AMPA receptor function is associated with the development of many cognitive and neurodegenerative diseases. Hence, it is crucial to understand the molecular mechanisms behind AMPA receptor activity in order to fully comprehend brain function and associated disorders.

In vivo, AMPA receptors interact intimately with transmembrane AMPA receptor regulatory proteins (TARPs). These proteins are divided into two different subfamilies according to their sequence homology and functionality. Type I-TARPs regulate nearly all of the receptor’s properties, including their pharmacology, trafficking, and gating. Their regulatory influence on AMPA receptors is profound, as they raise their agonist-induced currents by many fold. Since their discovery in the early 90s’ they have been extensively studied and, especially in heterologous expression systems, are now used as a reliable tool to increase AMPA receptor responses.

Despite their high sequence homology, the other members of the TARP family, γ5 and γ7, are distinguished as type II-TARPs. They have unusually short C-termini that lack the characteristic PDZ-binding domains that are conserved in type I-TARPs. Thus, they are unable to interact with scaffolding proteins that assist in AMPA receptor trafficking, clustering and synaptic delivery. Their role is therefore purely in the modulation of the biophysical properties of AMPA receptors, and it is more discrete. Consequently, they have been initially disregarded as modulatory proteins, and it was not until recently that they were classified as true TARPs. Hence, very little is known about type II-TARPs when compared to their type I relatives.

This thesis was aimed at investigating the different aspects of type II-TARP modulation of AMPA receptors. In addition to type II-TARPs, γ6, a homologous protein currently not classified as a TARP, was also considered as a potential TARP candidate. However, γ6 did not show any TARP-like behavior, confirming its classification as a
non-TARP. Despite its lack of AMPA receptor functional modulation, γ6 showed interaction with all the analyzed receptors in bimolecular fluorescence complementation (BiFC) assays. Furthermore, interaction of γ6 with calcium permeable receptors was at the same level than that of γ5 and γ7, while its interaction with calcium impermeable receptors was even stronger than that observed with the type II-TARPs, and comparable to that of γ2.

Both type II-TARPs successfully modulated currents of all the analyzed AMPA receptors, homomeric and heteromeric receptors alike. γ7 potentiated steady-state currents of all the receptors indiscriminately, much like a type I-TARP, although to different degrees. The ratios of kainate- to glutamate-induced currents were in general increased by γ7. The observed modification of these ratios indicates a slowing of the receptor’s desensitization kinetics and an increased agonist efficacy.

In contrast, γ5 modulation of AMPARs was strongly dependent on the type of receptor analyzed. Differential modulation was primarily determined by the amino acid at the Q/R editing site. Agonist-induced responses of receptors that express a glutamine at the Q/R editing site were decreased by γ5, while agonist-induced responses of receptors that instead contain an arginine at this position were potentiated. Furthermore, GluA1 mutants that express other amino acids at the Q/R editing site were regulated in a variety of ways, as their current responses were either potentiated or inhibited. However, the determinant feature of this specific amino acid at the Q/R editing site, that defines the direction and efficiency of γ5 modulation, continues to elude us. The data further indicates that γ5 influences ion channel kinetics in a very distinctive way. While all the other TARPs seem to slow down AMPA receptor desensitization and deactivation rates, γ5 has shown evidence that it might, on the contrary, accelerate channel kinetics. This TARP also showed a positive effect on channel conductance through an increase of its permeability to calcium, in a Q/R editing site independent manner. The data further supports the notion that γ5 is not involved in trafficking, since its co-expression with different Q/R editing site GluA1 mutants did not alter their membrane protein expression levels.

Both TARPs, but not γ6, attenuated rectification of calcium permeable receptors, suggesting a reduction of the receptor’s affinity for intracellular polyamines.

The unique γ5 properties in combination with the existence of five other TARPs as well as other auxiliary proteins offer the possibility of an intricate fine regulation of the diverse AMPA receptors found in the different neuronal cell types.
1 Introduction

1.1 Synapses and signal transmission in the CNS

We have come a long way since the father of modern neuroscience, Ramón y Cajal, first studied the brain at a more microscopic level (Ramón y Cajal 1909). He proposed that neurons, even though physically independent from each other, establish communication with each other through synapses. Via the synapse, neurons are able to communicate with thousands of other cells, forming complex neuronal networks.

Besides neurons, glial cells compose the nervous system. These cells fill the spaces between neurons and intimately cooperate with them, providing for support and protection.

Neurons are electrochemically excitable. Through the synapse, they receive information, process it and transmit it further along using a combination of chemical and electrical signals. This communication between neurons is only possible due to the selective passage of ions, across different ion channels at the plasma membrane.

While at electrical synapses a direct connection between the somata of two neurons through gap junctions allows for fast signal propagation, at chemical synapses, signal transmission is mediated by neurotransmitters. In this case, neurotransmitters are released by the presynaptic cell into the synaptic cleft, in response to an action potential.

1.1.1 Chemical synapses

Neurotransmitters are stored in synaptic vesicles at the resting presynaptic cell. Once the presynaptic cell becomes depolarized in response to an action potential, the voltage-sensitive Ca\(^{2+}\) channels in its membrane open, allowing for Ca\(^{2+}\) to enter the cell. The increase in concentration of intracellular Ca\(^{2+}\) triggers the exocytosis of synaptic vesicles, and consequently leads to neurotransmitter release into the synaptic cleft.
By diffusion, the neurotransmitter reaches a variety of specialized proteins, present at the postsynaptic membrane, which contain specific binding sites for the neurotransmitter. Upon neurotransmitter binding, a flux of ions enters the postsynaptic cell, either directly through ligand-gated ion channels, or indirectly by activation of G protein-coupled receptors. In theory, many ion channels can be simultaneously activated by the release of a single vesicle (Kandel et al 2000).

Depending on the type of neurotransmitter and receptor, a synapse can be either excitatory or inhibitory. In case of cell depolarization, the resulting synapse is excitatory and an action potential is propagated. When instead, there is a hyperpolarization of the cell’s membrane potential, the synapse is inhibitory and the action potentials is suppressed. A classic example of inhibitory synapses are the GABAergic synapses, while glutamatergic synapses are excitatory.

## 1.1.2 Synaptic plasticity

The difference in charge between the outside and the inside of a neuron defines its membrane potential. The strength of the synapse is thus defined by the number of ions that enter the post-synaptic cell changing its potential. Synapses are exceptionally plastic and able to get stronger or weaker as they become more or less active, as a consequence of repeated stimulation. The ability of the synapse to change its strength or efficacy is called synaptic plasticity. Synaptic plasticity is depends on both presynaptic and post-synaptic events. For example, it can be determined by the number of receptors at the post-synaptic cell (Gerrow & Triller 2010) and by the amount of neurotransmitters released by the pre-synaptic cell (Gaiarsa et al 2002).

The strength of synapse transmission can be modified for periods in the order of milliseconds to a few minutes (short-term plasticity) or as long as a few minutes to a few weeks or maybe even longer (long-term plasticity) (Cooke & Bliss 2006, Stevens & Wesseling 1999).

There are two main mechanisms of long-term synaptic plasticity. If the association between neurons becomes consistently stronger, there is long-term potentiation (LTP), if it becomes weaker there is long-term depression (LTD). LTP is thought to be the key event to the processes of learning and memory (Lynch 2004, Martin & Morris 2002, Park et al 2015)
1.2 Glutamate receptors

Glutamate is the main excitatory neurotransmitter in the vertebrate central nervous system (CNS). Additionally, it is also the precursor for GABA, its main inhibitory neurotransmitter (Petroff 2002).

Glutamate is responsible for the activation of two very different families of glutamate receptors (GluRs), which are classified based on the mechanism by which they are activated (Niswender & Conn 2010). The ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that open directly upon the binding of an agonist, mediating fast synaptic transmission. The relatively slow-acting metabotropic glutamate receptors (mGluRs) (Conn 2003, Ferraguti & Shigemoto 2006), on the other hand, are coupled to GTP-binding proteins, which indirectly activate ion channels by initiating an intracellular signaling cascade. Together, iGluRs and mGluRs are accountable for most of the excitatory signal transmission in the CNS, and play a core role in the modulation of synaptic plasticity (Debanne et al 2003), which is essential for the processes of memory formation and learning (Bliss & Collingridge 1993, Woolf & Salter 2000). Glutamate receptors are present at neuronal and non-neuronal cells and have been associated with various diseases (Bitanihirwe et al 2009, Bolton & Paul 2006, Cuomo et al 2009, Diguet et al 2004, Dorval et al 2007, Johnson et al 2009, Schmeisser et al 2012).

1.2.1 Ionotropic glutamate receptors

In mammals, the iGluR family is encoded by 18 different genes, which are shown in Figure 1.1 (Hollmann & Heinemann 1994). The encoded proteins are structurally related but have distinctive pharmacological profiles. They are all integral membrane proteins that vary in sequence identity to different degrees, but nonetheless share a set of core structural features that allow them to take part in fast glutamatergic synaptic transmission. iGluRs are characterized by their propensity to be activated by certain selective synthetic agonists, such as N-methyl-D-aspartate (NMDA) for NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) for
AMPA receptors, and kainic acid (KA) for kainate receptors. In addition, there is a fourth subfamily of iGluRs, classified as delta or orphan receptors, for which no agonist has been identified so far (Orth et al 2013).

NMDA receptors are further divided into seven receptor subunits (GluN1, GluN2A-D, GluN3A-B), AMPA receptors into four (GluA1-4), kainate receptors into five (the low affinity subunits GluK1-3 and the high affinity subunits GluK4-5), and delta receptors into two subunits (GluD1-2).

Additionally, a number of homologous proteins have been identified in invertebrates and plants (Tapken et al 2013, Tapken & Hollmann 2006).

Figure 1.1 The family of vertebrate iGluRs.

1.2.1.1 Structure

Functional iGluRs are membrane proteins complexes composed of four subunits, which form a central cation-selective ion pore. They first assemble as dimers and ultimately form a tetrameric structure via the association of two dimers (Ayalon & Stern-Bach 2001, Mayer 2006, Rosenmund et al 1998, Sobolevsky et al 2009, Wu et al 1996). Functional tetramers can only consist of subunits of the same subfamily (Hollmann & Heinemann 1994). The receptor can be homomeric, if composed of four identical subunits (Hollmann et al 1989), or heteromeric if composed of different subunits (Brose et al 1994, Herb et al 1992, Wentholt et al 1992). However, homomeric
combinations are only functional among AMPARs or the low affinity subunits of kainate receptors.

iGluR subunits are structurally similar. They all have a large extracellular amino-terminal domain (NTD) (Sobolevsky et al. 2009), an intracellular carboxyl-terminal domain (CTD) and three transmembrane domains (TMDA-C). The ion pore domain (P) is a hairpin-like structure between TMDA and TMDB that enters the membrane from the inside, without crossing it (Hollmann et al. 1994). Two discontinuous domains, S1 and S2, combine extracellularly to form the ligand-binding domain (LBD) (Stern-Bach et al. 1994). S1 can be found between the NTD and TMDA, and S2 between TMDB and TMDC (see Figure 1.2).

Figure 1.2 Schematic representation of the iGluR structure.
A: Topology of an iGluR subunit. Each subunit consists of three transmembrane domains (TMDA-C), a ligand binding domain (LBD), a large extracellular amino-terminal domain (-NH₂) and an intracellular carboxy-terminal domain (-COOH). Each subunit counts with a Q/R editing site at the re-entrant loop of the pore domain. B: The tetrameric iGluR. The ion pore is situated in the middle of the tetramer, involving the pore loop and transmembrane domain B (TMDB). For clarity, transmembrane domains and carboxy-termini of the two front subunits are not represented.
1.2.1.2 AMPA receptors

AMPA receptors are the most common receptors in the nervous system and mediate most of the signal transmission in the brain (Borges & Dingledine 1998, Dingledine et al 1999). Each of the four subunits comprises approximately 900 amino acids. Although the subunits are closely related, they differ from each other to different extents, predominantly at their CTD, which is critical for interaction with intracellular scaffolding proteins.

AMPA receptors offer great diversity, due to their flexible nature regarding subunit combination. All subunits can form functional homomeric receptors, but they can also combine amongst each other to form distinctive receptors with characteristic properties. Additionally, two different post-transcriptional events, alternative splicing and editing, decisively determine receptor variability and assembly (Greger et al 2006, Greger et al 2003), kinetics (Mosbacher et al 1994, Sommer et al 1990) and permeability to Ca\textsuperscript{2+} ions (Seeburg et al 1998, Tanaka et al 2000), contributing further to an even larger functional diversity. In addition to the aforementioned modifications, AMPA receptors can also undergo N-glycosylation, phosphorylation and palmitoylation (Hall et al 1997, Hayashi et al 2005, Jenkins et al 2014, Lee et al 2000).

1.2.1.2.1 Alternative splicing

Alternative splicing occurs in all AMPA subunits, at a 38 amino acid sequence near the LBD, immediately preceding TMDC, resulting in two variants called flip and flop (Sommer et al 1990). The flip variants are predominant in early developmental stages, while the flop variants become more prevalent in adulthood (Monyer et al 1991). Flip and flop variants have individual, distinctive desensitization and deactivation kinetics, and dissimilar sensitivity to allosteric modulators (Mosbacher et al 1994, Partin et al 1995, Pei et al 2009, Sommer et al 1990). The flip variant allows for larger currents upon agonist-binding due to delayed desensitization.
1.2.1.2.2 The Q/R editing site

RNA editing occurs in mRNA sequence coding for the pore loop of the receptor of both AMPARs and KARs, resulting in the change of an uncharged glutamine (Q) to a positively charged arginine (R) (Hume et al 1991, Seeburg 1996). Through the process of RNA editing, certain nucleotides in a codon can be modified, which may result in the substitution of a given amino acid. Editing of a glutamine to an arginine at a receptor’s Q/R editing site has a major impact on the channel’s ion permeability. In the adult CNS, nearly all GluA2 subunits are edited, in contrast to the other AMPA receptor subunits, making this subunit particularly interesting. During neuronal differentiation, editing occurs very quickly. Four and a half days after embryonic stem cells start their differentiation process into neuroepithelial precursors, GluA2 subunits are Q/R-edited by less than 10%. Nevertheless, only half a day later GluA2 is already fully edited (Pachernegg et al 2015).

In the GluA2 gene RNA editing leads to the conversion of the codon at position 586 from CAG to CGG, replacing the glutamine at this position by an arginine. Thereby, a double-stranded RNA adenosine deaminase (ADAR2) first converts an adenosine to an inosine by deamination (Rueter et al 1999). Subsequently, this inosine is read as a guanosine during translation.

Receptors lacking edited receptor subunits are permeable to Na⁺, K⁺ and Ca²⁺. Receptors that feature the edited GluA2, on the other hand, have a lower conductance and are nearly Ca²⁺ impermeable (Burnashev et al 1992, Hollmann et al 1991, Hume et al 1991, Swanson et al 1997). Furthermore, the edited GluA2 subunit plays an important role in receptor assembly and trafficking (Greger et al 2002, Isaac et al 2007). In vivo, most AMPA receptors are heteromers containing both GluA1 and GluA2 subunits (Lu et al 2009, Wenthold et al 1996). Physiologically, the prevention of Ca²⁺ influx is of vital importance in order to avoid excitotoxicity (Kim et al 2001).

Another receptor property that is altered, as a consequence of editing, is the shape of the current-voltage (I/V) curve, which changes from inwardly rectifying (unedited) to linear (edited) (Hollmann et al 1991, Liu & Zukin 2007). This change results from the fact that, at more positive membrane potentials and correspondingly less negative intracellular environments, the existence of an extra positive charge from the arginine side chain prevents the migration of polyamines to the plasma membrane.
where they normally would block the intracellular mouth of the channel, as observed for the Q variant (Bowie & Mayer 1995, Kamboj et al 1995).

1.2.1.2.3 Desensitization

Upon binding of at least one agonist molecule to a receptor’s LBD, a conformational change occurs that leads to the opening of the ion channel. This causes a certain instability at the dimer interface and, consequently, a second rearrangement can ensue in order to escape this energetically unfavorable state. Eventually, the dimer interface disintegrates causing the ion channel of the receptor to close, although the agonist is still bound to it, resulting in the discontinuation of the ionic flow (see Figure 1.3). This event is called desensitization (Armstrong et al 2006, Horning & Mayer 2004, Jin et al 2003, Jin et al 2005, Mayer 2006, Meyerson et al 2014, Sun et al 2002). Desensitization is a reversible event, with a dynamic equilibrium existing between receptors in the open and in the desensitized states. Alternatively, the LBD can reopen upon agonist dissociation, which triggers the return of the receptor to its closed state. Different receptors have specific activation, deactivation, and desensitization kinetics.

Desensitization can be successfully inhibited by a number of mutations. A point mutation at position 479 (GluA1) or 483 (GluA2) from a leucine to either a tyrosine or a phenylalanine is well known to almost fully abolish desensitization (Robert et al 2001, Stern-Bach et al 1998).

The use of certain allosteric modulators such as cyclothiazide (CTZ) and trichlormethiazide (TCM) or lectins such as concanavalin A (ConA), can also be used to stabilize the dimer interface and therefore block desensitization (Mitchell & Fleck 2007). Unsurprisingly, these modulators have little to no effect on AMPA receptor non-desensitizing mutants, the only exception being kainate-induced currents, which are still slightly increased by CTZ.
1.2 Glutamate Receptors

Upon binding of glutamate at the ligand binding domain (LBD), the channel opens allowing for cations to enter the cell. This leads to a destabilization of the dimer interface, which eventually causes the channel to desensitize.

In addition to their most obvious effects on desensitization, both CTZ and TCM have been reported to decelerate deactivation and to increase agonist potency of GluA1 receptors while doing the opposite to the L479Y non-desensitizing mutants, as has been shown in HEK293 cells (Mitchell & Fleck 2007). CTZ is more effective on the flip variants of AMPARs, and recovery from CTZ potentiation is faster for the flop variant (Partin et al 1994).

Moreover, different agonists induce desensitization differently. For example, the full agonist glutamate strongly desensitizes AMPARs, while the partial agonist kainate is known to be nearly non-desensitizing (Levchenko-Lambert et al 2011).

**Figure 1.3 Desensitization of glutamate receptors.**

Upon binding of glutamate at the ligand binding domain (LBD), the channel opens allowing for cations to enter the cell. This leads to a destabilization of the dimer interface, which eventually causes the channel to desensitize.
1.3 AMPAR auxiliary proteins

AMPARs are abundant at postsynaptic membranes, and play an important role in synaptic plasticity, as mentioned above. They interact with a number of proteins that modulate their function and assist their trafficking to the membrane (Haering et al 2014). Some of these proteins are specific for AMPARs and differ from other proteins that interact with members of other iGluRs subfamilies such as KARs (Pahl et al 2014) and NMDARs (Bard & Groc 2011). So far, seven distinct families of AMPAR auxiliary proteins have been identified. The most extensively characterized of these are the transmembrane AMPA receptor regulatory proteins (TARPs). Besides TARPs, suppressor of lurcher (SOL) (Walker et al 2006, Zheng et al 2006), cornichon homologues (CNIHs) (Brockie et al 2013, Jackson & Nicoll 2009, Schwenk et al 2009), synapse differentiation-induced gene I (SynDIG I) (Kalashnikova et al 2010), cysteine knot AMPAR modulating proteins 44 (CKAMP44) (Farrant & Cull-Candy 2010), germ cell-specific gene 1-like protein (GSG1L) (Shanks et al 2012) and claudins are among the proteins that so far have been described to regulate AMPARs (Haering et al 2017 (unpublished)).

1.3.1 TARPs

TARPs were the first AMPAR auxiliary proteins to be discovered. In the beginning of the 90s, the investigation of the stargazer mice led to the discovery of stargazin (Noebels et al 1990). These mice were reported to suffer from cerebellar ataxia and to experience periodic epilepsy episodes. The name stargazer was derived from the peculiar head movements, during epileptic seizures that made the mice look like they were gazing at the stars. Stargazin expression in these mice is disrupted due to a point mutation (Letts et al 1998). Later on, it was shown that, while the expression of AMPARs in cerebellar granule cells was at a normal level in stargazer mice, these cells lacked functional receptors in their plasma membranes. By contrast, trafficking and localization of other glutamate receptors, such as NMDA receptors were not affected (Hashimoto et al 1999). Interestingly, delivery of AMPARs to the membrane could be
rescued by co-expression with other type I-TARPs in both developing and mature CNS (Tomita et al 2003).

Stargazin is also known as γ2, a name derived from the structural homology with the voltage-dependent calcium channel subunit γ1. Subsequently discovered TARP subunits were named in accordance with this nomenclature (Letts et al 1998).

Functional AMPAR tetramers are believed to include different numbers of associated TARPs, depending on the TARP expression levels. Yet, this number is never above four for γ2 or γ3, and infrequently above two for γ8 (Hastie et al 2013). Furthermore, one TARP per receptor seems to be sufficient for modulation of AMPARs (Kim et al 2010)

1.3.1.1 The family of TARPs

So far, six members of the TARP family have been sequenced and identified. Based on sequence homology and functionality, they have been further divided into two different subfamilies. The type I-TARP subfamily includes γ3, γ4, and γ8, besides its original member stargazin. The type II-TARP subfamily consists of γ5 and γ7. Despite sharing a certain degree of homology with the members of the TARP family, γ1 and γ6 do not show any TARP-like function and are therefore not classified as TARPs (Kato et al 2007).

Both subfamilies of TARPs modulate AMPAR function, but only type I-TARPs are also involved in their trafficking (Kato et al 2008, Tomita et al 2003).
1.3.1.2 Topology

All members of the TARP family are integral membrane proteins that share a common topology (see Figure 1.5). They are composed of four transmembrane domains (TMDI-IV) with both NTD and CTD located intracellularly. The CTD is comparatively large and it contains either a typical PDZ-binding domain (-TTPV for type I-TARPs) or an atypical PDZ-binding domain (-TSPC for γ7, -SSPC for γ5) (Tomita 2010). The PDZ-binding domain of type I-TARPs is crucial for interaction with cytoskeletal proteins and incorporation of AMPARs at the membrane. Additionally, important phosphorylation and nitrosylation sites are located in the CTD.

Figure 1.4 Phylogenetic tree of TARP-related proteins.
Modified from Tomita et al. (2003).
1.3 AMPAR auxiliary proteins

Each subunit consists of four transmembrane domains (TMD-I-IV). Both the amino-terminal domain (-NH₂) and the carboxyl-terminal domain (-COOH) are intracellular. Type I-TARPs have a typical PDZ-binding domain (-TTPV) while type II-TARPs have atypical PDZ-binding domains (-S/TSPC).

1.3.1.3 Localization

TARPs are expressed in every neuronal cell type. However, expression of each subunit varies depending on the brain region.

Type I-TARPs are widespread all over the brain (Chu et al 2001, Green et al 2001, Klugbauer et al 2000, Sharp et al 2001, Tomita et al 2003) and the loss of a specific type I-TARP can be compensated by a different TARP of the same subfamily. Furthermore, except for cerebellar granule cells, all cells in the CNS express a minimum of two different type I-TARPs, the stargazer mutant is therefore the only TARP knockout with a phenotype (Menuz et al 2008).

While γ1 and γ6 are found predominantly in striated skeletal muscle (Burgess et al 2001, Chu et al 2001), γ5 and γ7 are found primarily in the brain. The type II-TARPs, γ5 and γ7, are present to different extents in distinct brain areas. γ5 is found mainly in the cerebellum, more specifically in Bergmann glia cells (BGCs) but also in the...
olfactory bulb, globus pallidus, hippocampal CA2 region, and thalamus (Fukaya et al 2005, Kato et al 2007). γ7 is also expressed predominantly in the cerebellum, but also in the olfactory bulb, neocortex, thalamus, and hippocampus (Kato et al 2007).

1.3.1.4 Trafficking

AMPARs assemble at the ER (Greger et al 2007). It is also at the ER that the interaction with the type I-TARPs takes place, after tetrameric assembly of the receptor (Coombs & Cull-Candy 2009, Gan et al 2015). The TARPs then assure the correct folding of the receptor and act as chaperones to export the AMPAR through the Golgi apparatus to the plasma membrane (Haering et al 2014, Tomita et al 2003, Ziff 2007). Type I-TARPs are responsible for the transport of AMPARs to the membrane, either by masking a retention signal at the receptor, or by providing its own export signal (Bedoukian et al 2008, Vandenbergh et al 2005, Ziff 2007). Consequently, the number of receptors at the postsynaptic membrane is drastically increased by type I-TARPs (Chen et al 2003, Schnell et al 2002).

Nevertheless, type I-TARPs traffic AMPARs selectively, depending on the subunits involved (Bats et al 2012, Menuz et al 2008, Yamazaki et al 2010). For example, it has been proposed that TARPs preferentially traffic Ca\textsuperscript{2+} permeable receptors to the membrane (Menuz et al 2008) and that Ca\textsuperscript{2+} permeable receptors rely more critically on type I-TARPs for transport than Ca\textsuperscript{2+} impermeable receptors (Bats et al 2013, Bats et al 2012). Likewise, transport of the flop variants of certain AMPARs is more profoundly enhanced than that of the correspondent flip variants (Coleman et al 2006).

By contrast, although type II-TARP are important modulators of several functional properties of AMPARs, they seem to have no effect on trafficking (Kato et al 2008, Tomita et al 2003).

1.3.1.5 Synaptic targeting

TARPs help the receptor to get correctly positioned at the active zone of the plasma membrane. After transport from the Golgi apparatus to extrasynaptic parts of
the plasma membrane by vesicular transport, AMPAR-containing vesicles fuse with the membrane and are further transported laterally through the lipid bilayer to the synaptic location, where they get anchored. All these latter steps are regulated by TARPs (Chen et al 1999, Chen et al 2000, Cuadra et al 2004, Hashimoto et al 1999, Schnell et al 2002, Tomita et al 2003). Via the CTD of type I-TARPs and their PDZ-binding domains, AMPARs, which lack such PDZ-binding motifs, can interact with scaffolding proteins at the postsynaptic density (PSD), including PSD-95 and other membrane-associated guanylate kinases (MAGUKs) (Bats et al 2007, Beique & Andrade 2003, Beique et al 2006, Chen et al 2000, Ehrlich & Malinow 2004, El-Husseini et al 2000, Schnell et al 2002, Tomita et al 2001). Accordingly, the CTD containing the PDZ-binding domain is essential for synaptic targeting but not for extrasynaptic delivery. In addition, the interaction with scaffolding proteins is particularly dependent on the phosphorylation status of the various phosphorylation sites at the CTD of $\gamma_2$ (Chen et al 2000, Chetkovich et al 2002, Choi et al 2002, Sager et al 2010, Sumioka et al 2010, Tomita et al 2005b).

Conversely, type II-TARPs lack standard PDZ-binding domains, and therefore do not promote clustering and anchoring of AMPARs at the synapse (Tomita 2010). However, $\gamma_7$, has been shown to assist $\gamma_2$ in doing so in a subunit-dependent manner, where the resulting number of Ca$^{2+}$ permeable receptors at the plasma membrane is preferentially enhanced, whilst that of Ca$^{2+}$ impermeable receptors is reduced (Studniarczyk et al 2013, Yamazaki et al 2010).

### 1.3.1.6 Electrophysiological properties

By regulating the biophysical properties of AMPARs, TARPs also modulate AMPARs electrophysiologically, which ultimately results in altered agonist-induced currents (Kott et al 2007, Priel et al 2005).

Association of type I-TARPs with AMPARs leads to slower deactivation and desensitization kinetics, resulting in an extended time interval in which the channel is in the open state, thus allowing for more current to pass across the membrane (Cho et al 2007, Kato et al 2010, Korber et al 2007, Milstein et al 2007, Turetsky et al 2005). Type I-TARPs also lead to higher ligand binding affinities and to a decrease in the
Introduction

effective agonist concentration (Cho et al 2007, Kato et al 2010, Korber et al 2007, Kott et al 2009, Milstein et al 2007, Priel et al 2005, Turetsky et al 2005). Furthermore, TARPs have been found to convert AMPAR antagonists such as CNQX into partial agonists (Menuz et al 2007). Additionally, the efficiency of partial agonists such as kainate, in comparison to glutamate, is improved in the presence of TARPs.

1.3.1.6.1 Type II-TARPs

While type I-TARPs have been extensively studied, type II-TARPs have been frequently neglected. Their modulation of AMPARs may be more modest than that of type I-TARPs, but it should not be ignored as they have a substantial impact on receptor function. Type II-TARPs are found widely spread in many brain regions, which implies they may have a role in modulating synaptic transmission (Fukaya et al 2005, Kato et al 2007). Yet, their lack of a typical PDZ-binding motif suggests interaction with different partners than those of type I-TARPs, which indicates that they are not involved in receptor trafficking (Kato et al 2008). Type II-TARPs also have shorter C-terminal domains, which contain only two out of ten phosphorylation sites present in the other TARPs, which are thought to be critical for trafficking.

It has been shown that both, $\gamma_5$ and $\gamma_7$, are highly expressed in the cerebellum, where $\gamma_7$ is able to selectively interact with certain AMPARs and PSD-95. In addition, $\gamma_7$ increases both peak and glutamate-induced steady-state current responses in HEK293T cells and slows down deactivation and desensitization kinetics in GluA1 homomeric receptors (Kato et al 2007). On the other hand, $\gamma_5$ appears to selectively modulate GluA2-containing receptors but not homomeric GluA1 receptors (Kato et al 2008, Kato et al 2007). Therefore, the editing status of the receptor appears to be a determinant for modulation of AMPARs by $\gamma_5$. It has been suggested that the effects of $\gamma_5$ on edited receptors include not only an increase in peak currents, but also a decrease in steady-state currents, acceleration of deactivation and desensitization kinetics, and a decrease in glutamate affinity (Kato et al 2008, Kato et al 2007).

Furthermore, when co-expressed with either GluA3 or GluA4, $\gamma_5$ and $\gamma_7$ were not able to change peak currents of GluA4 and even had a slight inhibitory effect on GluA3. Regarding steady-state currents, $\gamma_7$ had a potentiating effect on GluA3 but no effect on GluA4, while $\gamma_5$ had no major effect on either of them (Kato et al 2007). The
same authors reported that, in the presence of the non-desensitizing agent cyclothiazide (CTZ), both \( \gamma_5 \) and \( \gamma_7 \) enhanced responses of GluA2-containing receptors, while their effect on GluA2-lacking receptors was minimal.

Finally, it has been suggested that \( \gamma_5 \) modulates only the long form splice variants of AMPARs (Soto et al 2009). Soto’s results appear to be partly contradictory to those of Kato’s. The latter suggests that \( \gamma_5 \) modulation does not depend on the Q/R editing site, but acts selectively on the long forms of mostly Ca\(^{2+}\) permeable receptors by inhibiting them, and further interacts with all other AMPAR subunits, modulating their properties to different extents. They also found that \( \gamma_5 \) increases single-channel conductance and decreases the open probability of AMPAR channels.
1.4 Aims

AMPARs are key elements for fast excitatory transmission in the brain. Because of their core role in synaptic plasticity, which underlies the processes of memory and learning, they have been extensively studied over the past twenty-five years after the cloning of the first iGluR (Hollmann et al 1989). Consequently, we have now a broad knowledge of many aspects of AMPAR properties, such as assembly, trafficking, and gating (Anggono & Huganir 2012, Derkach et al 2007, Gan et al 2015).

Yet, with the discovery of TARPs (Hashimoto et al 1999, Letts et al 1998) and other auxiliary proteins (Haering et al 2014), a new and exciting perspective for the study of AMPARs was revealed. It is now evident that TARPs are involved in regulating almost every aspect of AMPAR structure and function (Milstein & Nicoll 2008, Ziff 2007). Furthermore, they serve as intermediates for interaction with other proteins at the PSD, such as PSD-95 (Bats et al 2007, Schnell et al 2002).

Nevertheless, not all TARPs are created equal. While type I-TARPs, which include stargazin, have long been known to modulate both trafficking and all of the channel properties, type II-TARPs, $\gamma_5$ and $\gamma_7$ have more modest and refined effects on gating and are likely not involved in trafficking (Kato et al 2010). Hence, despite being amply expressed in the brain and having a high structure and sequence homology to type I-TARPs, type II-TARPs have initially been disregarded as TARPs.

Consequently, while type I-TARPs have been subject of intense investigation in the past few years, type II-TARPs have been neglected; only very few studies have focused on them. Thus, there are many open questions concerning the type II-TARP’s role in modulating AMPARs. This thesis focuses on addressing some of these questions.

Electrophysiological recordings using the *Xenopus laevis* oocyte heterologous expression system were performed in order to analyze if and how $\gamma_6$ and type II-TARPs, especially $\gamma_5$, modulate current amplitudes of specific AMPARs, depending on receptor subunit composition. Channel properties such as desensitization, steady-state currents, ion permeability, and rectification were addressed, as were membrane expression levels and sensitivity to certain agonists.
2  Materials and Methods

2.1  Materials

2.1.1  Equipment

Amplifier  TURBO TEC-10CX, NPI electronics, Tamm, Germany

Autoclave  Certoclav CV-EL, Certoclave Stereliser GmbH, Traun, Austria

                       Varioklav, type 500E, H+P Labortechnik GmbH, Oberschleißheim, Germany

Blotting chamber  TE22, Haefer Scientific Instruments, Holliston, MA, USA

                       TE42, Haefer Scientific Instruments, Holliston, MA, USA

Centrifuges  Biofuge fresco, Heraeus, Hanau, Germany

                       Biofuge stratos, Heraeus, Hanau, Germany

                       Centrifuge 5415C, Eppendorf, Hamburg, Germany

                       Labofuge 400R, Heraeus, Hanau, Germany

                       UZ Discovery 90, Sorvall, Bad Nauheim, Germany

                       Z326K, Hermle, Stuttgart, Germany

Computer  Dell Vostro 220

                       Intel®Pentium®4
## Materials and Methods

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<th>Equipment</th>
<th>Manufacturer/Model</th>
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<td>Power Mac G4</td>
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<td>Confocal microscope</td>
<td>TCS SP2 AOBS, Leica, Mannheim, Germany</td>
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<td>Drying cabinet</td>
<td>T6120, Heraeus, Hanau, Germany</td>
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<td>Freezer (-80°C)</td>
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<td>MGU-202T (7x10 cm), C.B.S., Del Mar, CA, USA</td>
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<td>Gel documentation imager</td>
<td>INTAS Science Imaging, Göttingen, Germany</td>
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<td>Ice machine</td>
<td>AF-30, Scotsman Ice Systems, Vernon Hills, IL, USA</td>
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<td>Incubators</td>
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<td>Kelvitrion K, Heraeus, Hanau, Germany</td>
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<td>KS-15/TH-15, Edmund Büller, Hechingen, Germany</td>
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### 2.1 Materials

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<td>Stemi 2000, Carl Zeiss, Jena, Germany</td>
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<td>pH meter</td>
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<td>Photometer</td>
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### Materials and Methods

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<td>PowerPac 3000, Bio-Rad, Hercules, CA, USA</td>
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<td>Standard Power Pack P25, Biometra, Göttingen, Germany</td>
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<td>Kilomat, Sartorius, Göttingen, Germany</td>
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<td>P-120, Mettler, Greifensee, Switzerland</td>
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<td>Thermomixer 5436, Eppendorf, Hamburg</td>
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<td>Thermocycler</td>
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<td>PTC-200, MJ Research, Watertown, MA, USA</td>
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<td>Valves</td>
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<td>Vibraplane, Kinetic Systems, Boston, MA, USA</td>
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<td>Vortexes</td>
<td>MS2 Minishaker, IKA, Staufen, Germany</td>
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2.1 Materials

Vortex Genie 2, Bender & Hobein AG, Zürich, Switzerland

Water baths MP-5A, Julabo, Seelbach, Germany

Water distillation unit Ultra Clear, SG, Barsbüttel, Germany

2.1.2 Consumables

Bottle top filter Sarstedt, Nümbrecht, Germany

Cell culture flasks Sarstedt, Nümbrecht, Germany

Cell culture plates (glass bottom) WPI, Sarasota, FL, USA

Cell culture plates (variable sizes) Sarstedt, Nümbrecht, Germany

Centrifuge polypropylene tubes (14 mL) Greiner, Frickenhausen, Germany

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

PCR reaction tubes (0.2 mL) Starlab, Ahrensburg, Germany

Petri dishes Sarstedt, Nümbrecht, Germany

Petri dishes (3-section) Sarstedt, Nümbrecht, Germany

Petri dishes (4-section) VWR, Darmstadt, Germany

Pipette filter tips Starlab, Ahrensburg, Germany

Pipette tips Starlab, Ahrensburg, Germany

Reaction tubes (1.5 mL) Starlab, Ahrensburg, Germany

Recording borosilicate capillaries No. 1404501, Hilgenberg, Malsfeld, Germany
Materials and Methods

Screw cap micro tubes (1.5 mL) Sarstedt, Nümbrecht, Germany
Screw cap tubes (15 mL) Sarstedt, Nümbrecht, Germany
Screw cap tubes (50 mL) Sarstedt, Nümbrecht, Germany
Sterile reaction tubes (1.5 mL) Biozym, Hess. Oldendorf, Germany
24-well microtiter plates Starlab GmbH, Ahrensburg, Germany

2.1.3 Chemicals

The chemicals used were adequate for use in molecular biology experiments and were obtained from the suppliers listed below:

Amersham, Freiburg, Germany
Applichem, Darmstadt, Germany
Ascent Scientific, Weston-Super-Mare, United Kingdom
BD, Heidelberg, Germany
Bio-Budget, Krefeld, Germany
Biomol, Hamburg, Germany
Bio-Rad Laboratories, Munich, Germany
Biozym, Hess. Oldendorf, Germany
DuPont, Wilmington, DE, USA
Ega-Chemie, Steinheim, Germany
Fisher Scientific, Schwerte, Germany
Fluka, Buchs, Switzerland
2.1 Materials

FMC Bioproducts, Philadelphia, PA, USA
Hello Bio, Bristol, United Kingdom
ICN Biochemicals, Aurora, OH, USA
J.T. Baker, Deventer, Netherlands
Life Technologies, Darmstadt, Germany
Lonza, Basel, Switzerland
Merck, Darmstadt, Germany
Pierce, Rockford, IL, USA
Promega, Madison, WI, USA
RBI, Natick, MA, USA
Riedel de Haën, Seelze, Germany
Roth, Karlsruhe, Germany
Serva Feinbiochemica, Heidelberg, Germany
Sigma, Taufkirchen, Germany
Tocris, Bristol, United Kingdom
VWR, Darmstadt, Germany
2.1.4 Enzymes

All enzymes were obtained from the suppliers listed below:

Fermentas, St. Leon-Rot, Germany

Invitrogen/GibcoBRL, Karlsruhe, Germany

New England Biolabs, Frankfurt, Germany

Promega, Madison, WI, USA

QIAGEN, Hilden, Germany

Roche, Mannheim, Germany

Stratagene, La Jolla, CA, USA

Thermo Scientific, Waltham, MA, USA

2.1.5 Kits

JetQuick Gel Extraction Kit	Genomed, Löhne, Germany

JetStar 2.0 Plasmid Kit	Genomed, Löhne, Germany

mMessage mMACHINE T7 Transcription Kit	Life Technologies, Darmstadt, Germany

My-Budget Double Pure Kit	Bio-Budget, Krefeld, Germany

RNA Clean & Concentrator	Zymo Research, Irvine, CA, USA
2.1 Materials

2.1.6 Antibodies

2.1.6.1 Primary antibodies

**Anti-GluA1** antibody (RH95, Merck Millipore, Darmstadt, Germany) is a mouse monoclonal antibody directed against the N-terminus of rat GluA1. It was used for Western blot development at a dilution of 1:1000.

**Anti-GluA2** antibody (556341, BD Biosciences, Erembodegem, Belgium) is a mouse monoclonal antibody directed against the N-terminus of GluA2. It recognizes rat, monkey and dog GluA2. It was used for Western blot development at a dilution of 1:500.

**Anti-pan-Cadherine** antibody (71-7100, Invitrogen, Karlsruhe, Germany) is a rabbit polyclonal antibody, widely reactive against all members of the cadherin family, including N-cadherin, P-cadherin, and R-cadherin. It displays cross-reactivity for several species, including human, mouse, and rat, amongst others. It was used for Western blot development at a dilution of 1:250.

2.1.6.2 Secondary antibodies

**Anti-Mouse** antibody (32230, Pierce, Rockford, IL, USA) is a goat Poly-HRP polyclonal secondary antibody that recognizes the light and the heavy chains of mouse IgG. It was used for Western blot development at a dilution of 1:10000.

**Anti-Rabbit** antibody (32260, Pierce, Rockford, IL, USA) is a goat Poly-HRP polyclonal secondary antibody that recognizes the light and the heavy chains of mouse IgG. It was used for Western blot development at a dilution of 1:10000.
2.1.7 Oligonucleotides

All oligonucleotides were obtained from the suppliers listed below:

Microsynth AG, Balgach, Switzerland

Sigma, Taufkirchen, Germany

2.1.8 Plasmids

2.1.8.1 pSGEM

The plasmid vector pSGEM (Figure 2.1) was used for proteinexpression in *Xenopus laevis* oocytes. After subcloning of the desired cDNA clones into the vector, the plasmids were linearized for subsequent cRNA synthesis. For higher expression efficiency, the multiple cloning site (MCS) is flanked by untranslated regions of the *Xenopus laevis* β-globin gene (5’UTR and 3’UTR). T7 and SP6 RNA polymerase promoters allow for transcription of sense and antisense RNA products. Linearization is performed on one of many available linearization sites (LS) downstream of the gene of interest. Additionally, the vector contains a β-lactamase gene (Amp^R^) for ampicillin resistance for selection in *E.coli*. 
2.1 Materials

Represented are the T7 (3102-3) and the SP6 (442-461) promoters, the 5'- (34-77) and the 3'-untranslated regions (198-339), the multiple cloning site (93-186), linearization sites (392-434), the pUC origin of replication (849-1492), and the ampicillin resistance gene (1640-2500).

2.1.8.2 pSGEM-C-CGFP

The plasmid vector pSGEM-C-CGFP (Figure 2.2), used for protein expression in *Xenopus laevis* oocytes, was created by modification of pSGEM. Green fluorescent protein (GFP) is split in two parts at the residues Gln<sup>157</sup>/Lys<sup>158</sup>. The first part contains the N-terminus of the protein and it is here referred to as NGFP, the second part contains the C-terminus of the protein and therefore is referred to as CGFP. The vector pSGEM-C-CGFP is thus generated by insertion of CGFP downstream from the MSC.
Figure 2.2 Map of the pSGEM-C-CGFP plasmid vector.

Represented are the T7 (3348-3) and the SP6 (688-707) promoters, the 5’- (34-71) and the 3’- untranslated regions (444-585), CGFP (101-346), linearization sites (638-680), the pUC origin of replication (1095-1738), and the ampicillin resistance gene (1886-2746).

2.1.8.3 pSGEM-C-NGFP

The plasmid vector pSGEM-C-NGFP (Figure 2.3), used for protein expression in *Xenopus laevis* oocytes, was created by modification of pSGEM. The vector pSGEM-C-NGFP, was generated by insertion of NGFP downstream from the MSC.
2.1 Materials

Figure 2.3 Map of the pSGEM-C-NGFP plasmid vector.
Represented are the T7 (3576-3) and the SP6 (916-935) promoters, the 5’- (34-71) and the 3’-untranslated regions (672-813), NGFP (101-575), linearization sites (866-908), the pUC origin of replication (1323-1966), and the ampicillin resistance gene (2114-2974).

2.1.8.4 pIRES2-EGFP

The plasmid vector pIRES2-EGFP (Figure 2.4) was used for protein expression in HEK293 cells. By using the pIRES2-EGFP vector, simultaneous expression of the enhanced green fluorescence protein (EGFP) and the gene of interest is assured. While the EGFP gene is normally translated by recognition of the 5’-cap structure, of the mRNA translation of the gene of interest is mediated by the encephalomyocarditis virus internal ribosome entry site (IRES). For higher expression efficiency, the human Cytomegalovirus (CMV) promoter, optimized for expression in eukaryotic cells, is positioned upstream of the MCS. To increase the stability of mRNA by polyadenylation, the vector is provided downstream of the MCS with the simian virus-40 (SV40) poly adenylation sequence downstream of the EGFP gene. Additionally, the vector contains a kanamycin resistance gene (Kanr) and a pUC origin of replication, for improved prokaryotic growth, selection and replication.
Figure 2.4 Map of the pIRES2-EGFP plasmid vector.
Represented are the human Cytomegalovirus promoter (1-589), the multiple cloning site (591-665), IRES2 (666-1250), the EGFP (1254-1974), the SV40 polyadenylation sequence (2126-2160), the f1 origin of replication (2224-2679), the kanamycin resistance gene (3204-3998), and the pUC origin of replication (4583-5226).

2.1.8.5 pIRES2-DsRed-Express2

The plasmid vector pIRES2-DsRed-Express2 (Figure 2.5) was used for protein expression in HEK293 cells. By using the pIRES2-DsRed-Express2 vector, independent expression of the DsRed2 fluorescent protein (DsRed) and the gene of interest is assured. The general features of this vector are identical to the pIRES2-EGFP vector, but here EGFP is exchanged by the DsRed2 gene.
2.1 Materials

Represented are the human *Cytomegalovirus* promoter (1-589), the multiple cloning site (591-665), IRES2 (666-1250), the DsRed2 fluorescent protein (1254-1931), the SV40 polyadenylation sequence (2083-2117), the f1 origin of replication (2181-2636), the kanamycin resistance gene (3161-3955), and the pUC origin of replication (4540-5183).

2.1.9 Organisms

2.1.9.1 Prokaryotic cells

Two *Escherichia coli* (*E. coli*) strains were used for cloning purposes: the ER2925 and the XL1-Blue MRF’. The ER2925 (New England Biolabs, Frankfurt, Germany), has the genotype: *ara-14 leuB6 flhaA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2. The XI1-Blue MRF’ (Agilent Technologies, Santa Clara, CA, USA) has the genotype: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F’ proAB lacF° ZΔM15 Tn10(Tet’)].
Materials and Methods

2.1.9.2 Eukaryotic cells

HEK293 ACC635 cells (DSMZ, Braunschweig, Germany) were used for transient transfection, and subsequent patch-clamp recordings.

2.1.9.3 Animals

Oocytes were harvested from specimens of South African *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI, USA), and used for two-electrode voltage clamp (TEVC) recordings.

2.1.10 Software

For purposes of either evaluation or presentation of the data presented in this thesis, the software packages listed below were used:

- Adobe Acrobat XI: Adobe systems Inc., Mountain View, CA, USA
- Adobe Creative Suite 5.1: Adobe systems Inc., Mountain View, CA, USA
- Chemostar Imager: INTAS, Göttingen, Germany
- DNASTAR Lasergene 8: DNASTAR Inc., Madison, WI, USA
- EndNote X7: Thomson Reuters, Boston, MA, USA
- ImageJ: Wayne Rasband, National Institute of Health, Bethesda, MD, USA
- Leica Confocal Software: Leica, Mannheim, Germany
- Microsoft Office 2010: Microsoft Corporation, Redmont, WA, USA
- Prism 6: GraphPad Software Inc., San Diego, CA, USA
2.1 Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse</td>
<td>HEKA, Lambrecht, Germany</td>
</tr>
<tr>
<td>Sequencer Scanner</td>
<td>Applied Biosystems, Life Technologies, Darmstadt, Germany</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Molecular Biology

Proteins are extremely versatile macromolecules. They execute a wide range of functions in an organism. Each protein has a unique amino acid sequence that is determined by the nucleotide sequence on the corresponding gene. Thus, the genome holds in its deoxyribonucleic acid (DNA) all the information necessary to make all the proteins in an entire organism. During transcription, the information contained in the DNA is copied into ribonucleic acid (RNA), which is subsequently translated into proteins.

Next, the techniques applied for manipulation, isolation and characterization of either of these macromolecules, used for acquisition of the data presented in this thesis, will be described.

2.2.1.1 Molecular cloning

Through DNA cloning it is possible to obtain many copies of identical DNA molecules. The goal of the cloning process is the insertion of the DNA fragment of interest into a vector that is suitable for replication in a chosen host cell. The host cell is then responsible for the replication of the recombinant DNA, which can be subsequently isolated and purified.

2.2.1.2 Mutagenesis

Through mutagenesis, a desired mutation can be introduced to a gene, or small DNA fragments can be inserted or deleted. Hence, certain properties of a protein can be further analyzed.

Traditionally, site-specific mutagenesis begins with a polymerase chain reaction (PCR), followed by digestion with specific enzymes, ligation into an appropriated
vector, and lastly, transformation into a competent host for amplification of the newly generated DNA.

**2.2.1.2.1 Polymerase chain reaction**

The invention of the PCR technique was worth a Nobel Prize in Chemistry to Kary B. Mullis and Michael Smith in 1993 (Hutchison et al 1978, Mullis et al 1986). PCR can be used to directly amplify a defined DNA fragment *in vitro* (Figure 2.6).

![Polymerase chain reaction diagram]

Figure 2.6 The polymerase chain reaction.

A double-stranded DNA molecule is first melted (denatured) at 98°C into single strands. Next, two primers, which are small synthetic oligonucleotides complementary to the flanking 3’ end regions of the DNA fragment to be amplified, is hybridized with the complementary regions of the target DNA at a calculated temperature that depends on the length and sequence of the primers. In the presence of deoxynucleotides (dNTPs), a thermostable DNA polymerase next extends the primers according to the template
Materials and Methods

DNA sequence at 72°C. After extension is complete, the newly synthetized double-stranded DNA is again denatured at 98°C, starting a new cycle. The number of DNA copies is doubled after each cycle, and therefore an exponential growth of the number of DNA fragments results in an increase of approximately a million-fold after only 20 cycles, while the remaining DNA sequences of the original sample are not amplified and their number remains constant. Standard components and volumes of a PCR protocol are shown on Table 2.1.

Table 2.1 Volume and concentration of the components in a standard PCR reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phusion® polymerase (2 U/μL)</td>
<td>0.5 μL</td>
<td>0.02 U/μL</td>
</tr>
<tr>
<td>dNTP mix (10 mM)</td>
<td>1 μL</td>
<td>200 μM</td>
</tr>
<tr>
<td>Sense primer (10 μM)</td>
<td>5 μL</td>
<td>1 μM</td>
</tr>
<tr>
<td>Antisense primer (10 μM)</td>
<td>5 μL</td>
<td>1 μM</td>
</tr>
<tr>
<td>Reaction buffer (5x)</td>
<td>10 μL</td>
<td>1x</td>
</tr>
<tr>
<td>MgCl₂ (50 mM)</td>
<td>1 mM</td>
<td>1 μL</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 50 μL</td>
<td></td>
</tr>
</tbody>
</table>

The polymerase used for DNA amplification, during acquisition of the data presented in this thesis was the Phusion® High-fidelity DNA Polymerase, which is a Pyrococcus-like editing enzyme fused with a DNA binding domain. It is one of the fastest and most accurate polymerases available. The buffers used were the ones supplied with the enzyme by the manufacturer, which are optimized for the highest performance. DNA polymerases require divalent ions such as Mg²⁺ for activity, therefore, MgCl₂ was added to the reaction mixtures. Primers were designed with care to avoid formation of eventual secondary structures, with either themselves or other sequences on the template. The nearest neighbor method (Sugimoto et al 1996) was applied to calculate primer optimum annealing temperatures (Tm).
2.2 Methods

2.2.1.2.2 Overlap extension PCR

Overlap extension PCR was developed as a method to introduce a given mutation into a DNA fragment. In a first step two separate mutagenesis PCRs are performed simultaneously. Each of two complementary primers, containing the desired mutation, is combined with another primer, in different PCR reactions. The resulting DNA fragments have complementary ends that can be combined in an overlap extension PCR step, after purification. In this step, no further primers are added for the first few cycles, and therefore the amplicons are allowed to anneal through their complimentary ends. It is only after a number of cycles that the two flanking primers, used in the first two PCR reactions, are added in order to amplify the fused PCR product.

Figure 2.7 Overlap extension PCR.
2.2.1.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate and isolate mixed DNA fragments of different size and charge in an agarose gel making use of an electric field. DNA molecules are negatively charged due to their phosphate backbone, and therefore move towards the positively charged anode. Linear DNA molecules migrate through the gel at a rate inversely proportional to the logarithm of the number of base pairs. Additionally, migration rates will depend on the size of the gel pores, the applied voltage, and the ionic strength of the electrophoresis buffer. Ethidium bromide is a DNA-intercalating agent that allows visualization of DNA bands in an agarose gel when exposed to UV light.

Gels were cast by melting agarose in TBE buffer at a standard concentration of 0.8 – 1.2 % (w/v), depending on the size of the fragments to be separated. Lower concentrations result in larger pores and were thus used to separate larger fragments. Agarose was dissolved in TBE by heating in a microwave. Evaporation was compensated by addition of water until the initial volume was reached again. Ethidium bromide (1 μg/μL) was added after the agarose mixture was allowed to cool down to approximately 60 ºC. The molten agarose solution was then poured into a sealed cast and a comb was placed near one of the ends so that wells were molded, for later sample application. After the gel set, comb and seals were removed and the gel was placed in a TBE buffer-containing electrophoresis tank. DNA samples were mixed with loading buffer before loading into the wells. Loading buffers contain a dye, such as bromophenol blue, that helps tracking the progress of the electrophoresis, and a dense component such as glycerol to make sure DNA samples do not flow out of the wells. A suitable DNA ladder was always added to at least one of the pockets to allow estimation of fragment size in the gel. The GeneRuler 1 kb DNA ladder and the GeneRuler 100 bp DNA ladder (Thermo Scientific) were consistently used to estimate the length of DNA fragments.

After samples and markers were loaded on the gel, electrophoresis was started, at a voltage between 90 and 150 V, depending on the length of the DNA fragments. Compositions of the buffers used are listed below on Table 2.2.
2.2 Methods

### Table 2.2 Buffer composition for DNA electrophoresis.

<table>
<thead>
<tr>
<th>TBE buffer</th>
<th>5 x Loading buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 mM Tris-borate</td>
<td>50 % Glycerol</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>0.05 % Bromophenol blue</td>
</tr>
<tr>
<td>NaOH to pH 8.0</td>
<td>50 % 10x TBE buffer</td>
</tr>
</tbody>
</table>

#### 2.2.1.2.4 Extraction of DNA fragments from agarose gels

The DNA fragments separated by agarose gel electrophoresis (see section 2.2.1.2.3) can be easily extracted from the gel and used for further experiments. The desired bands can be visualized and cut out of the gel with a scalpel under UV light. Next, the gel is dissolved and the DNA fragments purified. A commercially available kit (JetQuick Gel Extraction Kit, Genomed) was used for this purpose. The instructions on the manual provided with the kit were strictly followed.

First, the excised gel slices were weighed and subsequently 3 μL of Gel Solubilization Buffer (L1) was added for each mg of gel. The gel-containing tubes were then incubated in a heating block, under constant agitation, at 50 ºC for 15 minutes or until the gel slices were completely dissolved. After incubation, samples were transferred to a spin column in a receiver tube, centrifuged at 12,000 x g for 1 minute and the flow-through was discarded. Ethanol-containing Wash Buffer (L2) was next added to the spin column, which was again centrifuged at 12,000 x g for another minute. The flow-through was again discarded and another centrifugation at maximum speed was performed in order to remove any residual Wash Buffer and ethanol. Finally, the spin column was placed into a clean tube, water or TE buffer pre-heated to 70 ºC was added and, after 1 minute incubation time, a final centrifugation at 12,000 x g was performed. The eluate contained the purified DNA.
Table 2.3 Buffer composition in the extraction of DNA from agarose gel slices.
Concentrations of the components present in buffers L1 and L2, supplied by the kit are not provided by the manufacturer.

<table>
<thead>
<tr>
<th></th>
<th>TE buffer</th>
<th>Buffer L1</th>
<th>Buffer L2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl</td>
<td></td>
<td>Guanidine isothiocyanate</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td></td>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td>pH 7.4</td>
<td></td>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EDTA</td>
</tr>
</tbody>
</table>

2.2.1.2.5 Restriction digestion of DNA fragments

Restriction enzymes were used to cleave DNA at specific sites for subsequent ligation, screening of recently cloned plasmids, or plasmid linearization prior to in vitro transcription.

Restriction enzymes are endonucleases that specifically recognize and cleave 4 to 8 bp DNA segments, generally palindromic, called restriction sites. For cloning purposes, both plasmid DNA and insert are usually cut with the same enzymes in order to generate complementary ends that can be ligated. Depending on the chosen restriction enzyme, the resulting fragments can have sticky (cohesive) or blunt ends. Sticky ends are characterized by a few base pairs of a 5’ or 3’ overhang that will anneal only with a complementary end during ligation. However, some enzymes generate blunt ends, characterized by the absence of protruding ends. Therefore, any blunt end will ligate to another blunt end, independently of the enzyme used. Blunt end ligations are less specific and less efficient than sticky end ligations.

Given the vast number of restriction enzymes available, in a given sequence there are several restriction sites that can be used for cloning purposes. Particularly, expression vectors usually contain several engineered restriction sites at their multiple cloning sites to facilitate insertion of foreign DNA, or at their linearization sites to enable linearization.
Besides distinct recognition sequences, different enzymes have characteristic properties that determine their optimal restriction conditions, such as buffer composition, temperature and reaction time. Commercially available enzymes are supplied with optimized reaction buffers. Many enzymes require bovine serum albumin (BSA), which helps to stabilize the enzyme, reducing enzymatic loss. Therefore BSA was often supplied to the reaction for an enhanced enzyme performance. The amount of enzyme needed depends on the amount and nature of the DNA, number of restriction sites per fragment, reaction time, and the enzyme itself. Enzyme efficiency is measured in units. By definition, 1 unit is the amount of enzyme necessary to completely digest 1 μg of highly pure DNA in 1 hour. Reagents and respective concentrations for a typical restriction reaction are shown on Table 2.4.

### Table 2.4 Volume and concentration of the components in a standard restriction digestion reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td></td>
<td>0.02 μg/μL</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td></td>
<td>0.2 U/μL</td>
</tr>
<tr>
<td>Reaction buffer (10x)</td>
<td>5 μL</td>
<td>1x</td>
</tr>
<tr>
<td>BSA (100x)</td>
<td>0.5 μL</td>
<td>1x</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 50 μL</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.2.1.2.6 Dephosphorylation of DNA fragments

Through dephosphorylation, the 5’ phosphate group of the restricted cloning vector is removed using a phosphatase. Vector dephosphorylation avoids vector religation during the ligation reaction and hence reduces vector background after transformation. It is particularly important when the restriction digestion is performed with two enzymes that generate compatible ends, such as blunt ends. The
Materials and Methods

dephosphorylated vector will therefore only ligate with inserts that contain a phosphate group at their 5’ end.

For dephosphorylation purposes the Antarctic phosphatase was used (New England Biolabs, Frankfurt, Germany). Samples were dephosphorylated for 15 minutes at 37 ºC, before enzyme inactivation at 70 ºC. Reagents and respective concentrations for a typical dephosphorylation reaction are shown on Table 2.5.

Table 2.5 Volume and concentration of the components in a standard dephosphorylation reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td></td>
<td>0.02 – 0.1 µg/µL</td>
</tr>
<tr>
<td>Antarctic phosphatase (5 U/ µL)</td>
<td>1 µL</td>
<td>0.1 U/µL</td>
</tr>
<tr>
<td>AP buffer (10X)</td>
<td>5 µL</td>
<td>1x</td>
</tr>
<tr>
<td>H2O</td>
<td>to 50 µL</td>
<td>1x</td>
</tr>
</tbody>
</table>

2.2.1.2.7 Purification of DNA fragments

The choice of a purification strategy depends on the level of purity intended and the downstream application for which the DNA is needed. In some cases purification through gel extraction was not necessary. Hence, purification using the my-Budget Double Pure Kit (Bio-Budget, Krefeld, Germany) was performed. The instructions in the manual provided with the kit were strictly followed.

First, up to 50 µL of sample volume to be purified were mixed with 500 µL of binding buffer and vortexed. The mixture was then transferred to a spin filter in a collection tube and centrifuged for 2 minutes at 10,000 x g. Flow-through was discarded and another 1 minute centrifugation at 10,000 x g was performed in order to remove any residual buffer or ethanol. The collection tube was discarded and the spin filter placed in a collection tube. Finally, water or elution buffer pre-heated to 50 ºC were added to the spin filter and, after 1 minute incubation time at room temperature, a final centrifugation at 10,000 x g was performed. The eluate contained the purified DNA.
2.2 Methods

2.2.1.2.8 Ligation

Through ligation, two DNA fragments can be combined by a ligase. A T4 DNA ligase (Promega, Madison, WI, USA) was used for this purpose. This enzyme catalyzes the joining of both sticky-ended and blunt-ended double stranded DNA, through formation of a phosphodiester bond, in the presence of ATP.

A molar ratio of insert to vector of 3:1 was generally used for cloning purposes. Samples were either incubated in a water bath at 4ºC overnight or subjected to a temperature-cycle ligation (Lund et al 1996), for higher efficiency. In the latter, samples were ligated in a thermocycler, cycling between 30 seconds at 10 ºC and 30 seconds at 30 ºC, overnight. As a control, a ligation reaction without insert was always performed to screen for background vector religation. Reagents and respective concentrations for a typical ligation reaction are shown on Table 2.6.

Table 2.6 Volume and concentration of the components in a standard ligation reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA insert</td>
<td></td>
<td>3x fmol/μL</td>
</tr>
<tr>
<td>DNA vector</td>
<td></td>
<td>n fmol/μL</td>
</tr>
<tr>
<td>T4 ligase (3 U/μL)</td>
<td>1 μL</td>
<td>0.3 U/μL</td>
</tr>
<tr>
<td>Ligase buffer (10x)</td>
<td>1 μL</td>
<td>1x</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 10 μL</td>
<td></td>
</tr>
</tbody>
</table>

2.2.1.2.9 Transformation of DNA into competent bacteria

Transformation is a molecular biology technique that allows incorporation of foreign DNA into competent bacteria. Chemical competence was induced artificially by chilling the cells in the presence of calcium ions. Subsequently, these cells are covered in a layer of positive ions that shield the negatively charged phospholipids at
the bacterial cell surface, and are thereby able to attract negatively charged DNA. Therefore, these competent bacteria have a higher probability of taking up alien DNA.

Freshly thawed chemically competent bacteria incorporated exogenous DNA after being exposed to a heat shock. After addition of plasmid DNA, cells were kept on ice for 30 minutes. Next, they were incubated at 42 °C for 45 seconds and placed back on ice for another 2 minutes. 900 μL of 2x YT medium were then added to the bacteria, before incubation at 37 °C for 1 hour. After incubation, the cells were pelleted by centrifugation at 4,000 x g for 4 minutes, and 900 μL of the supernatant were discarded. The cells were resuspended in the remaining 100 μL of supernatant and plated on LB agar plates that contained a suitable antibiotic for selection. Finally, plates were allowed to dry for a few minutes and incubated overnight at 37 °C. Compositions of the media used are shown in Table 2.7.

Table 2.7 Composition of media and antibiotics used in bacterial culture.

<table>
<thead>
<tr>
<th></th>
<th>2x YT medium</th>
<th>LB medium</th>
<th>LB plates</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>16 g/L</td>
<td>10 g/L</td>
<td>10 g/L</td>
<td>200 μg/mL Ampicillin</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10 g/L</td>
<td>5 g/L</td>
<td>5 g/L</td>
<td>75 μg/mL Kanamycin</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g/L</td>
<td>5 g/L</td>
<td>5 g/L</td>
<td>(liquid) 50 μg/mL Kanamycin</td>
</tr>
<tr>
<td>pH 7.4</td>
<td></td>
<td>pH 7.2</td>
<td>pH 7.2</td>
<td>(plates)</td>
</tr>
</tbody>
</table>

2.2.1.2.10 Colony polymerase chain reaction

Colony PCR is a fast and convenient technique that allows screening of many colonies simultaneously, with no requirement for further culturing or extensive DNA purification steps, to determine if the insert has been incorporated into the plasmid construct.
With a sterile pipette tip a single colony was first scratched from the agar plate. The pipette tip was rubbed against the bottom of a PCR tube and afterwards was introduced into a 2 x YT medium containing sterile reaction tube, which was incubated at 37 ºC for approximately 1 hour. The PCR tube was used for a regular PCR reaction, using the flanking primers designed for the cloning process. During PCR, the initial heating step results in the release of the plasmid from the heat-disrupted cells and can then serve as a template for the PCR reaction. The presence of the insert was investigated by agarose gel electrophoresis (see section 2.2.1.2.3). Insert-containing clones were used for subsequent miniature DNA preparation.

2.2.1.2.11 Plasmid DNA preparation

After growth of the bacteria culture, cells were harvested, lysed and finally purified. In order to extract and purify plasmid DNA from bacteria, alkaline lysis is commonly applied. First, cells were lysed by treatment with the anionic detergent sodium dodecyl sulfate (SDS). In the presence of SDS, the bacterial cell membrane is solubilized and the contents of the cell released into solution. Under alkaline conditions, by addition of a strong base such as sodium hydroxide, proteins, chromosomal DNA, and plasmid DNA are denatured. Addition of a neutralizing buffer, such as potassium acetate, leads to precipitation of both proteins and chromosomal DNA, while plasmid DNA is renatured and remains in solution. Furthermore, the potassium ions remove the detergent precipitating the SDS. The removal of unwanted RNA can be achieved by addition of RNase. A centrifugation step sediments the non-solubilized components, and the plasmid containing supernatant can be transferred to a clean tube and precipitated with isopropanol.

Based on the quantity and purity of the plasmid needed, the plasmid preparation was either done at a small scale (minipreparation) or medium scale (midipreparation).

2.2.1.2.11.1 Minipreparation

The day before the minipreparation was performed, cells were picked from a LB agar plate containing an appropriate antibiotic and used to inoculate 2 mL of 2x YT
medium supplemented with the same antibiotic, for a 37 °C overnight incubation. In some cases, the source of transformed bacteria was instead a glycerol stock or a bacterial colony that had been analyzed by colony PCR. A 1.5 mL aliquot of this culture was centrifuged at 16,000 x g for 1 minute, and the supernatant discarded. The remaining 0.5 mL of culture was saved at 4 °C for subsequent inoculation of a medium scale plasmid preparation. The bacterial pellet was resuspended in 200 μL of ice cold buffer P1 and incubated on ice for 5 minutes. Next, 400 μL of buffer P2 was added to lyse the cells and the tube carefully inverted a few times to homogenize the sample. After 5 minutes of incubation on ice, 300 μL of ice cold buffer P3 was added to neutralize the sample and the solution was again homogenized by carefully inverting the tube, before incubating on ice for another 5 minutes. After a 5 minute centrifugation at 16,000 x g, the supernatant was transferred to a clean reaction tube and the pellet containing cell debris and chromosomal DNA was discarded. Next, 700 μL of isopropanol was added to the supernatant to precipitate the plasmid DNA and centrifuged at 16,000 x g for 15 minutes. The supernatant was discarded and the pelleted DNA washed with 70 % ethanol. After another 5-minute centrifugation step at 16,000 x g, the supernatant was discarded and the DNA dried in the vacuum concentrator for a few minutes. Finally, the DNA was redissolved in 30 μL of TE buffer and the sample concentration was determined (see section 2.2.1.2.12). Compositions of the buffers used during the minipreparation are shown on Table 2.8.

**Table 2.8 Composition of the buffers used in minipreparation.**

<table>
<thead>
<tr>
<th>Buffer P1</th>
<th>Buffer P2</th>
<th>Buffer P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris-HCl</td>
<td>200 mM NaOH</td>
<td>3 M CH₃COOK</td>
</tr>
<tr>
<td>50 mM glucose</td>
<td>1 % (w/v) SDS</td>
<td>2 M CH₃COOH</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1.2.11.2 Midipreparation

The day before the midipreparation, 50 mL of LB medium containing an appropriate antibiotic were inoculated with bacteria overnight at 37 °C. A commercially available kit (JetStar 2.0 Plasmid Kit, Genomed) was used for the midipreparation. The bacterial culture was first centrifuged at 4,000 rpm for 20 minutes and the supernatant discarded. The pellet was resuspended in 4 mL of resuspension buffer E1. Next, 4 mL of lysis buffer E2 were added to the cell suspension and the tubes inverted a few times to homogenize the mixture. After 5 minutes of incubation at room temperature, 4 mL of precipitation buffer E3 were added, and again the tubes were gently inverted a few times for homogenization purposes, followed by 10 minutes of centrifugation at 11,000 rpm. The lipid layer on top of the sample and the pellet consisting of cell debris were discarded, while the supernatant was loaded into a column supplied with the kit, which was pre-equilibrated with equilibration buffer E4. The provided anion exchange columns consist of a diethylaminoethanol (DEAE)-coated silica matrix. The negatively charged DNA binds to the positively charged DEAE groups and can be eluted with a high salt concentration elution buffer, while proteins, RNA, and other contaminants flow through at lower salt concentrations. After the sample passed through the column by gravity, two washing steps were performed. For each washing step, 10 mL of washing buffer E5 were added to the column and allowed to flow through. Next, a 14 mL polypropylene tube was placed under the column and 5 mL of elution buffer E6 were added to the column. The flow-through contained the eluted DNA, which was precipitated by addition of 3.5 mL of isopropanol. After centrifuging the sample for 30 minutes at 11,000 rpm, the supernatant was discarded and 70% ethanol was added to wash the pellet, which was again centrifuged at 11,000 rpm for 10 minutes. The supernatant was again discarded and the DNA dried in the vacuum concentrator for a few minutes. Finally, the DNA was redissolved in 100 μL of TE buffer and its concentration was determined (see section 2.2.1.2.12). Compositions of the buffers used during the minipreparation are shown on Table 2.9.
### Table 2.9 Compositions of the buffers used in midipreparation.

<table>
<thead>
<tr>
<th>Buffer E1</th>
<th>Buffer E2</th>
<th>Buffer E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl</td>
<td>200 mM NaOH</td>
<td>3.1 M CH₃COOK</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>1 % (w/v) SDS</td>
<td>CH₃COOH to pH 5.5</td>
</tr>
<tr>
<td>100 μg/mL RNaseA</td>
<td></td>
<td>pH 8.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer E4</th>
<th>Buffer E5</th>
<th>Buffer E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 mM NaCl</td>
<td>800 mM NaCl</td>
<td>1250 mM NaCl</td>
</tr>
<tr>
<td>100 mM CH₃COONa</td>
<td>100 mM CH₃COONa</td>
<td>100 mM Tris-HCl</td>
</tr>
<tr>
<td>0.15 % Triton X-100</td>
<td>CH₃COOH to pH 5.0</td>
<td>pH 8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH₃COOH to pH 5.0</td>
</tr>
</tbody>
</table>

### 2.2.1.2.12 Quantification of nucleic acids

By means of spectrophotometry, solubilized DNA and RNA can be quantified, and their respective purity level estimated. Nucleic acids are exposed to UV light in a spectrophotometer at different wavelengths. A photo detector then determines the amount of light that passes through.

Nucleic acids have an absorption peak at 260 nm ($A_{260}$), and therefore, their concentration directly correlates with the absorption at this wavelength, which can be calculated using the Beer-Lambert Law:

$$A = \varepsilon \times l \times c$$

where $A$ = absorbance; $\varepsilon$ = attenuation coefficient (L/g x cm); $l$ = light path length (cm); $c$ = concentration (g/L).
Since proteins absorb at a wavelength peak of 280 nm and phenol at 270 nm, sample purity can be assessed by calculation of the absorbance ratio at 260 and 280 nm. Pure DNA samples have $A_{260}/A_{280}$ ratios of approximately 1.8, pure RNA samples have $A_{260}/A_{280}$ ratios of approximately 2.0. Protein or phenol contaminations lead to low $A_{260}/A_{280}$ ratios.

2.2.1.2.13 DNA sequencing

The sequence of all DNA constructs generated was confirmed by DNA sequencing. Sanger sequencing (Sanger et al 1977) relies on the incorporation of chain-terminating dideoxynucleoside triphosphates (ddNTPs) during a PCR reaction. ddNTPs can not form phosphodiester bonds with other nucleotides, due to the absence of the 3’-OH group, and therefore, when incorporated, they discontinue DNA extension by the polymerase. ddNTPs are added to the mixture in a much lower concentration than that of the normal dinucleotides (dNTPs), hence, statistically, at the end of the PCR reaction all possible termination fragments will have been created. The fragments can then be separated according to their length by capillary electrophoresis conducted on the sequencer apparatus. The different ddNTPs (ddATP, ddGTP, ddCTP and ddTTP) have distinct fluorescent labels that allow for detection and identification.

All DNA sequencing was performed by Björn Peters (Department of Biochemistry I, Ruhr University, Bochum) or Anette Tolle and Sabine Laerbusch (Department of Biochemistry II, Ruhr University, Bochum).

2.2.1.2.14 DNA linearization

Before transcription of the cloned genes, DNA samples were linearized. Linearization is performed in order to avoid the generation of different concatemers during transcription. It was performed by one of the many restriction enzymes that cleave at the plasmid linearization site. The recognition site of the chosen enzyme must be unique in the plasmid. Consequently, the RNA polymerase generates same-size transcripts that start downstream of the promoter sequence and end at the linearization.
Materials and Methods

site. The linearization reaction was always performed overnight and analyzed by electrophoresis (see section 2.2.1.2.3). Reagents and respective concentrations used for a standard linearization reaction are shown on Table 2.10.

Table 2.10 Volume and concentration of the components in a standard linearization reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td></td>
<td>0.2 μg/μL</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td></td>
<td>0.3 U/μL</td>
</tr>
<tr>
<td>Reaction buffer (10x)</td>
<td>5 μL</td>
<td>1x</td>
</tr>
<tr>
<td>BSA (100x)</td>
<td>0.5 μL</td>
<td>1x</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 50 μL</td>
<td></td>
</tr>
</tbody>
</table>

2.2.1.2.15 *In vitro* transcription

The *in vitro* transcription technique was used to generate cRNA molecules from a DNA template. The linearized DNA plasmid (see section 2.2.1.2.14) used as a template must include at least a double-stranded RNA polymerase promoter sequence. The pSGEM vector used here has two promoter regions, one for the T7 RNA polymerase and another for the SP6 RNA polymerase. These promoter sequences flank both sides of the multiple cloning site, so transcription of sense and antisense cRNA is possible. Additionally, the vector was engineered to contain both 3’ and 5’ untranslated regions of the *Xenopus laevis* β-globin gene, between the promoter region and the linearization site, in order to enhance protein expression in oocytes.

A kit (mMessage mMachne T7 Transcription Kit, Life Technologies) was used to perform the transcription reaction. The reaction was assembled according to the recommendations of the kit’s manufacturer. The kit provides a mixture (NTP/CAP) consisting of all four ribonucleotides and a capping analog [m⁷G(5')ppp(5')G]. The capping analog provides protection against degradation by RNases. Furthermore, besides the T7 RNA polymerase and a cap-transferring enzyme, the enzyme mix
contains an RNase inhibitor that confers further protection against eventual contaminating ribonucleases.

The \textit{in vitro} reaction was incubated for 2h at 37 °C. Subsequently, the DNA template was digested by addition of 1 μL of TURBO DNase to the reaction tube and further incubation for 15 min at 37 °C. Reagents and respective concentrations used for a standard \textit{in vitro} reaction are shown on Table 2.11.

Table 2.11 Volume and concentration of the components in a standard \textit{in vitro} reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearized DNA</td>
<td>x μL</td>
<td>0.01 – 0.1 μg/μL</td>
</tr>
<tr>
<td>NTP/CAP (2X)</td>
<td>5 μL</td>
<td>1x</td>
</tr>
<tr>
<td>Reaction buffer (10X)</td>
<td>1 μL</td>
<td>1x</td>
</tr>
<tr>
<td>Enzyme mix</td>
<td>1 μL</td>
<td></td>
</tr>
<tr>
<td>Nuclease-free H₂O</td>
<td>to 10 μL</td>
<td></td>
</tr>
</tbody>
</table>

2.2.1.2.16 Purification of cRNA

Purification of cRNA samples was performed with the RNA Clean & Concentrator kit (Zymo Research). Before purification, the sample volume was adjusted to 50 μL, as recommended by the manufacturer. The RNA sample was then mixed with 100 μL of a 1:1 mixture of RNA binding buffer and 100 % ethanol. Next, the sample was transferred to a Zymo spin column provided with the kit and centrifuged for 30 s at 12,000 x g. The flow-through was discarded. 400 μL of RNA Prep Buffer were added to the column and allowed to flow through during a new 30 s centrifugation at 12,000 x g. The flow-through was again discarded. A first washing step was performed by addition of 700 μL of RNA washing buffer to the column, which was then subjected to a new centrifugation for 30 s at 12,000 x g. The flow-through was discarded again before addition of 400 μL of RNA Wash Buffer to the column for a second washing step. Another centrifugation step at 12,000 x g for 30 s was performed and the flow-through was discarded. To ensure that no wash buffer was left in the column, a final
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centrifugation step was performed for 2 min at 12,000 x g. The column was transferred to an RNase-free tube, and 25 μL of RNase-free water was added to elute the purified cRNA. After resting for 1 min at RT, the column was centrifuged for 30 s at 10,000 x g. An aliquot of the eluted cRNA was used for agarose gel electrophoresis analysis (see sections 2.2.1.2.3 and 2.2.1.2.17) and determination of sample concentration (see section 2.2.1.2.12). The remaining cRNA was stored at -80 °C.

2.2.1.2.17 Qualitative analyses of cRNA

Agarose gel electrophoresis (see section 2.2.1.2.3) was used to analyze RNA samples in the same way it was applied to DNA samples. However, a different buffer, loading dye, and molecular marker were used. The RiboRuler High range RNA ladder (Thermo Scientific) was used to estimate the length of RNA fragments. The gel was stained, before casting, by addition of a highly sensitive fluorescent stain (GelStar nucleic acid gel stain 10,000 x, Lonza). To avoid secondary structures and hence aberrant migration, RNA denaturation was assured by a 15-minute incubation step at 75 °C with the 2x loading buffer supplied with the mMessage mMachine T7 Transcription Kit (Life Technologies), before loading samples to the gel. The loading buffer contains formamide as a denaturing agent and a combination of xylene cyanol and bromophenol blue, in order to track migration. Since Tris contains a primary amino group, Tris-based buffers such TBE, normally used for running DNA gels, cannot be treated with DEPC, used to inactivate RNases in water. Therefore, 3-(N-morpholino)propanesulfonic acid (MOPS) was used as an alternative to Tris-borate in gels. The composition of the buffer is listed below on Table 2.12.
2.2 Methods

Table 2.12 Composition of the running buffer in RNA electrophoresis.

<table>
<thead>
<tr>
<th>MOPS buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM MOPS</td>
</tr>
<tr>
<td>50 mM C₂H₃NaO₂</td>
</tr>
<tr>
<td>10 mM EDTA</td>
</tr>
<tr>
<td>NaOH to pH 6.5</td>
</tr>
</tbody>
</table>

2.2.2 Expression Systems

2.2.2.1 Xenopus laevis oocytes

The oocytes of the South African frog *Xenopus laevis* possess all the internal machinery required for eukaryotic synthesis of proteins and are therefore well suited for expression of heterologous proteins (Dascal 1987). In addition to a high translation rate of RNA, low RNase activity in these cells contributes to a high efficiency system. Furthermore, post-translational modifications of newly synthetized proteins in mature oocytes is successfully achieved and they get correctly transported and localized.

The robustness and the large size of the oocyte facilitate cell manipulation. Both pharmacological agents and RNA can be directly injected into the oocyte; therefore, a stoichiometrically defined mixture of various RNAs can be introduced in a single step. In addition, the big cell size also facilitates insertion of the two electrodes used in two-electrode voltage clamp (TEVC) recordings.

However, its large size is also the main disadvantage of the oocyte system. Ligand-gated ion channel responses are too fast to be resolved by TEVC. The agonist application is not fast enough to reach all the channels at the same time. Hence, when the last channels are activated, previously activated channels may already be desensitized. Consequently, recording of fast gating kinetics is not possible in oocytes.

Even though oocytes do not express a large number of endogenous channels,
which could compromise the recordings, they express Ca\(^{2+}\)-activated chloride channels, which can be activated by incoming Ca\(^{2+}\). For instance, Ca\(^{2+}\) can enter the cell through exogenous Ca\(^{2+}\) permeable iGluRs once they are expressed. This is particularly important when measuring ion channel permeability to Ca\(^{2+}\). To circumvent this problem, oocytes can be injected with a Ca\(^{2+}\)-chelating agent, such as ethylene glycol tetraacetic acid (EGTA), before measurements.

### 2.2.2.1.1 Frog surgery and oocyte preparation

Frog surgery and oocyte preparation were performed by Björn Peters and Stefanie Nolte, who are technicians at the department of Biochemistry I. A female *Xenopus laevis* frog was first anesthetized with tricaine. Once the frog was anesthetized it was moved into ice and the surgery was performed. The ovaries were pulled out through a small cut in the abdomen of the frog and placed in a Petri dish containing Ca\(^{2+}\)-free Barth medium. The cut on the frog's abdomen was closed and disinfected, and the frog was allowed to recover. The ovaries were cut into small lumps with a scissor and defolliculated by treatment with a 10 mg/mL solution of collagenase type II (385 U/mg, Worthington), for about 2 h at 18 °C, under gentle but constant agitation. After incubation, oocytes were washed several times with Barth solution containing increasing amounts of Ca\(^{2+}\). Oocytes were kept in Ca\(^{2+}\)-containing Barth medium at 17 °C. Compositions of the buffers used during oocyte preparation are shown on Table 2.13.
2.2 Methods

Table 2.13 Composition of the buffers for oocyte preparation.

<table>
<thead>
<tr>
<th></th>
<th>Barth medium without Ca(^{2+})</th>
<th>Barth medium with Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM HEPES</td>
<td></td>
<td>15 mM HEPES</td>
</tr>
<tr>
<td>88 mM NaCl</td>
<td></td>
<td>88 mM NaCl</td>
</tr>
<tr>
<td>1.1 mM KCl</td>
<td></td>
<td>1.1 mM KCl</td>
</tr>
<tr>
<td>2.4 mM NaHC(_3)</td>
<td></td>
<td>2.4 mM NaHC(_3)</td>
</tr>
<tr>
<td>0.8 mM MgSO(_4)</td>
<td></td>
<td>0.8 mM MgSO(_4)</td>
</tr>
<tr>
<td>100 µg/mL Gentamycin</td>
<td></td>
<td>0.4 mM CaCl(_2)</td>
</tr>
<tr>
<td>63 µg/mL Penicillin-G</td>
<td></td>
<td>0.3 mM Ca(NO(_3))(_2)</td>
</tr>
<tr>
<td>40 µg/mL Streptomycin</td>
<td></td>
<td>100 µg/mL Gentamycin</td>
</tr>
<tr>
<td>pH 7.6</td>
<td></td>
<td>63 µg/mL Penicillin-G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 µg/mL Streptomycin</td>
</tr>
</tbody>
</table>

2.2.1.2 Injection of cRNA into oocytes

cRNA was injected into the oocytes with the help of a mechanical injector. The borosilicate glass micropipettes required for injection were prepared with a pipette puller in a two-step procedure. During the first step, the capillary is molten in the middle by a heat filament while the ends are slowly being pulled apart. This results in the narrowing of the middle part of the capillary, which easily brakes into two identical capillaries by applying a stronger pull, in a second step. The length and the diameter of the tip are dependent on the temperature and force applied by the puller in both steps. After pulling, the tip of the capillary was broken back manually to a diameter of approximately 15 µm by pressing it against a glass block, under a microscop.. Successfully broken capillaries were filled with a 1:1 mixture of light and heavy mineral oils and fixed to the injector piston.
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The cRNA was allowed to thaw on ice and briefly centrifuged at 4 ºC. The pre-diluted cRNA mixture was subsequently drawn into the injection capillary through its tip, with care to avoid introducing any air bubbles.

Oocytes were pre-sorted based on their developmental stage. Only healthy stage V and stage VI oocytes, with well-defined animal and vegetal poles were used for injection. Selected oocytes were placed in an injection chamber filled with Barth medium, and injected one by one under a microscope, by gently inserting the tip of the capillary into the oocyte, with care not to pierce the oocyte too deep. Once inside the oocyte, a pre-defined volume of the RNA solution was discharged and the capillary removed. Injected oocytes were kept in an incubator at 17 ºC for 4-5 days, in a 4-section 10-cm dish filled with Barth medium. Oocytes were checked regularly, and any eventually damaged oocytes were discarded.

2.2.2.2 HEK293 cells

Human Embryonic Kidney 293 cells (HEK 293) are easy to maintain, grow and manipulate and are therefore extensively used for cell biology. Successfully transfected cells (see section 2.2.2.2.2) were used for patch clamp experiments (see section 2.2.3.2).

2.2.2.2.1 Cell passaging

HEK293 cells were kept in Dulbecco’s Modified Eagle Medium (DMEM), which was renewed frequently. When cells get highly confluent it is necessary to split them into new dishes, in order to keep them in the exponential growth phase. Confluent cells were washed with PBS before a 5 min incubation step with trypsin at 37 ºC to dissociate the cells. Trypsin can be inhibited by serum, Ca²⁺, or Mg²⁺, therefore, the reaction was stopped by the addition of fresh serum-containing DMEM. After a mild centrifugation step at 2,000 rpm for 2 min, pelleted cells were resuspended in DMEM and cell density was determined with a Neubauer chamber. An aliquot of the cell suspension was seeded in a new cell culture flask containing DMEM and incubated at 37 ºC and 8 % CO₂. For patch clamp recordings, 50,000 cells were plated in a 35 mm dish, three days before transfection.
2.2 Methods

All described procedures were performed by Tobias Strasdeit and Christina Klein-Schmidt (Department of Biochemistry I, Ruhr University, Bochum).

2.2.2.2 Transfection

HEK293 cells were transfected with Metafectene (Biontex). Before transfection, fresh DMEM was added to the cells. For the transfection, a total of 3 µg of DNA was diluted in 100 µL of phosphate-buffered saline (PBS) and 3 µL of Metafectene was added to the same volume of PBS in a separate reaction tube. The DNA solution was then added to the Metafectene solution and the mixture was incubated at RT for 15 min. The transfection mixture was added to the cells and incubated overnight at 37 ºC and 8 % CO₂. Thereafter, the transfection mixture was removed and the cells supplemented with fresh DMEM. For patch clamp recordings, 50,000 cells were split into a glass bottom dish and incubated overnight at 37 ºC and 8 % CO₂.

Cell transfection was performed by Tobias Strasdeit and Christina Klein-Schmidt (Department of Biochemistry I, Ruhr University, Bochum).

The composition of the PBS buffer used for cell transfection is shown in Table 2.14.

Table 2.14 Composition of the PBS buffer used for transfection.

<table>
<thead>
<tr>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mM NaCl</td>
</tr>
<tr>
<td>8 mM Na₂HPO₄</td>
</tr>
<tr>
<td>2 mM KH₂HPO₄</td>
</tr>
<tr>
<td>pH 7.4</td>
</tr>
</tbody>
</table>
2.2.3 Electrophysiology

2.2.3.1 Two-Electrode Voltage Clamp

TEVC was applied to investigate ion channels of iGluRs in oocytes. This technique consists of the use of two intracellular electrodes, which are connected to both an amplifier and a computer. The voltage (potential) electrode continuously measures the intracellular potential against an extracellular reference electrode. Channel activity, caused for instance by application of an agonist, leads to fluctuations in the oocyte membrane potential. The detected changes are compensated by injection of current through the current electrode in order to maintain the membrane potential at a fixed value.

Recording glass capillaries were prepared on a pipette puller, in the same fashion as the injection capillaries. However, after the pulling procedure, capillaries were not broken back against a glass block. Optimal capillaries showed resistance values between 0.1-1.5 MΩ for the current electrode, and between 0.5-5.0 MΩ for the potential electrode. Capillaries were filled with a 3 M KCl solution before being fixed to the Ag/AgCl electrodes. Before each measurement, capillaries were calibrated in the Ringer solution, and their respective resistances were checked.

The oocyte was placed in the recording chamber, the electrodes were introduced into opposite sides of the oocyte, and normal frog Ringer (NFR) was allowed to perfuse the chamber. The selection of solutions perfusing the chamber was controlled by an 8-way Hamilton valve connected to eight 50 mL reservoirs. Because the flow is controlled by hydrostatic pressure, the flow rate can be regulated by adjusting the position of the reservoirs, the volume contained in the reservoirs, and both length and diameter of the tubes that connect the reservoirs to the valve. The overflow was continuously removed from the chamber by suction. The membrane potential was set to -70 mV, and 1-minute recordings were performed. During the first 10 s of the recording no agonist was applied and the resulting leak current was used as a baseline reference to calculate current amplitudes. Subsequently, agonist was applied for a period of 20 s, after which the agonist was washed out with NFR. If different agonists were applied to the same oocyte, the oocyte was allowed to recover for approximately 2 minutes before the next agonist
application, or until the leak current was stable. The composition of the NFR is shown on Table 2.15.

**Table 2.15 Composition of the NFR used in TEVC.**

<table>
<thead>
<tr>
<th>NFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES</td>
</tr>
<tr>
<td>115 mM NaCl</td>
</tr>
<tr>
<td>2.5 mM KCl</td>
</tr>
<tr>
<td>1.8 mM CaCl₂</td>
</tr>
<tr>
<td>pH to 7.2 with NaOH</td>
</tr>
</tbody>
</table>

**2.2.3.1.1 I/V curves**

I/V curves were recorded by first subjecting the oocyte to a hyperpolarizing potential of -150 mV, then gradually increasing the applied voltage until the depolarizing potential of +50 mV was reached. The whole recording was completed within 2 s. I/V curves were recorded in Ca\(^{2+}\)-free magnesium Ringer (MgR). First, an I/V curve was recorded in the absence of agonist. The resulting curve characterizes unspecific conductance endogenous to the oocyte and has to be subtracted from the I/V curve recorded in the presence of agonist, which was recorded afterwards in the same oocyte. The composition of the MgR is shown on Table 2.16.
Table 2.16 Composition of the MgR used for I/V curve recording.

<table>
<thead>
<tr>
<th>MgR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES</td>
</tr>
<tr>
<td>115 mM NaCl</td>
</tr>
<tr>
<td>2.5 mM KCl</td>
</tr>
<tr>
<td>1.8 mM MgCl₂</td>
</tr>
<tr>
<td>pH to 7.2 with NaOH</td>
</tr>
</tbody>
</table>

2.2.3.1.2 Calcium permeability

In order to determine Ca²⁺ permeability of AMPARs, recordings of agonist-induced currents were performed in extracellular solutions that contained Ca²⁺ as the only permeable ion. The large impermeable cation N-methyl-D-glucamine (NMDG) was added to the Ca²⁺ solutions to adjust the osmolarity to that of NFR.

To quench endogenous Ca²⁺ and thus avoid activation of Ca²⁺-activated chloride channels, 10 nmol of EGTA were injected in each oocyte 30 min before recording.

By recording reversal potentials in an external solution where Ca²⁺ is the only permeable ion, it is possible to calculate the relative conductance of Ca²⁺ to monovalent ions and therefore calculate Ca²⁺ permeability values. A shift of the reversal potential to less negative values indicates an increase in permeability. Hence, given that Ca²⁺ is the only permeable ion present extracellularly, and that the monovalent ions Na⁺ and K⁺ can be assumed to be the only intracellular ions and have identical permeability values, a modified Goldman-Hodgkin-Katz equation can be used to calculate permeability ratios (Iino et al 1990):
2.2 Methods

\[
\frac{P_{Ca}}{P_{Mono}} = \frac{[Mono]_i e^{E_{rev} \frac{F}{RT}} (e^{E_{rev} \frac{F}{RT}} + 1)}{4 [Ca^{2+}]_o}
\]

\([Mono]_i = \text{concentration of intracellular monovalent cations (mM)}
\]

\([Ca^{2+}]_o = \text{concentration of extracellular calcium cations (mM)}
\]

\(E_{rev} = \text{reversal potential (mV)}
\]

\(F = \text{Faraday constant (9.65 x 10^4 C mol}^{-1}\)

\(R = \text{Avogadro number (8.314 J mol}^{-1} K^{-1}\)

\(T = \text{temperature (K)}
\]

To attest for reliability of the calculated values, two individual low-\(Ca^{2+}\) concentration solutions (4 mM and 8 mM) were used in parallel to determine \(Ca^{2+}\) permeability. The \(P_{Ca}/P_{Mono}\) value of the wild type GluK2 is known from the literature (Egebjerg & Heinemann 1993). Therefore, reversal potentials for GluK2 were recorded in every experiment, in both 4 mM and 8mM \(Ca^{2+}\) Ringer solutions. This allowed to estimate the concentration of intracellular monovalent cations ([Mono]_i), which was then used to calculate \(P_{Ca}/P_{Mono}\) values. Activity values were used to replace concentrations in the equation, and reversal potentials were corrected for the junction potential (Shatkay 1968). The composition of the \(Ca^{2+}\) Ringer solutions used for calculation of \(Ca^{2+}\) permeability is shown in Table 2.17.

**Table 2.17 Composition of the Ca\(^{2+}\) Ringer solutions used in the determination of Ca\(^{2+}\) permeabilities.**

<table>
<thead>
<tr>
<th>4 mM CaR</th>
<th>8 mM CaR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES</td>
<td>10 mM HEPES</td>
</tr>
<tr>
<td>114.2 mM NMDG</td>
<td>108.6 mM NMDG</td>
</tr>
<tr>
<td>4 mM CaCl(_2)</td>
<td>8 mM CaCl(_2)</td>
</tr>
<tr>
<td>pH to 7.2 with HCl</td>
<td>pH to 7.2 with HCl</td>
</tr>
</tbody>
</table>
2.2.3.2 Patch clamp

Transfected HEK cells were analyzed by patch clamp (Neher & Sakmann 1976). This technique allowed analyzing fast receptor kinetics that were unresolvable in oocytes using TEVC.

Patch clamp involves the use of two electrodes: a recording electrode and an extracellular reference electrode, which are connected to both an amplifier and a computer. The recording electrode is controlled by a micromanipulator that allows precise positioning, and a piezo-electric element that can effect fast movement of the capillary in the case of recording from an excised membrane patch. The glass capillary is filled with intracellular saline, which mimics the cytoplasmatic ionic composition, and fixed to the recording electrode. The assembled recording electrode is then brought into near proximity of the cell, gentle suction is applied and a tight seal is generated between cell and electrode tip, which keeps the cell attached to the electrode for the duration of the recording. The electrical resistance generated is ideally between 2-10 MΩ. The diameter of the tip of the recording glass electrode is only a few micrometers and, therefore, the area of the membrane patched contains only a few ion channels.

This system involves fast agonist application. Therefore, it was absolutely crucial to be able to change between background solution and agonist on a millisecond time scale. This is enabled by the use of a θ-capillary. The θ-capillary is divided longitudinally by a septum, creating two parallel channels that can be filled with different solutions. One of the channels is connected to a reservoir that contains background solution (extracellular saline), while the other channel is controlled by an 8-way Hamilton valve connected to eight different reservoirs that can be filled with different agonist solutions. If enough pressure is applied, a laminar flow is created, and therefore the incoming solutions do not mix outside the capillary.

Different variants exist of this technique, permitting analysis of ion channels at many different levels. For instance, they can be focused on either single-patch or whole-cell recordings, depending on whether an excised membrane patch or a whole cell is used. Some involve ligand application to the extracellular part of the membrane while others involve ligand application to the intracellular side. The patch clamp recordings performed for this thesis were done in whole-cell mode. In this variation, after attachment of the capillary to the cell membrane, extra suction is applied to the cell.
leading to rupture of the patch while the seal to the cell is not disrupted. This allows access to the cytosol of the cell and, consequently, clamping of the membrane potential. The current measured upon agonist application in this configuration is the result of the simultaneous response of all ion channels across the whole cell membrane.

The pIRES2-EGFP and the pIRES2-DsRed-Express2 vectors were used for expression in cells intended for patch clamp recordings. As a result, independent expression of either EGFP or DsRed was used to screen for successfully transfected cells. Ion channel current recordings were performed at a clamped potential of -60 mV.

Patch clamp recordings were performed by Tobias Strasdeit (Department of Biochemistry I, Ruhr University, Bochum).

Compositions of the solutions used for patch clamp are shown in Table 2.18.

**Table 2.18 Composition of the solutions used in patch clamp.**

<table>
<thead>
<tr>
<th>Extracellular saline</th>
<th>Intracellular saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES</td>
<td>10 mM HEPES-KOH</td>
</tr>
<tr>
<td>140 mM NaCl</td>
<td>130 mM CsCl</td>
</tr>
<tr>
<td>4 mM KCl</td>
<td>1 mM CaCl₂</td>
</tr>
<tr>
<td>2 mM CaCl₂</td>
<td>2 mM MgCl₂</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>11 mM EGTA</td>
</tr>
<tr>
<td>pH to 7.3 with NaOH,</td>
<td>pH to 7.3 with NaOH,</td>
</tr>
<tr>
<td>285-295 mosm/kg with NaCl or H₂O</td>
<td>285-295 mosm/kg with CsCl or H₂O</td>
</tr>
</tbody>
</table>
2.2.4 Protein Biochemistry

2.2.4.1 Membrane preparation

For membrane protein analysis, it was important to distinguish between the membrane-expressed receptors and the intracellular receptor pools. With that in mind, intact oocytes were initially incubated with the biotin-labeled lectin concanavalin A (ConA). ConA binds to glycosylated asparagine residues of proteins. On intact oocytes, only membrane proteins are available for interaction with ConA and therefore they can be successfully separated from intracellular receptors based on their selective biotinylation.

Oocytes were first injected with the selected RNA (see section 2.2.2.1.2) and allowed to express for four days in normal frog Ringer’s solution (NFR) at 17 °C. Between 20 to 50 oocytes were then transferred into one well of a 24-well plate, containing 600 μL of 10 μM biotinyl-ConA and incubated for 30 min at RT under gentle but constant agitation. After incubation, the ConA solution was carefully removed and the oocytes were washed five times, in 10-min periods under gentle agitation, with 1 mL of sterile NFR, always with caution not to rupture the oocytes. Damaged oocytes were removed immediately to avoid eventual labeling of intracellular receptors and consequently false results. After washing, intact oocytes were moved into a 1.5 mL reaction tube and excess NFR carefully removed. Next, a volume of 20 μL of H-buffer per oocyte was added and oocytes were thoroughly homogenized. Homogenized oocytes were incubated under agitation at 4 °C for 1h to ensure complete membrane solubilization.

Meanwhile, 20 μL of homogenized streptavidin-agarose beads per sample were washed four times in H-buffer. Each washing step consisted of a 2-min centrifugation period at 20,000 x g, removal of supernatant and addition of 1 mL of fresh H-buffer. After washing, the initial supernatant volume was reestablished with fresh H-buffer.

The homogenized oocytes were centrifuged at 20,000 x g for 15 min at 4 °C, and the resultant supernatant transferred to a new 1.5 mL reaction tube, with care to avoid the upper lipid layer. The supernatant was submitted to another 15 min centrifugation step at 20,000 x g at 4 °C, and transferred to a fresh 1.5 mL reaction tube. A 20 μL aliquot of the supernatant was at this point stored as “total fraction” at -80 °C.
The remaining supernatant was incubated overnight with 20 µL of the washed streptavidin-agarose beads, under rotation at 4 ºC. Next, the mixture was centrifuged at 20,000 x g for 5 min at 4 ºC. The supernatant, which contains both intracellular and unlabeled membrane proteins, was stored as “supernatant fraction” at -80 ºC. The pelleted beads were washed three times with 1 mL of ice-cold H-buffer, and the supernatant discarded after each of the 5-min centrifugation steps at 20,000 x g at 4 ºC. Afterwards, all the H-buffer was carefully removed and 20 µL of 2X SDS-PAGE loading buffer were added to the washed beads. Samples were incubated in a heating block for 10 min at 95 ºC and centrifuged for 2 min at 16,000 x g. The supernatant was either used immediately for SDS-PAGE (see section 2.2.4.2) or was moved into a new reaction tube and stored at -80 ºC. Compositions of the buffers used during the membrane preparation are shown on Table 2.19.

Table 2.19 Composition of the buffers used in membrane preparation.

<table>
<thead>
<tr>
<th>H-Buffer</th>
<th>2X SDS loading buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl</td>
<td>25 mM Tris-HCl</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>6 % (w/v) SDS</td>
</tr>
<tr>
<td>1 % (v/v) Triton X-100</td>
<td>0.8 M ß-Mercaptoethanol</td>
</tr>
<tr>
<td>1 tablet/50 mL cOmplete™ protease inhibitor cocktail (Roche)</td>
<td>4 M Urea</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>20 % (w/v) Glycerol</td>
</tr>
<tr>
<td></td>
<td>0.05 % (w/v) Bromophenol blue</td>
</tr>
</tbody>
</table>

2.2.4.2 SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to separate proteins according to their molecular weight. Naturally, proteins would separate on a polyacrylamide (PA) gel according to their size, charge and conformation. However, a combination of heat treatment and use of ß-mercaptoethanol during membrane preparation (see section 2.2.4.1) and further use of (SDS), during both
membrane preparation and SDS-PAGE, results in protein denaturation. Also, SDS is negatively charged, thus it’s binding to proteins masks their charge and creates a uniform mass-to-charge ratio. Therefore, protein migration on the PA gel becomes solely a function of amino acid chain length.

PA gels are the result of the polymerization of acrylamide cross-linked with N,N’-methylenediacylamide. Polymerization is initiated by ammonium persulfate (APS), which works as a source of free radicals, and is catalyzed by tetramethylethylenediamine (TEMED), which stabilizes the free radicals contributing to a more uniform polymerization. Pore size is determined by the concentration of acrylamide and cross-linker, and therefore can be easily optimized with respect to the proteins to be analyzed.

For better resolution discontinuous gels were casted. The stacking gel consists of a lower acrylamide concentration and a lower pH value, compared to the running gel. The sudden pH change between the two gels, from 6.8 to 8.8, leads to deprotonation of the N-terminal amino groups of proteins, so they are more negative in the running gel when compared to the stacking gel. Also, the running buffer contains glycine, which is protonated at the lower pH characteristic of the stacking gel, losing its negative nature and thus not moving towards the anode. Yet, at this pH chloride ions remain negative and move towards the anode. Consequently, proteins get crammed between glycine and chloride ions. With the higher pH of the running gel, glycine becomes deprotonated and starts moving towards the anode and subsequently proteins can move unrestrained. In addition, the lower ionic strength in the stacking gel implies a higher electrical resistance and a higher electrical field, resulting in a faster migration of every charged particle in the gel. Furthermore, the large pore size, consequence of the low acrylamide concentration, allows proteins to migrate freely despite their size. The combination of all these factors results in the fact that proteins get concentrated in a single band upon reaching the gel interface and can therefore initiate migration into the running gel at the same time, leading to sharper bands, and in the running gel (also called resolving gel) finally get separated according to their molecular weights.

Considering the relatively high molecular weights of AMPARs, a 10 % acrylamide concentration was routinely used to assemble the running gel. All components were mixed as described in Table 2.20.
2.2 Methods

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Running gel (10 %)</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>3 mL</td>
<td>4.3 mL</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>1.9 mL</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris pH 6.8</td>
<td>-</td>
<td>1.9 mL</td>
</tr>
<tr>
<td>30 % Acrylamide (37:5:1)</td>
<td>2.5 mL</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>150 µL</td>
<td>75 µL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>37.5 µL</td>
<td>25 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>12.5 µL</td>
</tr>
</tbody>
</table>

After addition of TEMED, which was added last to start polymerization, the gel mixture was immediately poured into the pre-assembled gel chamber, leaving between 1 to 1.2 cm to the edge of the small glass. To achieve a smooth gel surface, this space was filled immediately with isopropanol, with care to not leave any air bubbles. After polymerization was complete, isopropanol was poured out of the chamber and the gel was rinsed with distilled water, and the space in between was dried with care not to touch the gel. The stacking gel was prepared according to Table 2.14, adding TEMED last, and poured directly into the casting chamber on top of the running gel. A comb was inserted immediately into the casting chamber with care not to leave any air pockets between comb and stacking gel. After full polymerization, the comb was removed and the gel rinsed with running buffer. The gel was inserted into the electrophoresis chamber, which was then filled with running buffer. Samples prepared as described in section 2.2.4.1 were loaded into the gel pockets. PageRuler Pre-stained Protein Ladder (Thermo Scientific) was loaded into the pockets flanking the samples, and a constant voltage of 100 V was applied until samples reached the running gel, after which the voltage was increased to 150 V until the samples were sufficiently resolved. The composition of the running buffer used for SDS-PAGE is shown on Table 2.21.
Table 2.21 Composition of the running buffer used in SDS-PAGE.

<table>
<thead>
<tr>
<th>Running Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris-HCl</td>
</tr>
<tr>
<td>190 mM Glycine</td>
</tr>
<tr>
<td>0.1 % (w/v) SDS</td>
</tr>
<tr>
<td>pH 8.8</td>
</tr>
</tbody>
</table>

2.2.4.3 Western Blot

After SDS-PAGE, Western blot was used to transfer the proteins from the gel to a nitrocellulose membrane (Towbin et al 1979).

First, the PA gel was removed from the electrophoresis chamber and the stacking gel part was cut off and discarded. A nitrocellulose membrane and two filter papers were cut to about the same size as the PA gel and soaked in blotting buffer together with two sponges and the gel. The blotting cassette was next assembled following the order: sponge, filter paper, nitrocellulose membrane, gel, filter paper, sponge. Eventual air bubbles were carefully squeezed out and the cassette was firmly closed and moved into the blotting tank, which had previously been filled with blotting buffer and a magnetic stirrer. Because proteins are negatively charged in the presence of SDS, the cassette was arranged in a way that the nitrocellulose membrane faced the anode while the gel faced the cathode in the blotting apparatus. The blotting was performed at 4 °C overnight at 100 mA, under constant stirring. The composition of the blotting buffer is shown on Table 2.22.
2.2 Methods

Table 2.22 Composition of the blotting buffer used in Western blotting.

<table>
<thead>
<tr>
<th>Blotting buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris-HCl</td>
</tr>
<tr>
<td>190 mM Glycine</td>
</tr>
<tr>
<td>20 % (v/v) Ethanol</td>
</tr>
<tr>
<td>0.01 % (w/v) SDS</td>
</tr>
<tr>
<td>pH 8.8</td>
</tr>
</tbody>
</table>

2.2.4.4 Western blot analysis

After Western blotting, the presence of the protein of interest on the membrane was detected and analyzed with antibodies.

The membrane was removed from the blotting cassette and washed three times in PBS-T, for periods of at least 5 min at RT and under constant agitation. Antibodies are also proteins and therefore, due to the unspecific binding of proteins to the nitrocellulose membrane, the membrane at first had to be saturated with milk proteins, to avoid unspecific binding by antibodies. In order to do that, the membrane was incubated at RT in a milk blocking solution for 60 min under constant agitation. Afterwards, the membrane was incubated with a primary antibody, which binds to a specific antigen on the protein of interest. The primary antibody was diluted in the blocking solution, for an optimal period of time, temperature, and dilution factor, which can vary depending on the antibody. Yet, for the primary antibodies used in this thesis, an overnight incubation at 4 °C was ideal in all cases. Next, the membrane was again washed three times in PBS-T, to remove any unbound primary antibody, for periods of at least 5 min at RT and under constant agitation. The membrane was subsequently incubated with the secondary antibody, at the recommended dilution, for 90 min at RT, under constant agitation. The secondary antibody is directed against an immunoglobulin of the species from which the primary antibody was originated. In addition, the secondary antibody is linked to a reporter enzyme such as horseradish peroxidase.
(HRP). In the presence of hydrogen peroxide and using luminol as a substrate, HRP catalyzes a chemiluminescence reaction that emits light in direct proportion to the amount of protein probed and which can be easily detected and quantified. After incubation, the membrane was washed three times in PBS-T, to remove any unbound secondary antibody, for periods of minimum 5 min at RT and under constant agitation. Finally, right before imaging, luminol based chemiluminescence substrate (SuperSignal West, Thermo Scientific) was prepared and added directly to the nitrocellulose membrane. Detection times depended on the amount of protein to be analyzed and were therefore optimized for every membrane. Compositions of the buffers used for western blot analysis are shown on Table 2.23.

Table 2.23 Composition of the buffers used in Western blot analysis.

<table>
<thead>
<tr>
<th>PBS-T</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mM NaCl</td>
<td>5 % (w/v) Nonfat dry milk</td>
</tr>
<tr>
<td>3 mM KCl</td>
<td>in PBS-T</td>
</tr>
<tr>
<td>8 mM Na$_2$HPO$_4$</td>
<td></td>
</tr>
<tr>
<td>2 mM KH$_2$PO$_4$</td>
<td></td>
</tr>
<tr>
<td>0.05 % (v/v) Tween 20</td>
<td></td>
</tr>
<tr>
<td>pH 7.4</td>
<td></td>
</tr>
</tbody>
</table>

2.2.4.5 Regeneration of membranes

In order to be able to compare the amount of protein between lanes, the protein level was always normalized to $\alpha$-cadherin expression levels. $\alpha$-cadherin is constitutively expressed at the membrane of oocytes and is evenly expressed amongst oocytes from the same batch, independently of the heterologously expressed iGluRs and TARPs. Thus, the nitrocellulose membranes had to be probed a second time. Hence, HRP was quenched by hydrogen peroxidase, before incubation with a new primary
antibody (Upadhaya et al 2011). Since the primary antibodies were in no case from the same species, it was not necessary to preabsorb primary and secondary antibodies.

The nitrocellulose membrane was incubated in quenching buffer for 20 min at RT under constant agitation. Subsequently, the membrane was washed in PBS-T for a minimum of 5 min at RT under constant agitation. After washing, the membrane was incubated with new primary and secondary antibodies and analyzed, as described in section 2.2.4.4. The composition of the quenching buffer is shown on Table 2.24.

<table>
<thead>
<tr>
<th>Quenching buffer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>27 % (v/v) H₂O₂</td>
<td></td>
</tr>
<tr>
<td>in PBS-T</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.5 Microscopy

#### 2.2.5.1 Confocal microscopy

Confocal microscopy is a high-resolution optical imaging technique. The existence of a pinhole in the confocal microscope permits the visualization of small sample zones at a time, eliminating out of focus background fluorescence, characteristic to conventional fluorescence microscopes. The size of the pinhole can be adjusted and therefore depth of field can be regulated. Additionally, scanning through different sections of the object allows for the creation of a three dimensional image.
2.2.5.1.1 Bimolecular fluorescence complementation

In order to investigate interaction between two proteins, a bimolecular fluorescence complementation (BiFC) assay was employed. Two complementary fragments of GFP were fused to the C-terminus of each interaction candidate and both fusion proteins were subsequently expressed in oocytes. None of the GFP fragments is able to fluoresce by itself, nor do they interact on their own when not fused to another protein. However, if the proteins of interest interact, the complimentary fragments are in close proximity and consequently, GFP's native structure will be reestablished and fluorescence can be observed. Fluorescence does not necessarily mean that the fusion proteins interact directly, but that they are at least part of the same complex.

For BiFC analysis, oocytes were injected with RNA and incubated at 17 °C for 5 to 6 days before analysis. The long expression period assures that expression has reached a plateau and is therefore at its maximum level, reducing variability between oocytes. After incubation, fluorescence at the cell membrane was analyzed by confocal microscopy in living oocytes.
3 Results

Type II-TARPs $\gamma 5$ and $\gamma 7$ associate with AMPARs in neurons modulating the receptors’ biophysical properties. However, both TARPs were until recently categorized as non-TARPs, together with $\gamma 1$ and $\gamma 6$, due to a perceived lack of AMPA receptor modulation. Such classification was justly modified after both $\gamma 5$ and $\gamma 7$ proved to be both valuable auxiliary subunits to AMPARs (Kato et al 2008). Still, very little is known about these TARPs when compared to type I-TARPs, which have been extensively studied. This study aims to fill that gap, by analyzing type II-TARP interaction with all members of the AMPAR subfamily in close detail. Furthermore, we have looked into the possibility of $\gamma 6$ having a role in regulating AMPARs and therefore joining the TARP family.

3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

To analyze the contribution of $\gamma 5$ and $\gamma 7$ to AMPA receptor agonist-induced currents, AMPA receptors were co-expressed with TARPs in X. laevis oocytes. For expression in oocytes, the designated cRNA combinations were injected into oocytes and after 4 to 5 days of incubation, the expressed ion channels were electrophysiologically analyzed by TEVC. All cDNAs used in the in-vitro transcription procedure had previously been subcloned in the pSGEM vector.

Both splice variant and editing state of the receptor were taken into consideration. Hence, homomeric receptors composed of either flip or flop variants were analyzed for all four AMPAR subunits, each in their edited (R) and non-edited (Q) variants, even in those cases where edited variants do not exist physiologically. Additionally, different receptor subunits were combined to form various heteromeric receptors.
For all combinations, currents induced by two different agonists were investigated: the full agonist glutamate and the partial agonist kainate. Furthermore, current voltage (I/V) relationships were recorded for all homomeric and heteromeric receptors.

3.1.1 Homomeric AMPA receptors

3.1.1.1 GluA1

The first set of receptors to be electrophysiologically characterized included the flip and flop variants of GluA1 in both, its Q/R-edited and non-edited forms, although the edited variant does not occur physiologically. Homomeric GluA1 was co-expressed in oocytes with either one of the type II-TARPs or with γ6. For comparison, GluA1 was additionally co-expressed with the well-characterized type I-TARP γ2 and recorded in parallel for each experiment. Representative traces of both glutamate and kainate-induced currents for every recorded combination of expressed subunits, the resultant glutamate and kainate-induced responses, as well as the calculated ratio between kainate and glutamate-induced currents are shown in Figures 3.1, 3.2, 3.3 and 3.4. This ratio is well known to be altered by type I-TARPs as a direct consequence of reduced receptor desensitization and increased kainate efficacy (Kott et al 2007, Levchenko-Lambert et al 2011, Turetsky et al 2005). Hence, we wondered if a similar effect was observed as a result of co-expression of the receptor with type II-TARPs.

Much like γ2, γ7 was able to significantly increase both glutamate- and kainate-induced currents of all GluA1 receptors. γ7 also significantly increased the kainate-to-glutamate current ratio of the flip variants, although only slightly. Potentiation values are shown in table 3.1.

Reinforcing its non-TARP classification, γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist, except for GluA1(Q)flop, whose current amplitudes for both glutamate and kainate were only very mildly decreased (to 70 % and 90 % of the original current), although the statistic test showed significance, due to the very high number of oocytes and a very low current variance between recorded oocytes.
More interestingly, γ5 had a potentiating effect on edited receptors while it reduced current amplitudes of unedited receptors, indicating a discriminatory regulation of AMPARs by γ5 depending on the amino acid at this very specific location, position which also defines the receptor permeability to cations. The resulting potentiation values are shown in Table 3.1.

Furthermore, γ5 was able to very strongly increase kainate-to-glutamate current ratios of edited receptors, while it had a much less prominent effect on that ratio of GluA1(Q)flip ratio and no effect at all at GluA1(Q)flop.
Results

Figure 3.1 Current responses of GluA1(Q)flip homomeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(Q)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA1 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 3 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.2 Current responses of GluA1(Q)flop homomeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(Q)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA1 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 3 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
**Results**

**Figure 3.3 Current responses of GluA1(R)flip homomeric receptors ± γ5, γ6, γ7, or γ2.**
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(R)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA1 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

![Graph](image)

**Figure 3.4 Current responses of GluA1(R)flop homomeric receptors ± γ5, γ6, γ7, or γ2.** A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(R)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA1 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Table 3.1 Modulation of GluA1 homomeric receptors.

Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of homomeric GluA1 receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>0.4 ± 0.03</td>
<td>0.9 ± 0.14</td>
<td>9.8 ± 0.95</td>
<td>12.3 ± 1.16</td>
</tr>
<tr>
<td>GluA1(Q)flip KA</td>
<td>0.7 ± 0.04</td>
<td>1.1 ± 0.13</td>
<td>12.2 ± 1.00</td>
<td>39.3 ± 4.69</td>
</tr>
<tr>
<td>GluA1(Q)flip KA/Glu</td>
<td>1.6 ± 0.05</td>
<td>1.4 ± 0.13</td>
<td>1.3 ± 0.09</td>
<td>3.4 ± 0.47</td>
</tr>
<tr>
<td>Glu</td>
<td>0.5 ± 0.04</td>
<td>0.7 ± 0.05</td>
<td>8.4 ± 1.18</td>
<td>32.2 ± 4.13</td>
</tr>
<tr>
<td>GluA1(Q)flop KA</td>
<td>0.4 ± 0.02</td>
<td>0.9 ± 0.05</td>
<td>6.9 ± 0.57</td>
<td>53.2 ± 6.68</td>
</tr>
<tr>
<td>GluA1(Q)flop KA/Glu</td>
<td>0.9 ± 0.05</td>
<td>1.3 ± 0.10</td>
<td>1.0 ± 0.10</td>
<td>1.7 ± 0.23</td>
</tr>
<tr>
<td>Glu</td>
<td>6.7 ± 0.77</td>
<td>1.1 ± 0.12</td>
<td>31.3 ± 2.16</td>
<td>51.1 ± 6.12</td>
</tr>
<tr>
<td>GluA1(R)flip KA</td>
<td>28.8 ± 3.01</td>
<td>1.0 ± 0.11</td>
<td>40.3 ± 2.43</td>
<td>133.4 ± 16.57</td>
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<tr>
<td>GluA1(R)flip KA/Glu</td>
<td>4.3 ± 0.23</td>
<td>0.9 ± 0.07</td>
<td>1.2 ± 0.03</td>
<td>2.4 ± 0.06</td>
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<td>Glu</td>
<td>2.6 ± 0.21</td>
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<td>GluA1(R)flop KA</td>
<td>15.4 ± 2.63</td>
<td>1.1 ± 0.10</td>
<td>176.9 ± 32.30</td>
<td>6702 ± 756.5</td>
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<td>GluA1(R)flop KA/Glu</td>
<td>4.7 ± 0.54</td>
<td>1.1 ± 0.08</td>
<td>1.6 ± 0.28</td>
<td>1.6 ± 0.21</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were recorded for all the calcium permeable receptor combinations (Figure 3.5). Calcium impermeable receptors do not yield sufficiently high currents, even in the presence of desensitization inhibitors such as TCM, to allow for reliable I/V relationship recordings. Therefore, these receptors will be analyzed in section 3.5.2, as heteromeric combinations. I/V relationships are known to be altered by type I-TARPs like stargazin, which attenuates polyamine-induced rectification in calcium permeable receptors (Jackson et al 2011, Soto et al 2014, Soto et al 2007). Typically, the non-Q/R-edited variants of GluA1 show a typical inwardly rectifying curve while the edited variants show a characteristic linear curve. None of the TARPs or γ6 was able to change the inwardly
rectifying profile of calcium permeable receptors. However, except for γ6, which yielded I/V relationships that were virtually undistinguishable from those of the TARP-less receptors, all of the TARPs were able to attenuate rectification to a certain extent. Stargazin led to a considerable attenuation of rectification, a classic feature of type I-TARPs. Stargazin was followed by γ7, whose I/V curve had a very similar profile to that of a type I-TARP. γ5 showed a more modest effect on rectification, but still clearly discernible from that of the TARP-less receptors.

![Figure 3.5](image)

**Figure 3.5 Normalized I/V relationships of GluA1(Q) homomeric receptors ± γ5, γ6, γ7, or γ2.**

Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). A GluA1(Q)flip, B GluA1(Q)flop. Traces of TARP-less GluA1 are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

Type II-TARPs regulate GluA1 homomeric receptors in a unique way, which distinguishes them from type I-TARPs. γ5 could be shown to selectively modulate GluA1 depending on its editing status, being the only TARP with the ability to discriminate amongst edited and unedited receptors, and either inhibit or potentiate current amplitudes of AMPARs in a distinctive fashion. Moreover, γ7 was able to increase current amplitudes of all the receptors and to alter the ratio of kainate-to-glutamate-induced currents, similarly to γ2, only less prominently. γ6 did not modulate GluA1, except for a very mild inhibitory effect on the GluA1(Q)flop variant. All the TARPs, but not γ6 were able to attenuate polyamine block of unedited receptors to different extents.
3.1.1.2 GluA2

GluA2 receptors to be electrophysiologically characterized included the flip and flop variants, in both their Q/R-edited and non-edited forms. Homomeric GluA2 was co-expressed in oocytes with either one of the type II-TARPs or γ6. For comparison, GluA2 was co-expressed with the well-characterized γ2 and recorded in parallel for every experiment. The resulting glutamate- and kainate-induced currents and the calculated ratio between kainate- and glutamate- induced currents are shown in Figures 3.6, 3.7, 3.8, and 3.9, as are representative traces of both glutamate- and kainate-induced currents for every recorded subunit combination. The respective potentiation values are shown in Table 3.2.

As for GluA1, γ7 was able to significantly increase both glutamate- and kainate-induced currents of all GluA2 receptors. However, it significantly increased the kainate-to-glutamate current ratio only for GluA2(R)flop.

Consistently, γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist. Additionally, γ6 also did not change kainate-to-glutamate current ratios of edited variants. However, it significantly altered these ratios for calcium permeable receptors, although only slightly (decreased to 80 % and increased to 120 % of the original currents, for the flip and the flop variants, respectively).

Furthermore, γ5 had a potentiating effect on edited receptors while it reduced current amplitudes of unedited receptors, similarly to GluA1. Moreover, also resembling its effect on GluA1, γ5 was able to strongly increase kainate-to-glutamate current ratios of unedited receptors, while it had a much less prominent effect on that ratio at GluA2(Q)flip and no effect on GluA2(Q)flop.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.6 Current responses of GluA2(Q)flip homomeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA2(Q)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of GluA2 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.7 Current responses of GluA2(Q)flop homomeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA2(Q)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA2 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.8 Current responses of GluA2(R)flip homomeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA2(R)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA2 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.9 Current responses of GluA2(R)flop homomeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA2(R)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA2 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 3 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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Table 3.2 Modulation of GluA2 homomeric receptors.
Glutamater-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of homomeric GluA2 receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>0.3 ± 0.03</td>
<td>1.1 ± 0.10</td>
<td>2.2 ± 0.20</td>
<td>2.8 ± 0.37</td>
</tr>
<tr>
<td>GluA2(Q)flip KA</td>
<td>0.3 ± 0.02</td>
<td>0.9 ± 0.09</td>
<td>2.0 ± 0.09</td>
<td>5.1 ± 0.26</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>1.3 ± 0.08</td>
<td>0.8 ± 0.07</td>
<td>1.0 ± 0.09</td>
<td>2.1 ± 0.24</td>
</tr>
<tr>
<td>Glu</td>
<td>0.1 ± 0.02</td>
<td>0.9 ± 0.07</td>
<td>2.3 ± 0.28</td>
<td>6.0 ± 1.33</td>
</tr>
<tr>
<td>GluA2(Q)flo KA p</td>
<td>0.1 ± 0.01</td>
<td>1.1 ± 0.05</td>
<td>2.2 ± 0.09</td>
<td>6.8 ± 0.96</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>1.0 ± 0.06</td>
<td>1.2 ± 0.08</td>
<td>1.1 ± 0.14</td>
<td>1.9 ± 0.72</td>
</tr>
<tr>
<td>Glu</td>
<td>2.9 ± 0.26</td>
<td>0.9 ± 0.11</td>
<td>122.9 ± 27.45</td>
<td>1386 ± 122.6</td>
</tr>
<tr>
<td>GluA2(R)flip KA</td>
<td>7.0 ± 0.88</td>
<td>1.0 ± 0.09</td>
<td>139.4 ± 18.02</td>
<td>2449 ± 361.8</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>2.4 ± 0.13</td>
<td>1.1 ± 0.08</td>
<td>1.5 ± 0.31</td>
<td>1.7 ± 0.12</td>
</tr>
<tr>
<td>Glu</td>
<td>1.9 ± 0.15</td>
<td>1.1 ± 0.09</td>
<td>40.6 ± 5.72</td>
<td>1684 ± 175.5</td>
</tr>
<tr>
<td>GluA2(R)flo KA p</td>
<td>4.6 ± 0.74</td>
<td>1.0 ± 0.06</td>
<td>87.3 ± 7.67</td>
<td>3349 ± 355.9</td>
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<tr>
<td>KA/Glu</td>
<td>2.5 ± 0.48</td>
<td>0.9 ± 0.08</td>
<td>2.4 ± 0.30</td>
<td>1.9 ± 0.05</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were recorded for all the calcium permeable receptors combinations (Figure 3.10). Calcium impermeable receptors will be analyzed in section 3.5.2, as heteromeric combinations.

The non-Q/R-edited variants of GluA2 show a typical inwardly rectifying curve. Again, none of the TARPs nor γ6 were able to change this feature. However, all of them but not γ6 were able to attenuate rectification of GluA2 receptors to different extents. As it was the case for GluA1 receptors, stargazin strongly attenuated rectification. However, γ7 had a more modest effect on the rectification profile when compared to GluA1, while, to the contrary, γ5 did so more strongly. γ6 again showed I/V profiles that were undistinguishable from the TARP-less receptors.
Results

Figure 3.10 Normalized I/V relationships of GluA2(Q) homomeric receptors ± γ5, γ6, γ7, or γ2.

Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). A GluA2(Q)flip, B GluA2(Q)flop. Traces of TARP-less GluA2 are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

The modulation of GluA2 homomeric receptors by type II-TARPs resembles that of GluA1 receptors. Also here, γ5 showed an editing-discriminatory behavior, potentiating currents of edited receptors, while inhibiting those of unedited receptors. Additionally, γ7 was able to increase current amplitudes of all of the receptor variants while γ6 did not modulate any of them. Both TARPs, but not γ6, attenuated polyamine block of Ca2+ permeable receptors.
3.1.1.3 GluA3

GluA3 receptors to be electrophysiologically characterized included the flip and flop variants, in both its Q/R-edited and non-edited forms, although edited GluA3 does not occur physiologically. Homomeric GluA3 was co-expressed in oocytes with either one of the type II-TARPs or γ6. For comparison, GluA3 was co-expressed with the well-characterized γ2 and recorded in parallel for every experiment. The resulting glutamate- and kainate-induced currents and representative traces of both glutamate- and kainate-induced currents for every recorded combination are shown in Figures 3.11, 3.12, 3.13, and 3.14, as is the calculated ratio between kainate- and glutamate-induced currents. The respective potentiation values are shown in Table 3.3.

As for GluA1 and GluA2, γ7 was able to significantly increase both glutamate- and kainate-induced currents of all GluA3 receptors. However, it did increase the kainate-to-glutamate current ratio significantly only for the Q/R-edited receptor variants, while it had no effect on the calculated ratio of GluA3(Q)flip and even led to a significant decrease on the ratio of GluA3(Q)flop.

As before, γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist. Accordingly, γ6 did also not change any of the receptors’ kainate-to-glutamate current ratios.

Consistent with the observations obtained from the analysis of GluA1 and GluA2 receptors, γ5 had a potentiating effect on Q/R-edited receptors while it reduced current amplitudes of unedited receptors. Moreover, also as observed hitherto, γ5 was able to consistently increase kainate-to-glutamate current ratios of unedited receptors, while it had a less prominent effect on the ratio of GluA3(Q)flip and further led to a significant decrease of the ratio to GluA3(Q)flop.
Results

Figure 3.11 Current responses of GluA3(Q)flip homomeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA3(Q)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA3 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 4 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.12 Current responses of GluA3(Q)flop homomeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA3(Q)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of GluA3 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

**Figure 3.13 Current responses of GluA3(R)flip homomeric receptors ± γ5, γ6, γ7, or γ2.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA3(R)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA3 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.14** Current responses of GluA3(R)flop homomeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA3(R)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA3 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 3 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.3 Modulation of GluA3 homomeric receptors.

GluA3 receptors were modulated by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
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<tr>
<th></th>
<th>GluA3(Q)flip</th>
<th>GluA3(Q)flo</th>
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<td>γ5</td>
<td>γ6</td>
<td>γ7</td>
<td>γ2</td>
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<tr>
<td>Glu</td>
<td>0.2 ± 0.03</td>
<td>1.0 ± 0.08</td>
<td>9.4 ± 1.22</td>
<td>36.8 ± 4.06</td>
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<tr>
<td>KA/Glu</td>
<td>1.5 ± 0.11</td>
<td>0.9 ± 0.04</td>
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<td>3.8 ± 0.92</td>
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<td>0.3 ± 0.06</td>
<td>0.9 ± 0.06</td>
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<td>158.3 ± 43.1</td>
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<td></td>
<td>0.7 ± 0.02</td>
<td>1.1 ± 0.18</td>
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<td>Glu</td>
<td>3.8 ± 0.26</td>
<td>1.1 ± 0.10</td>
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<td>3341 ± 312.3</td>
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<td>KA/Glu</td>
<td>2.4 ± 0.21</td>
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<td>1.1 ± 0.11</td>
<td>8.2 ± 0.85</td>
<td>3.8 ± 0.29</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were recorded for all the calcium permeable receptor combinations (Figure 3.15). Calcium impermeable receptors will be analyzed in section 3.5.2, as heteromeric combinations.

The non-Q/R-edited variants of GluA3 show a typical inwardly rectifying curve. None of the TARPs nor γ6 was able to change this feature. However, all of them, but not γ6, were able to attenuate rectification of GluA3 receptors to different extents. As observed for GluA1 receptors, stargazin and γ7 had the strongest effects on the rectification profile of the receptor, while γ5 showed a more modest effect. The I/V profiles of GluA3 receptors co-expressing γ6 were again identical to the ones observed with the TARP-less receptors.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.15 Normalized I/V relationships of GluA3(Q) homomeric receptors ± γ5, γ6, γ7, or γ2.

Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). A GluA3(Q)flip, B GluA3(Q)flop. Traces of TARP-less GluA3 are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

GluA3 homomeric receptors were modulated by type II-TARPs in a way similar to GluA1 and GluA2 receptors. Once more, γ5 modulation was dependent on the Q/R editing status of the receptor. Current amplitudes of all receptors were potentiated by co-expression with γ7 and were not affected by γ6. Polyamine block of Ca²⁺ permeable receptors was attenuated by TARPs but not γ6.
3.1.1.4 GluA4

GluA4 receptors to be electrophysiologically characterized included the flip and flop variants, in both its Q/R-edited and non-edited forms, although edited GluA4 does not occur physiologically. Homomeric GluA4 was co-expressed in oocytes with either one of the type II-TARPs or γ6. For comparison, GluA4 was co-expressed with the well-characterized type I-TARP γ2 and recorded in parallel for every experiment. The resulting glutamate- and kainate-induced currents and representative traces of both glutamate- and kainate-induced currents for every recorded combination are shown in Figures 3.16, 3.17, 3.18, and 3.19. The respective potentiation values are shown in Table 3.4.

With the exception of the glutamate-induced currents of GluA4(Q)flop, γ7 was able to significantly increase both glutamate- and kainate-induced currents of all the remaining GluA4 receptors. Additionally, although γ7 had no effect on the kainate-to-glutamate current ratio of GluA4(R)flip, it significantly potentiated the calculated ratios of the remaining GluA4 receptors, but only very weakly.

γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist. Furthermore, γ6 did not change GluA4 kainate-to-glutamate current ratios.

As already observed for all the other AMPARs, γ5 had a potentiating effect on Q/R-edited receptors while it reduced current amplitudes of unedited receptors. Moreover, and also resembling its effect on the remaining AMPARs, γ5 was able to increase kainate-to-glutamate current ratios of unedited receptors. Additionally, γ5 potentiated the ratio of GluA4(Q)flip and decreased that of GluA4(Q)flop.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.16 Current responses of GluA4(Q)flip homomeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA4(Q)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA4 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

**Figure 3.17** Current responses of GluA4(Q)flop homomeric receptors ± γ5, γ6, γ7, or γ2.  
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA4(Q)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA4 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 3 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.18** Current responses of GluA4(R)flip homomeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA4(R)flip homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of GluA4 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

![Graphs A, B, C, D, and E showing current responses.]

**Figure 3.19 Current responses of GluA4(R)flop homomeric receptors ± γ5, γ6, γ7, or γ2.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA4(R)flop homomeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of GluA4 alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Table 3.4 Modulation of GluA4 homomeric receptors.

Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of homomeric GluA4 receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
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<td>Glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>0.1 ± 0.02</td>
<td>1.2 ± 0.11</td>
<td>2.5 ± 0.14</td>
<td>5.0 ± 0.55</td>
</tr>
<tr>
<td>GluA4(Q)flip</td>
<td>KA</td>
<td>0.2 ± 0.02</td>
<td>1.0 ± 0.07</td>
<td>2.8 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>KA/Glu</td>
<td>1.6 ± 0.10</td>
<td>0.9 ± 0.04</td>
<td>1.1 ± 0.04</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>0.2 ± 0.04</td>
<td>1.1 ± 0.18</td>
<td>1.2 ± 0.31</td>
<td>3.2 ± 0.82</td>
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<tr>
<td>GluA4(Q)flop</td>
<td>KA</td>
<td>0.1 ± 0.02</td>
<td>1.1 ± 0.10</td>
<td>1.6 ± 0.14</td>
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<tr>
<td>p</td>
<td>KA/Glu</td>
<td>0.4 ± 0.09</td>
<td>1.0 ± 0.15</td>
<td>1.4 ± 0.13</td>
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<tr>
<td>Glutamate</td>
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<tr>
<td>Glu</td>
<td>2.6 ± 0.24</td>
<td>1.2 ± 0.10</td>
<td>85.6 ± 8.39</td>
<td>867.6 ± 134.5</td>
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<tr>
<td>GluA4(R)flip</td>
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<td>3.9 ± 0.46</td>
<td>1.0 ± 0.08</td>
<td>55.2 ± 6.12</td>
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<tr>
<td></td>
<td>KA/Glu</td>
<td>1.4 ± 0.10</td>
<td>0.9 ± 0.08</td>
<td>0.7 ± 0.12</td>
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<tr>
<td>Glutamate</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glu</td>
<td>2.1 ± 0.13</td>
<td>1.0 ± 0.10</td>
<td>30.3 ± 3.63</td>
<td>535.0 ± 90.95</td>
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Additionally, I/V relationships were recorded for all the calcium permeable receptor combinations and are shown in Figure 3.20. Calcium impermeable receptors will be analyzed in section 3.5.2, as heteromeric combinations.

The non-Q/R-edited variants of GluA4 show a typical inwardly rectifying curve. Again, none of the TARPs nor γ6 were able to change that feature. However, all of them, but not γ6, were able to attenuate rectification of GluA4 receptors to different extents. Stargazin and γ7 had the strongest effect on the rectification profile of GluA4(Q)flop, while γ5 showed a more modest effect. However, the effect of γ5 on the rectification profile of GluA4(Q)flip was more prominent than that of γ7. The I/V
Results

Relationships of both GluA4 receptors co-expressing γ6 were again indistinguishable from the ones observed with the TARP-less receptor.

Figure 3.20 Normalized I/V relationships of GluA4(Q) homomeric receptors ± γ5, γ6, γ7, or γ2.

Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). A GluA4(Q)flip, B GluA4(Q)flop. Traces of TARP-less GluA4 are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

All AMPA receptors responded to type II-TARPs in a similar fashion. GluA4 underwent the same electrophysiological changes as all the other AMPARs when co-expressed with type II-TARPs or γ6. Co-expression with γ5 resulted in larger current amplitudes for the Q/R-edited variants of the receptor, and smaller currents for the unedited forms, when compared to the receptor alone. γ7 potentiated all the receptors indiscriminately, with the sole exception of GluA4(Q)flop, whose glutamate-induced currents were not affected by co-expression with the TARP, while γ6 had no reproducible effect on any of the receptors. Also for GluA4, polyamine block of Ca²⁺ permeable receptors was weakened by the TARPs but not by γ6.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

3.1.2 Heteromeric AMPA receptors

In vivo, most AMPA receptors are heteromeric subunit combinations that include the edited GluA2 subunit, and are therefore Ca\(^{2+}\) impermeable. In addition, given the results with homomeric receptors presented above into account, it becomes evident that modulation of AMPARs by type II-TARPs, and especially γ5, strongly depends on the Q/R-editing status of the receptor. Thus, the next obvious step for the electrophysiological characterization of these TARPs involves their co-expression with different heteromeric AMPAR subunit combinations.

Heteromeric receptors were co-expressed in oocytes with either one of the type II-TARPs or γ6. In order to isolate a potential influence of alternative splicing in the analyses of the heteromers, receptors were recorded as pure flop and pure flip combinations. For comparison, the type II-TARP γ2 was co-expressed with every receptor combination.

3.1.2.1 GluA1(Q)/GluA2(R)

At first, the flip and flop variants of the GluA1(Q)/GluA2(R) heteromer were electrophysiologically characterized. This heteromer occurs as the most common AMPA receptor combination at the PSD and is therefore, physiologically, of high relevance. Both flip and flop heteromeric combinations were co-expressed in oocytes with either one of the type II-TARPs or with γ6. The edited GluA2 subunit cRNA was injected into oocytes in excess when compared to the GluA1 subunit (1:5 molar ratio Q:R cRNAs). The resulting glutamate- and kainate-induced responses, as well as the calculated ratios between kainate- and glutamate-induced currents for these combinations are shown in Figures 3.21 and 3.22. Representative traces of both glutamate- and kainate-induced currents for every recorded subunit combination are depicted in the same figures. The respective potentiation values are shown in Table 3.5.

As observed for homomeric receptors and, more importantly, for GluA1(Q) and GluA2(R), γ7 was able to significantly increase both glutamate- and kainate-induced currents of both heteromeric combinations by orders of magnitude that resemble the Q variants more closely. However, while γ7 had a positive impact on the kainate-to
glutamate-induced current ratios of the homomeric combinations, it significantly decreased the calculated ratios of the heteromers.

Remarkably, although it is well known that the R variant determines receptor permeability to calcium in heteromers, and also considering the molar excess of the R subunit cRNA injected into the oocyte, γ5 reduced current amplitudes of both receptor combinations even more strongly than it did with the homomers. Such an observation suggests a regulatory preference of the TARP for the Q variant over the R variant in heteromeric combinations. Additionally, the impact of γ5 on the kainate-to glutamate induced-current ratios of the heteromers is comparable to the one already observed for the homomeric Q variants.

Again, γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.21 Current responses of GluA1(Q)flip/GluA2(R)flip heteromeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(Q)flip/GluA2(R)flip heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

![Graphs showing normalized current responses](image)

**Figure 3.22 Current responses of GluA1(Q)flop/GluA2(R)flop heteromeric receptors ± γ5, γ6, γ7, or γ2.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(Q)flop/GluA2(R)flop heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.5 Modulation of GluA1(Q)/GluA2(R) heteromeric receptors.
Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of heteromeric GluA1(Q)/GluA2(R) receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluA1(Q)flip/</td>
<td>0.3 ± 0.03</td>
<td>0.9 ± 0.06</td>
<td>12.0 ± 2.84</td>
<td>57.0 ± 12.33</td>
</tr>
<tr>
<td>GluA2(R)flip</td>
<td>0.5 ± 0.05</td>
<td>0.9 ± 0.04</td>
<td>5.7 ± 0.75</td>
<td>49.0 ± 5.77</td>
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<tr>
<td>KA/Glu</td>
<td>1.5 ± 0.05</td>
<td>1.0 ± 0.04</td>
<td>0.7 ± 0.10</td>
<td>1.1 ± 0.10</td>
</tr>
<tr>
<td>GluA1(Q)flop/</td>
<td>0.2 ± 0.03</td>
<td>1.0 ± 0.11</td>
<td>17.8 ± 2.43</td>
<td>47.39 ± 7.21</td>
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<tr>
<td>GluA2(R)flop</td>
<td>0.1 ± 0.02</td>
<td>1.1 ± 0.12</td>
<td>3.4 ± 0.26</td>
<td>10.7 ± 0.70</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>0.7 ± 0.10</td>
<td>1.1 ± 0.14</td>
<td>0.2 ± 0.02</td>
<td>0.3 ± 0.06</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were taken for both receptor combinations and are shown in Figure 3.23.

Calcium impermeable GluA1(Q)/GluA2(R) heteromers are presumably composed of two unedited GluA1 subunits and two edited GluA2 subunits and therefore show a characteristic linear curve. Consistent with the analysis of I/V relationships of homomeric calcium permeable receptors, none of the TARPs nor γ6 were able to change the linear nature of the I/V curves elicited by the TARP-less receptors.
Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). A GluA1(Q)flip/GluA2(R)flip, B GluA1(Q)flop/GluA2(R)flop. Traces of TARP-less heteromers are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

As observed for homomeric receptors, current amplitudes of the analyzed heteromeric AMPA receptor combinations were potentiated by γ7. However, co-expression with γ5 resulted in a decrease in current amplitudes in receptors containing both edited and unedited subunits, resembling receptors composed by homomeric unedited subunits. This observation points to a regulatory preference for the unedited Q variants by this TARP. γ6 had no effect on any of the combinations analyzed.
3.1.2.2 GluA1(R)/GluA2(Q)

Next, the flip and flop variants of the GluA1(R)/GluA2(Q) heteromer were electrophysiologically characterized. This heteromer does not occur in vivo, and is therefore of no physiological significance. However, editingwise, is the inverse of the heteromer analyzed previously in section 3.1.2.2. Hence, it was used to investigate if the TARP effects observed with GluA1(Q)/GluA2(R) heteromers remain, even if the edited residue is present at the wrong subunit, or else if the TARPs are able to discriminate between the two receptor combinations.

The heteromeric combinations were co-expressed in oocytes with either one of the type II-TARPs or γ6. The edited GluA1 cRNA subunit was injected into oocytes in excess when compared to the unedited GluA2 cRNA (5:1 ratio of R:Q cRNAs). The resulting glutamate- and kainate-induced responses, as well as the calculated ratios between kainate- and glutamate induced currents for these combinations are shown in Figures 3.24 and 3.25. Representative traces of both glutamate- and kainate-induced currents for every recorded subunit combination are depicted in the same figures. The respective potentiation values are shown in Table 3.6.

As observed for homomeric receptors and, more importantly, for GluA1(R) and GluA2(Q), γ7 was able to significantly increase kainate-induced currents of both heteromeric combinations and glutamate-induced currents of the flip variant, by orders of magnitude that resemble the Q variants more closely, similar to what had been observed for the GluA1(Q)/GluA2(R) heteromers. The small increase observed for the glutamate-induced currents of the flop-type heteromer was not statistically significant. These observations indicate a regulatory preference of the TARP for the non-Q/R-edited variant rather than for a specific receptor subunit. However, while γ7 had a negative impact on the kainate-to glutamate-induced current ratios of the physiologically occurring heteromer, it had no effect on the flip variant of the GluA1(Q)/GluA2(R) heteromer ratios and significantly increased the calculated ratios of the flop-type heteromer.

Again, γ5 reduced current amplitudes of both receptor combinations as it did with the homomers, reinforcing the idea that this TARP also shows a preference for the Q variant over the R variant in heteromeric combinations. Additionally, the γ5 impact
Results

on the kainate- to glutamate induced-current ratios of the heteromers is comparable to the one already observed for the homomeric Q variants.

Once more, γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist.

Figure 3.24 Current responses of GluA1(R)flip/GluA2(Q)flip heteromeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(R)flip/GluA2(Q)flip heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.25 Current responses of GluA1(R)flop/GluA2(Q)flop heteromeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(R)flop/GluA2(Q)flop heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.6 Modulation of GluA1(R)/GluA2(Q) heteromeric receptors.
Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of heteromeric GluA1(R)/GluA2(Q) receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

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<thead>
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<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
</tr>
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<td>GluA1(R)flip/</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>GluA2(Q)flip</td>
<td>0.2 ± 0.01</td>
<td>1.1 ± 0.09</td>
<td>2.0 ± 0.11</td>
<td>44.0 ± 6.56</td>
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<td>KA</td>
<td>0.2 ± 0.02</td>
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<td>KA/Glu</td>
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<td>1.1 ± 0.08</td>
<td>0.6 ± 0.07</td>
</tr>
<tr>
<td>GluA1(R)flop/</td>
<td>0.1 ± 0.02</td>
<td>0.9 ± 0.14</td>
<td>1.2 ± 0.09</td>
<td>57.1 ± 9.75</td>
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<tr>
<td>GluA2(Q)flop</td>
<td>0.1 ± 0.01</td>
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<td>11.0 ± 1.66</td>
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<td>KA</td>
<td>0.9 ± 0.10</td>
<td>1.0 ± 0.10</td>
<td>1.5 ± 0.04</td>
<td>0.2 ± 0.02</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were recorded for both receptor combinations and are shown in Figure 3.26.

Calcium impermeable GluA1(R)/GluA2(Q) heteromers are presumably composed of two unedited GluA2 subunits and two edited GluA1 subunits and therefore show a characteristic linear curve. Consistent with the analysis of the former receptors, none of the TARPs nor γ6 were able to change the linear nature of the I/V curves elicited by the TARP-less receptors.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.26 Normalized I/V relationships of GluA1(R)/GluA2(Q) heteromeric receptors ± γ5, γ6, γ7, or γ2. Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). A GluA1(R)flip/GluA2(Q)flip, B GluA1(R)flop/GluA2(Q)flop. Traces of TARP-less heteromers are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

Current amplitudes of GluA1(R)/GluA2(Q) heteromers were modulated by both type II-TARPs to an extent that is comparable to what had already been observed for the unedited GluA2 homomeric assemblies. The preference for the Q variant had already been observed for GluA1(Q)/GluA2(R) heteromers and appears to be independent of receptor subunit composition. This observation indicates that Q/R-editing of AMPA receptors is critical for TARP function and supersedes the importance of the constitutive receptor subtypes.
3.1.2.3 GluA2(R)/GluA3(Q)

Next, the flip and flop variants of the GluA2(R)/GluA3(Q) heteromer were electrophysiologically characterized. The heteromeric combinations were co-expressed in oocytes with either one of the type II-TARPs or γ6. The edited GluA2 cRNA was injected into oocytes in excess when compared to the unedited GluA3 cRNA (5:1 ratio of R:Q cRNAs). The resulting glutamate- and kainate-induced responses, as well as the calculated ratios between kainate- and glutamate-induced currents for these combinations are shown in Figures 3.27 and 3.28. Representative traces of both glutamate- and kainate-induced currents for every recorded combination are depicted in the same figures. The respective potentiation values are shown in Table 3.7.

As observed for homomeric GluA3(Q) and GluA2(R) receptors, γ7 was able to significantly increase both glutamate- and kainate-induced currents of both heteromeric combinations, by orders of magnitude that resemble the Q variants more closely. Furthermore, γ7 had a similar impact on the kainate- to glutamate-induced current ratios of homomeric GluA3(Q) and GluA3(Q)-containing heteromers.

Additionally, γ5 reduced current amplitudes of both heteromers by identical factors as homomeric GluA3(Q). Moreover, γ5 impact on the kainate- to glutamate induced-current ratios of the heteromers is comparable to the effects already observed for the homomeric Q variants.

γ6 did not significantly alter current amplitudes for any of the receptors analyzed with either agonist.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.27 Current responses of GluA2(R)flip/GluA3(Q)flip heteromeric receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA2(R)flip/GluA3(Q)flip heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.28 Current responses of GluA2(R)flop/GluA3(Q)flop heteromeric receptors ± \( \gamma_5, \gamma_6, \gamma_7, \) or \( \gamma_2 \).

A: Normalized 300 \( \mu \)M glutamate-induced currents (± SEM). B: Normalized 150 \( \mu \)M kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA2(R)flop/GluA3(Q)flop heteromeric receptors in the presence or absence of \( \gamma_5, \gamma_6, \gamma_7, \) or \( \gamma_2 \), elicited by 300 \( \mu \)M glutamate (D) or 150 \( \mu \)M kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of \( \gamma_5, \gamma_6, \gamma_7, \) or \( \gamma_2 \). Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.005 \), ****\( p < 0.001 \)).
Table 3.7 Modulation of GluA2(R)/GluA3(Q) heteromeric receptors.

Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of heteromeric GluA2(R)/GluA3(Q) receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Glu</th>
<th>KA</th>
<th>KA/Glu</th>
<th>Glu</th>
<th>KA</th>
<th>KA/Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluA2(R)flip/GluA3(Q)flip</td>
<td>0.2 ± 0.01</td>
<td>0.3 ± 0.02</td>
<td>1.6 ± 0.05</td>
<td>0.2 ± 0.03</td>
<td>0.1 ± 0.02</td>
<td>0.4 ± 0.06</td>
</tr>
<tr>
<td>GluA2(R)flop/GluA3(Q)flop</td>
<td>1.0 ± 0.09</td>
<td>1.0 ± 0.10</td>
<td>1.0 ± 0.02</td>
<td>1.0 ± 0.16</td>
<td>1.0 ± 0.09</td>
<td>1.0 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>8.9 ± 1.25</td>
<td>5.3 ± 0.44</td>
<td>0.8 ± 0.09</td>
<td>20.3 ± 4.29</td>
<td>6.0 ± 0.74</td>
<td>0.4 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>37.0 ± 6.99</td>
<td>30.8 ± 3.95</td>
<td>1.0 ± 0.07</td>
<td>261.0 ± 36.29</td>
<td>37.7 ± 7.38</td>
<td>0.2 ± 0.05</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were recorded for both receptor combinations and are shown in Figure 3.29.

Calcium impermeable GluA2(R)/GluA3(Q) heteromers are presumably composed of two unedited GluA3 subunits and two edited GluA2 subunits and therefore show a characteristic linear curve.

Consistent with the analysis of the former receptors, none of the TARPs or γ6 was able to change the linear nature of the I/V curves elicited by the TARP-less receptors.
Results

Figure 3.29 Normalized I/V relationships of GluA2(R)/GluA3(Q) heteromeric receptors ± γ5, γ6, γ7, or γ2.

Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). **A** GluA2(R)flip/GluA3(Q)flip, **B** GluA2(R)flop/GluA3(Q)flop. Traces of TARP-less heteromers are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

The influence of type II-TARP on GluA2(R)/GluA3(Q) heteromers was identical to that on homomeric GluA3. Hence, the results suggest once more that the unedited Q variant determines the type and extent of modulation prompted by these TARPs, when both variants are combined.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

3.1.2.4 GluA1(Q)/GluA3(Q)

Here, the flip and flop variants of the GluA1(Q)/GluA3(Q) heteromer were the next receptors to be electrophysiologically characterized. The heteromeric combinations were co-expressed in oocytes with either one of the type II-TARPs or γ6. The two subunit cRNAs were injected into oocytes in a 1:1 molar ratio. The resulting glutamate- and kainate-induced responses, as well as the calculated ratios between kainate- and glutamate-induced currents for these combinations are shown in Figures 3.30 and 3.31. Representative traces of both glutamate- and kainate-induced currents for every recorded combination are depicted in the same figures. The respective potentiation values are shown in Table 3.8.

As observed for GluA1(Q) and GluA3(Q), γ7 was able to significantly increase both glutamate- and kainate-induced currents of both heteromeric combinations, by comparable factors. Moreover, γ7 had a positive impact on the kainate- to glutamate-induced current ratio of the homomeric flip combination, similarly to homomeric GluA1, and a negative impact on the kainate- to glutamate-induced current ratio of the homomeric flop combination, similar to homomeric GluA3.

γ5 modulation of the heteromers was comparable to the extent of modulation observed for GluA1(Q) and GluA3(Q) homomeric receptors. The TARP significantly decreased both glutamate- and kainate-induced currents of both heteromeric combinations. Additionally, the impact of γ5 on the kainate- to glutamate-induced current ratios of the heteromers was comparable to the one already observed for the GluA1 variants.

γ6 did not significantly altered current amplitudes for any of the receptors analyzed with either agonist.
Results

Figure 3.30 Current responses of GluA1(Q)flip/GluA3(Q)flip heteromeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(Q)flip/GluA3(Q)flip heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.31 Current responses of GluA1(Q)flop/GluA3(Q)flop heteromeric receptors ± γ5, γ6, γ7, or γ2.

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluA1(Q)flop/GluA3(Q)flop heteromeric receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.8 Modulation of GluA1(Q)/GluA3(Q) heteromeric receptors.

Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of heteromeric GluA1(Q)/GluA3(Q) receptors by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluA1(Q)flip/</td>
<td>0.3 ± 0.03</td>
<td>1.0 ± 0.10</td>
<td>10.8 ± 2.08</td>
<td>18.5 ± 2.26</td>
</tr>
<tr>
<td>GluA3(Q)flip</td>
<td>0.6 ± 0.13</td>
<td>1.2 ± 0.17</td>
<td>12.2 ± 1.10</td>
<td>34.0 ± 1.54</td>
</tr>
<tr>
<td>GluA1(Q)flop/</td>
<td>0.4 ± 0.05</td>
<td>0.9 ± 0.15</td>
<td>20.0 ± 3.02</td>
<td>12.3 ± 1.56</td>
</tr>
<tr>
<td>GluA3(Q)flop</td>
<td>0.2 ± 0.03</td>
<td>0.9 ± 0.15</td>
<td>5.5 ± 0.38</td>
<td>11.9 ± 0.78</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>1.9 ± 0.20</td>
<td>1.2 ± 0.07</td>
<td>1.5 ± 0.29</td>
<td>2.0 ± 0.22</td>
</tr>
<tr>
<td>KA</td>
<td>0.8 ± 0.12</td>
<td>1.1 ± 0.07</td>
<td>0.3 ± 0.03</td>
<td>1.0 ± 0.12</td>
</tr>
</tbody>
</table>

Additionally, I/V relationships were recorded for both receptor combinations and are shown in Figure 3.32.

Calcium permeable GluA1(Q)/GluA3(Q) heteromers are composed of four unedited subunits and therefore show a characteristic inwardly rectifying I/V curve. Consistent with the analysis of the I/V relationships of homomeric calcium permeable receptors, none of the TARPs nor γ6 were able to change the rectifying nature of the curve, but once again all the TARPs, but not γ6, induced a certain relief of the rectification of the TARP-less receptors. Stargazin and γ7 showed the strongest influence on rectification, while γ5 had a more subtle effect.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.32 Normalized I/V relationships of GluA1(Q)/GluA3(Q) heteromeric receptors ± γ5, γ6, γ7, or γ2.

Currents were evoked by 300 µM glutamate recorded by ramping of the membrane potential from -150 mV to 50 mV, within 2 s (n ≥ 3). **A** GluA1(Q)flip/GluA3(Q)flip, **B** GluA1(Q)flop/GluA3(Q)flop. Traces of TARP-less heteromers are shown in black whereas colored traces show currents induced in the presence of γ5 (blue), γ6 (green), γ7 (orange) or, γ2 (red).

As already observed for all the other AMPARs that contain at least one unedited Q subunit, homomeric or heteromeric alike, current amplitudes of GluA1(Q)/GluA3(Q) heteromers were potentiated by γ7, inhibited by γ5, and not affected by γ6.
3.1.3 Kainate receptors

Kainate receptors are very closely related to AMPA receptors, and therefore a set of experiments was designed in order to test if type II-TARP modulation extends to this iGluR subfamily. Due to the unexpected dependence on AMPAR editing observed for these TARPs, particularly γ5, four distinct editing variants of the GluK2 subunit were investigated GluK2(VCQ), GluK2(ICQ), GluK2(ICR), and GluK2(IYR).

KARs were co-expressed in oocytes with either one of the type II-TARPs or γ6. Stargazin (= γ2) is known not to modulate any other subfamily of iGluRs but AMPARs, and was therefore used as a negative control. Because KARs desensitize very quickly, their steady state currents are too small to be recorded in oocytes. Hence, oocytes were incubated for 8 minutes with 10 µM ConA before recordings. ConA is known to abolish desensitization of KARs very efficiently.

The resulting glutamate- and kainate-induced responses, the calculated ratio between kainate- and glutamate-induced currents, as well as representative traces of both glutamate- and kainate-induced currents for every recorded combination are shown in Figures 3.33, 3.34, and 3.35.

None of the TARPs nor γ6 were able to rescue currents of the GluK2(IYR) homomeric receptor, which normally does not show any currents in response to glutamate or kainate, even after treatment with ConA. Furthermore, neither type II-TARPs nor γ6 showed any major effect on the remaining analyzed KARs. However, γ7 was able to potentiate both glutamate- and kainate-induced currents of GluK2(ICQ), but only weakly by a factor of 1.4 and 1.3, respectively. Additionally, γ7 and γ5 both had a slight inhibitory effect on GluK2(ICR), decreasing glutamate-induced currents slightly (to 70% and 80% of the original current, respectively). Furthermore, the results also showed a small but barely significant modulation of GluK2(VCQ) and GluK2(ICQ) by γ2, which is a highly specific regulator of AMPARs. Thus, a larger n would likely eliminate the observed effects, which are inconsistent with the literature (Chen et al 2003, Shanks et al 2012). The calculated potentiation values are shown in detail on Table 3.9.

Since type II-TARPs had no noteworthy effect on KA receptors, which are so closely related to AMPARs, it was deemed unlikely that they would regulate other more distantly related receptors of the iGluR family. Thus, priority was given to a detailed
characterization of the modulation of AMPARs, rather than the extensive search for additional potential targets for type II-TARP regulation.

**Figure 3.33 Current responses of GluK2 (VCQ) receptors ± γ5, γ6, γ7, or γ2.**

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluK2(VCQ) receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.34 Current responses of GluK2 (ICQ) receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluK2(ICQ) receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 µM glutamate (D) or 150 µM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from 1 batch of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.35 Current responses of GluK2 (ICR) receptors ± γ5, γ6, γ7, or γ2.
A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). The number at the bottom of each bar refers to the number of oocytes recorded for this subunit combination. Representative current traces of GluK2(ICR) receptors in the presence or absence of γ5, γ6, γ7, or γ2, elicited by 300 μM glutamate (D) or 150 μM kainate (E). Traces of the receptor alone are shown in black, whereas colored traces show currents induced in the presence of γ5, γ6, γ7, or γ2. Grey bars indicate the duration of agonist application. Each set of data was collected from 1 batch of oocytes. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.9 Modulation of KARs.
Glutamate-induced currents, kainate-induced currents, and kainate-induced current to glutamate-induced current ratios of heteromeric KARs by γ5, γ6, γ7, or γ2. Colored numbers specify the combinations that were significantly regulated. Values were normalized to currents obtained in the absence of γ subunits.

<table>
<thead>
<tr>
<th></th>
<th>γ5</th>
<th>γ6</th>
<th>γ7</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>0.8 ± 0.11</td>
<td>0.8 ± 0.11</td>
<td>1.0 ± 0.08</td>
<td>1.1 ± 0.04</td>
</tr>
<tr>
<td>GluK2(VCQ) KA</td>
<td>0.9 ± 0.10</td>
<td>0.9 ± 0.11</td>
<td>1.1 ± 0.06</td>
<td><strong>1.3 ± 0.03</strong></td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.04</td>
<td><strong>1.1 ± 0.06</strong></td>
<td>1.1 ± 0.05</td>
<td>1.1 ± 0.03</td>
</tr>
<tr>
<td>Glu</td>
<td>1.0 ± 0.02</td>
<td>1.1 ± 0.17</td>
<td><strong>1.4 ± 0.06</strong></td>
<td><strong>1.6 ± 0.13</strong></td>
</tr>
<tr>
<td>GluK2(ICQ) KA</td>
<td>1.0 ± 0.01</td>
<td>1.1 ± 0.16</td>
<td><strong>1.3 ± 0.06</strong></td>
<td>1.4 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.01</td>
<td>1.0 ± 0.02</td>
<td>1.0 ± 0.01</td>
<td>0.8 ± 0.10</td>
</tr>
<tr>
<td>Glu</td>
<td>1.5 ± 0.20</td>
<td>1.2 ± 0.48</td>
<td><strong>0.7 ± 0.07</strong></td>
<td>0.8 ± 0.23</td>
</tr>
<tr>
<td>GluK2(ICR) KA</td>
<td>1.2 ± 0.17</td>
<td>1.0 ± 0.41</td>
<td>0.7 ± 0.10</td>
<td>1.0 ± 0.30</td>
</tr>
<tr>
<td></td>
<td><strong>0.8 ± 0.05</strong></td>
<td>0.8 ± 0.07</td>
<td>1.0 ± 0.11</td>
<td>1.2 ± 0.07</td>
</tr>
</tbody>
</table>

Type II-TARPs showed no noteworthy regulatory influence on GluK2 receptors, the closest AMPAR-related iGluRs. This observation supports the notion of exclusivity concerning type II-TARPs as AMPA receptors auxiliary proteins.
3.1.4 Bimolecular fluorescence complementation

The assumption that all type II-TARPs interact with all members of the AMPA receptor subfamily of iGluRs, is supported by the fact that there are diverse functional consequences upon co-expression of both, type II-TARPs and AMPARs, in oocytes. However, γ6 was proven not to have any major effect on AMPARs; therefore, the possibility of an interaction can neither be presumed nor excluded. In order to address this matter, oocytes were injected with varied AMPA receptor combinations that carry half a GFP molecule at their C-termini. The different TARPs and γ6 in this experiment were tagged with the other half of the GFP molecule, also at their C-termini, and co-expressed with the tagged AMPARs. If both molecules come together close enough to allow a direct interaction of the two fluorophore halves, green fluorescence can be observed.

Representative confocal images of the analyzed combinations are shown in Figure 3.36. Uninjected oocytes were used as a negative control. Stargazin is known to have a strong affinity for all AMPA receptor subunit’s cRNA and, therefore, was used as a positive control. For heteromeric combinations, only one subunit’s cRNA carried the CGFP tag, and this subunit was injected in a molar ratio of 1:5 when compared to the untagged subunit cRNA. The excess of untagged receptor cRNA makes the amount of homomeric, tagged receptor that will be formed negligible. The amount of homomeric untagged receptors that will be formed, however, will be considerable, though they will not fluoresce upon interaction with the TARPs or γ6 and can therefore be ignored for the purpose of bimolecular fluorescence complementation analysis.

Oocytes were controlled electrophysiologically to confirm that there was no loss of function due to the GFP tag. The electrophysiological performance of oocytes injected with the tagged versions of the receptors and TARPs was undistinguishable from that of oocytes injected with their wild type counterparts.
Results

Figure 3.36 Co-expression of AMPARs with \( \gamma 5, \gamma 6, \gamma 7, \) or \( \gamma 2, \) in \( X. \) laevis oocytes.
Representative confocal microscopy pictures of CGFP-tagged AMPARs and NGFP-tagged \( \gamma 5, \gamma 6, \gamma 7, \) or \( \gamma 2. \) Oocytes co-expressing \( \gamma 2 \)-C-NGFP are shown in every picture at the lower left corner, as a positive control. The lower right corner of every picture features uninjected oocytes, as a negative control. The oocyte on top of each picture shows oocytes co-expressing CGFP tagged AMPARs with \( \gamma 5 \)-C-NGFP (left), \( \gamma 6 \)-C-NGFP (middle), or \( \gamma 7 \)-C-NGFP (right). A, B, and C: GluA1(Q)flip-C-CGFP. D, E, and F: GluA2(R)flip-C-CGFP. G, H, and I: GluA1(Q)flip-C-CGFP/GluA2(R)flip. J, K, and L: GluA1(Q)flip-C-CGFP/GluA2(Q)flip. Comparable data were collected from 2 independent batches of oocytes.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Although BiFC is not an inherently quantitative method, it gives good indication whether or not two proteins interact, and an estimate for the strength of the interaction by comparison to a given control.

Oocytes expressing GluA1(Q)flip-C-CGFP showed the strongest fluorescence when co-expressed with γ2-C-NGFP, and no fluorescence on uninjected oocytes, as expected for effective positive and negative controls, respectively. Surprisingly, the fluorescence observed with NGFP-tagged γ5, γ6, and γ7 was comparable, indicating that all three interact with the receptor with similar binding affinities.

Moreover, the fluorescence induced by co-expression of GluA2(R)flip-C-CGFP with γ6-C-NGFP was as strong as the fluorescence caused by co-expression of the receptor with γ2-C-NGFP, and stronger than that induced by co-expression with γ5-C-NGFP or γ7-C-NGFP, whose signals were in the same intensity range. The same pattern was observed with the heteromer GluA1(Q)flip-C-CGFP/GluA2(R)flip.

Conversely, the fluorescence profile of oocytes expressing heteromeric GluA1(Q)flip-C-CGFP/GluA2(Q)flip and NGFP-tagged γ5, γ6, γ7, or γ2 resembles the one observed for homomeric GluA1(Q)flip-C-CGFP.

All the oocytes co-expressing CGFP-tagged AMPARs and NGFP-tagged γ5, γ6, γ7, or γ2 show fluorescence. This observation is not surprising for the TARPs, since they are all effective auxiliary regulators of AMPARs, and functionality requires interaction. However, γ6 lacks the modulatory competence observed with the TARPs during their electrophysiological characterization. The results indicate that the absence of modulation is therefore not a consequence of the lack of interaction. Furthermore, the binding affinity of γ6 to Ca\textsuperscript{2+}-impermeable AMPARs seems to be stronger than to Ca\textsuperscript{2+}-permeable receptors, and it is in this case at the same level as that of γ2. By contrast, γ5 and γ7 seem to interact with AMPARs with fairly similar affinities, independently of the type of receptor analyzed.
3.1.5 GluA1 editing variants

The observation that γ5 modulation of AMPARs strongly depends on the amino acid at the Q/R editing site, making γ5 the only TARP that is able to selectively inhibit agonist-induced currents of receptors composed by unedited receptor subunits, shows how unique and versatile this TARP is, when compared to the other five members of the TARP family. Naturally, we wanted to know more about the molecular mechanisms behind the plasticity exhibited by γ5, and its dependence on editing. Hence, we point-mutated the amino acid at the Q/R editing site of GluA1 to all the remaining 20 amino acids, and each mutant was co-expressed with γ5 in X. laevis oocytes. After four to five days, oocytes were recorded by TEVC. Currents were elicited by either glutamate or kainate.

A potential effect of γ5 on desensitization was assessed by addition of TCM to either agonist. Hence, agonist-induced currents were first measured upon addition of TCM-free agonist solutions; afterwards, current amplitudes were recorded by application of TCM-containing agonist solutions to the same oocytes. A change in the ratio of agonist + TCM-induced currents to TCM-free agonist-induced currents by γ5 suggests a probable role of the TARP in regulating channel desensitization kinetics.

The flop splice variants of the twenty GluA1 mutants were originally characterized. The analysis of these mutants implied a role of γ5 in the modulation of channel kinetics. The flip splice variants of AMPARs have very distinct kinetic properties and are known to yield higher steady state currents upon agonist binding. Therefore, all twenty mutant flip counterparts were analyzed.

After the comparative analysis of γ5 modulation of agonist-induced currents with and without TCM, for the various Q/R-site mutants, it became clear that TARP function is somehow linked to receptor desensitization. Besides its obvious effect on desensitization, TCM has been described to also slow down deactivation and to increase agonist potency of GluA1, independently of desensitization, likely by affecting agonist dissociation kinetics (Mitchell & Fleck 2007).

To further explore the influence of γ5 on desensitization, we proceeded to introduce a point mutation at position 479, changing a leucine to a tyrosine in all twenty GluA1 Q/R flip mutants. This point mutation is known to stabilize the dimer interface, abolishing desensitization. Therefore, it is not expected that the use of allosteric
modulators, such as TCM, on these double mutants significantly impacts agonist-induced currents of the mutants.

For clarity, the combined results of both flip and flop splice variants and the non-desensitizing mutants will be considered simultaneously in this section, for each of the twenty Q/R mutants. Mutants were further divided into different groups considering the nature of the amino acid at the Q/R editing site.

Experiments were performed in numerous oocyte batches, which can introduce small variations to the results, the most obvious being protein expression levels. In consequence, the same RNA combinations injected in different batches of oocytes often produce currents that differ in magnitude, and hence absolute current amplitudes cannot be directly compared. To compensate for this variability, and therefore to be able to compare among the different experiments, current responses were always normalized to the responses obtained from the TARP-less receptor, in every individual recording.

3.1.5.1 Positively charged Q/R-site mutants

In this group are included the mutants GluA1(R), GluA1(H) and GluA1(K). For simplicity, the term “positively charged mutants” will be used for reference to GluA1 mutants that have a positively charged amino acid at the Q/R editing site.

3.1.5.1.1 GluA1(R)

As discussed earlier, γ5 was able to potentiate both glutamate and kainate-induced currents, as well as the ratios of kainate-induced to glutamate-induced currents of both GluA1(R) splice variants, although γ5 potentiation of both agonist-induced currents was considerably higher for the flip variant.

Moreover, the addition of TCM to kainate significantly potentiated induced currents even further yet, also in this case, more drastically for the flip variant. Distinguishing both splice variants was the ability of γ5 to potentiate the ratio of glutamate + TCM-induced to glutamate-induced currents only for the flip variant.

Glutamate-induced currents of the GluA1(R) double mutant were potentiated more strongly by γ5 than those of their desensitizing counterpart. This enhanced effect
on the non-desensitizing receptor hints at a role of γ5 on regulating GluA1(R) desensitization kinetics and is consistent with the results obtained when desensitization was abolished by co-application of TCM. Conversely, for the double mutant, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by γ5, when compared to the desensitizing mutant. The perceived difference between the two agonists largely impacts the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant. This ratio, which was amplified by γ5 for the single mutants, was significantly reduced by this TARP for the double mutant.

Furthermore, an unexpected observation was made when analyzing currents elicited by kainate and TCM for GluA1(R)flip-L479Y. Although addition of TCM did not significantly alter kainate-induced currents of oocytes expressing the receptor alone, as expected for non-desensitizing receptors, TCM considerably potentiated kainate-induced currents of oocytes co-expressing γ5. Consequently, the resulting ratio of kainate + TCM-induced to kainate-induced current, was substantially enlarged by γ5.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(R) mutants are shown in Figures 3.37, 3.38, and 3.39. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.10.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.37 Current responses of GluA1(R)flip ± γ5.**

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). D: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). E: Representative current traces of GluA1(R)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 μM glutamate + 600 μM TCM-induced currents to 300 μM glutamate-induced currents ratio (± SEM). H: Normalized 150 μM kainate + 600 μM TCM-induced currents to 150 μM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.38 Current responses of GluA1(R)flop ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(R)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.39 Current responses of GluA1(R)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(R)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.10 Modulation of GluA1(R) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(R) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(R)flip</th>
<th>GluA1(R)flop</th>
<th>GluA1(R)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>6.7 ± 0.77</td>
<td>2.6 ± 0.21</td>
<td>17.5 ± 1.33</td>
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<tr>
<td>KA</td>
<td>28.8 ± 3.01</td>
<td>15.4 ± 2.63</td>
<td>14.3 ± 0.95</td>
</tr>
<tr>
<td>Glu + TCM</td>
<td>51.1 ± 7.00</td>
<td>3.2 ± 0.47</td>
<td>20.9 ± 2.28</td>
</tr>
<tr>
<td>KA + TCM</td>
<td>64.3 ± 6.09</td>
<td>38.3 ± 7.24</td>
<td>30.2 ± 1.96</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>4.3 ± 0.23</td>
<td>4.7 ± 0.54</td>
<td>0.8 ± 0.04</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>7.0 ± 0.56</td>
<td>0.7 ± 0.07</td>
<td>1.2 ± 0.01</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>2.4 ± 0.12</td>
<td>1.2 ± 0.02</td>
<td>2.3 ± 0.06</td>
</tr>
</tbody>
</table>

3.1.5.1.2 GluA1(H)

Also for GluA1(H) mutants, γ5 was able to potentiate both glutamate- and kainate-induced currents, as well as the ratios of kainate-induced to glutamate-induced currents of both splice variants, although γ5 potentiation of these variables was also in this case considerably higher for the flip variant.

Moreover, the addition of TCM to both agonists significantly potentiated induced currents even further, yet in this case, only for the flip variant. For the flop variant, addition of TCM to glutamate further abolished the boosting effect of the TARP, which resulted in a reduction of the ratio of agonist + TCM-induced to agonist-induced currents. Moreover, kainate + TCM-induced currents were potentiated by γ5 to a similar extent as kainate-induced currents, and therefore, there was no change in the ratio of kainate + TCM-induced to kainate-induced currents, for the flop variant.
As it was the case for GluA1(R)-L479Y, glutamate-induced currents of the GluA1(H) double mutant were potentiated more strongly by γ5 than those of their desensitizing counterpart. This enhanced effect on the non-desensitizing receptor is consistent with the results obtained when desensitization was abolished by co-application of TCM, to GluA1(H)flip. Conversely, for the double mutant, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by γ5, when compared to the desensitizing mutant.

The perceived difference between the two agonists largely impacts the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant. This ratio, which was amplified by γ5 for the single mutants, was significantly reduced by the TARP, for the double mutant. These observations are consistent with what had previously been observed for GluA1(R).

Again, TCM considerably potentiated kainate-induced currents of oocytes co-expressing γ5 only, which resulted in a considerable inflation of the ratio of kainate + TCM-induced to kainate-induced currents by γ5.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios of agonist + TCM-induced to agonist-induced currents, for GluA1(H) mutants are shown in Figures 3.40, 3.41, and 3.42. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.11.
Results

Figure 3.40 Current responses of GluA1(H)flip ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(H)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced current ratios to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced current ratios to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.41 Current responses of GluA1(H)flop ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(H)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.42 Current responses of GluA1(H)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(H)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Table 3.11 Modulation of GluA1(H) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(H) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(H)flip</th>
<th>GluA1(H)flop</th>
<th>GluA1(H)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>1.9 ± 0.12</td>
<td>1.5 ± 0.12</td>
<td>12.4 ± 2.35</td>
</tr>
<tr>
<td>KA</td>
<td>4.2 ± 0.38</td>
<td>1.7 ± 0.14</td>
<td>2.8 ± 0.48</td>
</tr>
<tr>
<td>Glu + TCM</td>
<td>6.5 ± 1.02</td>
<td>0.9 ± 0.04</td>
<td>16.2 ± 3.48</td>
</tr>
<tr>
<td>KA + TCM</td>
<td>9.4 ± 1.67</td>
<td>1.9 ± 0.13</td>
<td>11.1 ± 2.35</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>2.3 ± 0.18</td>
<td>1.1 ± 0.04</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>3.5 ± 0.63</td>
<td>0.7 ± 0.01</td>
<td>0.9 ± 0.05</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>1.8 ± 0.13</td>
<td>1.1 ± 0.07</td>
<td>3.7 ± 0.18</td>
</tr>
</tbody>
</table>

3.1.5.1.3 GluA1(K)

Glutamate was not able to induce any measurable currents for GluA1(K)flop, with or without γ5, unless applied with TCM. The receptor alone also showed no response to kainate, but co-expression with γ5 raised kainate-induced currents above the limit of detection. In the presence of TCM, there was no difference for glutamate-induced currents of oocytes co-expressing GluA1(K)flop and γ5, when compared to the TARP-less receptor. However, co-expression with γ5 resulted in a significant potentiation of kainate + TCM-induced currents.

Contrary to the flop variant, both glutamate and kainate were able to produce measurable currents for GluA1(K)flip, which were in both cases potentiated by γ5. Moreover, γ5 significantly increased the ratio of kainate-induced to glutamate-induced
currents of GluA1(K)flip, as it did for the remaining positively charged desensitizing mutants.

Nonetheless, in great contrast to GluA1(K)flop, whose glutamate + TCM-induced currents were not affected by co-expression with \( \gamma 5 \), for GluA1(K)flip this agonist combination resulted in a drastic potentiation of the amplitude of the elicited currents by \( \gamma 5 \), which was yet much higher than the potentiating effect observed upon solo glutamate application. Furthermore, the co-expression of both splice variants with \( \gamma 5 \) resulted in a significant potentiation of kainate + TCM-induced currents, although this effect was a few hundred fold higher for the flip variant, and again much higher than the potentiation observed when kainate was applied without TCM. The aforementioned observations translate into an enhancement of the ratio of agonist + TCM-induced to agonist-induced currents by \( \gamma 5 \) for the flip variant, for both glutamate and kainate.

As it was observed for the other positively charged mutants, glutamate-induced currents of the GluA1(K) double mutant were potentiated more strongly by \( \gamma 5 \) than those of their desensitizing counterpart. This enhanced effect on the non-desensitizing receptor is consistent with the results obtained when desensitization was abolished by co-application of TCM, to GluA1(K)flip. Conversely, and also analogously to the remaining positively charged mutants, for the double mutant, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by \( \gamma 5 \), when compared to the desensitizing mutant, and therefore the perceived difference between the two agonists largely impacts the ratios of kainate-induced to glutamate-induced currents of the non-desensitizing mutant, which was severely reduced by the TARP. The addition of TCM to glutamate led, as expected, to no significant alteration of GluA1(K)flip-L479Y glutamate-induced currents, with or without \( \gamma 5 \), and therefore the ratio of glutamate + TCM-induced to glutamate-induced currents, remained unaffected for this mutant. Nevertheless, TCM considerably potentiated kainate-induced currents of oocytes co-expressing \( \gamma 5 \) only, which resulted in a considerable inflation of the kainate + TCM-induced currents to kainate-induced currents ratio by \( \gamma 5 \). These observations are consistent with what had previously been observed for GluA1(R) and GluA1(H).

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratio of agonist + TCM-induced to agonist-induced currents, for GluA1(K) mutants are
shown in Figures 3.43, 3.44 and 3.45. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.12.

**Figure 3.43 Current responses of GluA1(K)flip ± γ5.**
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(K)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application.

F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.44 Current responses of GluA1(K)flop ± γ5.
A: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). B: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). C: Representative current traces of GluA1(K)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.45 Current responses of GluA1(K)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(K)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Table 3.12 Modulation of GluA1(K) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(K) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(K)flip</th>
<th>GluA1(K)flop</th>
<th>GluA1(K)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>2.3 ± 0.21</td>
<td>_</td>
<td>128.5 ± 22.63</td>
</tr>
<tr>
<td>KA</td>
<td>9.9 ± 1.26</td>
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<td>3.9 ± 0.51</td>
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<td>Glu + TCM</td>
<td>68.0 ± 14.04</td>
<td>1.0 ± 0.20</td>
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<td>KA + TCM</td>
<td>253.8 ± 39.89</td>
<td>4.4 ± 0.68</td>
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<td>KA/Glu</td>
<td>2.9 ± 0.32</td>
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<td>Glu + TCM/Glu</td>
<td>22.1 ± 5.98</td>
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<td>KA + TCM/KA</td>
<td>30.9 ± 3.42</td>
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<td>14.2 ± 1.81</td>
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3.1.5.1.4 Overview

The type II-TARP γ5 potentiates agonist-induced currents of all assessable positively charged Q/R-site mutants. The observed effect is more evident for the flip variants, and stronger when kainate is applied as an agonist. Addition of TCM to either agonist further amplifies the factor for γ5 potentiation on the flip variants, suggesting a potential role of the TARP on receptor desensitization kinetics. This conclusion is further reinforced by the observation that γ5 regulates glutamate-induced currents of non-desensitizing, positively charged mutants more effectively than their desensitizing counterparts, while the effect of the TARP on currents induced by the non-desensitizing partial agonist kainate on the same receptors is less evident. Intriguingly, TCM considerably potentiates kainate-induced currents of oocytes co-expressing non-desensitizing mutants and γ5.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

3.1.5.2 Negatively charged Q/R-site mutants

In this group are included the mutants GluA1(D) and GluA1(E)flip. For simplicity, the term “negatively charged mutants” will here be used for reference to GluA1 mutants that have a negatively charged amino acid at the Q/R editing site.

3.1.5.2.1 GluA1(E)

Both glutamate- and kainate-induced currents of GluA1(E) were potentiated by $\gamma_5$. Nonetheless, while glutamate-induced responses of the flip variant were more obviously affected by the TARP, much like it happened with positively charged receptors, the massive potentiation seen for kainate-induced currents was in this case comparable for both splice variants. Moreover, $\gamma_5$ increased the ratio of kainate-induced to glutamate-induced currents of GluA1(E)flip even more evidently than it did for positively charged mutants.

Furthermore, addition of TCM to either agonist resulted in a further increase of the potentiation values measured upon application of glutamate or kainate alone, which translates into an enhancement of the ratio of agonist + TCM-induced to agonist-induced currents by $\gamma_5$. The effect of TCM was again higher on the agonist-induced responses of the flip variant.

As already observed for the positively charged mutants, glutamate-induced currents of the GluA1(E) double mutant were potentiated more strongly by $\gamma_5$ than those of their desensitizing counterpart. This enhanced effect on the non-desensitizing receptor is consistent with the results obtained when desensitization was abolished by co-application of TCM to GluA1(E)flip. Conversely, and also analogously to the remaining analyzed mutants, for the double mutant, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by $\gamma_5$, when compared to the desensitizing mutant, and therefore the perceived difference between the two agonists largely impacts the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant, which was greatly reduced by the TARP. The addition of TCM to glutamate led, as expected, to no significant alteration of GluA1(E)flip-L479Y glutamate-induced currents, with or without $\gamma_5$, and therefore the ratio of glutamate + TCM-induced to glutamate-induced currents, remained unaffected.
for this mutant. Nevertheless, TCM considerably potentiated kainate-induced currents of oocytes co-expressing \( \gamma_5 \) only, which resulted in a considerable inflation of the ratio of kainate + TCM-induced to kainate-induced currents by \( \gamma_5 \). These observations coincide with what was formerly observed for the positively charged mutants.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(E) mutants are shown in Figures 3.46, 3.47 and 3.48. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.13.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

![Diagram showing current responses of GluA1(E)flip ± γ5.](image)

**Figure 3.46** Current responses of GluA1(E)flip ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(E)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.47 Current responses of GluA1(E)flop ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(E)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

![Figure 3.48 Current responses of GluA1(E)flip-L479Y ± γ5.](image)

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(E)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Table 3.13 Modulation of GluA1(E) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(E) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
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<th>GluA1(E)flip</th>
<th>GluA1(E)flop</th>
<th>GluA1(E)flip-L479Y</th>
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<td>Glu</td>
<td>24.9 ± 2.68</td>
<td>3.8 ± 0.65</td>
<td>1216 ± 156.9</td>
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<td>KA</td>
<td>333.8 ± 37.50</td>
<td>360.1 ± 94.67</td>
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<td>Glu + TCM</td>
<td>549.0 ± 67.83</td>
<td>10.7 ± 0.84</td>
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<td>KA + TCM</td>
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<td>896.6 ± 63.52</td>
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<td>KA/Glu</td>
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<td>50.8 ± 12.83</td>
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<td>Glu + TCM/Glu</td>
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<td>1.1 ± 0.13</td>
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<td>KA + TCM/KA</td>
<td>4.7 ± 0.51</td>
<td>1.2 ± 0.05</td>
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</tbody>
</table>

3.1.5.2.2 GluA1(D)

Neither glutamate nor kainate were able to induce any measurable currents from GluA1(D)flip, with or without γ5, unless the agonists were applied with TCM. Also GluA1(D)flip showed no currents in response to glutamate, with or without the TARP. However, although kainate also did not elicit currents above the detection limit from the TARP-less receptor, co-expression of γ5 was able to rescue kainate-induced currents. Furthermore, in the presence of TCM, there was a significant increase of both glutamate- and kainate-induced currents of oocytes co-expressing either GluA1(D) variant and γ5, when compared to the receptor expressed alone. Potentiation by γ5 was stronger for kainate + TCM-induced currents and higher for the flip variant.

As neither glutamate nor kainate were able to induce any measurable currents for GluA1(D)flip in the absence of TCM, it is not possible to exhaustively compare it to the non-desensitizing mutant. Nevertheless, glutamate-induced responses of these double mutants are much higher than those elicited by kainate, which resembles the
tendency observed for the remaining non-desensitizing mutants, including the negatively charged GluA1(E)flip-L479Y. Also resembling the preceding results is the strikingly low ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant.

The addition of TCM to glutamate led, as expected, to no significant increase of GluA1(D)flip-L479Y glutamate-induced currents, with or without γ5, and therefore the ratio of glutamate + TCM-induced to glutamate-induced currents, was not significantly altered.

However, as already observed for the remaining non-desensitizing mutants, although addition of TCM did not significantly alter kainate-induced currents of oocytes solely expressing the receptor, TCM drastically potentiated kainate-induced currents of oocytes co-expressing the mutant and γ5. Consequently, the resulting ratio of kainate + TCM-induced to kainate-induced currents was drastically enlarged by γ5.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(D) mutants are shown in Figures 3.49, 3.50 and 3.51. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.14.
Results

Figure 3.49 Current responses of GluA1(D)flip± γ5.
A: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). B: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). C: Representative current traces of GluA1(D)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.50 Current responses of GluA1(D)flop ± γ5.
A: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). B: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). C: Representative current traces of GluA1(D)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.51 Current responses of GluA1(D)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(D)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.14 Modulation of GluA1(D) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(D) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
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<tr>
<th></th>
<th>GluA1(D)flip</th>
<th>GluA1(D)flop</th>
<th>GluA1(D)flip-L479Y</th>
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<tr>
<td>Glu</td>
<td>_</td>
<td>_</td>
<td>182.7 ± 24.00</td>
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<tr>
<td>KA</td>
<td>_</td>
<td>_</td>
<td>2.7 ± 0.26</td>
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<td>Glu + TCM</td>
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<td>1.7 ± 0.16</td>
<td>249.5 ± 36.88</td>
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<td>56.8 ± 7.67</td>
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<td>59.2 ± 11.22</td>
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<td>KA/Glu</td>
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<td>0.02 ± 0.003</td>
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<td>Glu + TCM/Glu</td>
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<td>_</td>
<td>0.7 ± 0.18</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>_</td>
<td>_</td>
<td>20.1 ± 3.41</td>
</tr>
</tbody>
</table>

3.1.5.2.3 Overview

γ5 potentiated agonist-induced currents of the only negatively charged Q/R-site mutant that exhibited measurable currents in response to glutamate or kainate. Also, for this mutant the modulation was more evident for the flip variant. Addition of TCM to the agonists increased the γ5 potentiating effect, predominantly on the flip variant. Furthermore, the TARP impacted glutamate-induced currents of the non-desensitizing mutant more efficiently than the desensitizing version. The strong reduction of the ratio of kainate-induced to glutamate-induced currents of non-desensitizing receptors becomes an apparent hallmark of γ5 modulation. Furthermore, also for negatively charged mutants, TCM enhanced kainate-induced currents of oocytes co-expressing non-desensitizing mutants and γ5.
3.1.5.3 Polar uncharged Q/R-site mutants

In this group are included GluA1(Q), and the mutants GluA1(N), GluA1(S), GluA1(T) and GluA1(C). For simplicity, the term “polar uncharged mutants” will be used here for reference to GluA1 mutants that have a polar uncharged amino acid at the Q/R editing site.

3.1.5.3.1 GluA1(Q)

As observed earlier, $\gamma_5$ was able to reduce agonist-induced currents of both splice variants of GluA1(Q) and to prompt a boost on the ratio of kainate-induced to glutamate-induced currents of the flip variant only.

Interestingly, for the flip variant, addition of TCM to the agonists completely abolished the inhibitory effect of $\gamma_5$ on glutamate-induced responses while it inverted the inhibitory tendency on kainate-induced responses, which were in these circumstances promptly amplified by the TARP. This result differs from the one observed for the flop variant, whose current amplitudes were both reduced in co-expression with $\gamma_5$, independently of TCM. Consequently, for the flip variant, the ratios of agonist + TCM-induced to agonist-induced currents were both enlarged by $\gamma_5$, as it was the case for the formerly analyzed flip mutants. For the flop variant, only the kainate-related ratio was increased and, although by a small factor, the glutamate ratio was decreased.

Consistently, glutamate-induced currents of the GluA1(Q) double mutant were potentiated by $\gamma_5$ in contrast to its desensitizing counterpart. This enhanced effect was stronger than that seen for the single mutants when desensitization was abolished with TCM. Contrariwise, currents induced by kainate were not significantly affected by $\gamma_5$ for these mutants. Hence, the dissimilarity between the two agonist-induced responses, once again, largely impacted the ratio of kainate-induced to glutamate-induced currents of the double mutants, which was critically reduced by the TARP.

In agreement with the analysis of previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was dramatically enlarged for GluA1(Q)flip-L479Y by $\gamma_5$. 
The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(Q) mutants are shown in Figures 3.52, 3.53 and 3.54. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.15.
Figure 3.52 Current responses of GluA1(Q)flip ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(Q)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.53 Current responses of GluA1(Q)flop ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(Q)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.54 Current responses of GluA1(Q)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(Q)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.15 Modulation of GluA1(Q) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(Q). Colored fonts specify the combinations that were significantly regulated.

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<th>GluA1(Q)flip-L479Y</th>
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<tr>
<td>Glu</td>
<td>0.4 ± 0.03</td>
<td>0.5 ± 0.04</td>
<td>2.5 ± 0.13</td>
</tr>
<tr>
<td>KA</td>
<td>0.7 ± 0.04</td>
<td>0.4 ± 0.02</td>
<td>0.8 ± 0.11</td>
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<td>Glu + TCM</td>
<td>1.1 ± 0.03</td>
<td>0.3 ± 0.03</td>
<td>3.2 ± 0.24</td>
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<td>KA + TCM</td>
<td>1.8 ± 0.04</td>
<td>0.5 ± 0.04</td>
<td>7.0 ± 0.91</td>
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<td>KA/Glu</td>
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<td>Glu + TCM/Glu</td>
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<td>3.6 ± 0.25</td>
<td>1.4 ± 0.09</td>
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</table>

3.1.5.3.2 GluA1(N)

Unexpectedly, in contrast to the results obtained for GluA1(Q), co-expression of γ5 with GluA1(N) mutants, which have an asparagine at the Q/R editing site that is so closely related to glutamine, resulted in a many fold increase of both glutamate- and kainate-induced currents, for both splice variants, as it was the case with the further related arginine-containing Q/R mutants. Both GluA1(N) mutants exhibit a characteristic increase of the ratio of kainate-induced to glutamate-induced currents, upon co-expression with the TARP.

Co-application of TCM with glutamate did not significantly alter the extent of γ5 potentiation, while kainate + TCM induced responses were augmented for the flop variant and diminished for the flip variant, when compared to kainate-elicted currents in the absence of TCM. This translated in the corresponding variation of the ratio of kainate + TCM-induced to kainate-induced currents for both forms.
Nonetheless, glutamate-induced currents of GluA1(N)flip-L479Y were potentiated more robustly by \( \gamma_5 \) than those of its desensitizing counterpart, as already observed for the remaining analyzed mutants. However, currents induced by kainate were not increased by \( \gamma_5 \), when compared to the desensitizing type. Thus once again, the expressive difference between the outcome prompted by the two agonists ultimately results in a reduction of the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant, by the TARP.

In agreement with the analysis of previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was considerably improved by \( \gamma_5 \) for GluA1(N)flip-L479Y.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(N) mutants are shown in Figures 3.55, 3.56 and 3.57. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.16.
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.55 Current responses of GluA1(N)flip ± γ5.**
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(N)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

**Figure 3.56 Current responses of GluA1(N)flop ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(N)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.57 Current responses of GluA1(N)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(N)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.16 Modulation of GluA1(N) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(N) mutants. Colored fonts specify the combinations that were significantly regulated.

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<th>GluA1(N)flop</th>
<th>GluA1(N)flip-L479Y</th>
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<td>Glu</td>
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<td>KA</td>
<td>14.6 ± 0.65</td>
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<td>Glu + TCM</td>
<td>6.9 ± 0.33</td>
<td>14.3 ± 2.64</td>
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<td>78.8 ± 11.28</td>
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<tr>
<td>KA/Glu</td>
<td>2.6 ± 0.21</td>
<td>2.1 ± 0.17</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>1.4 ± 0.27</td>
<td>0.8 ± 0.02</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>0.6 ± 0.06</td>
<td>1.8 ± 0.14</td>
<td>5.3 ± 0.60</td>
</tr>
</tbody>
</table>

3.1.5.3.3 GluA1(S)

In contrast to GluA1(Q) but similarly to GluA1(N), co-expression of γ5 with GluA1(S) mutants resulted in a many fold increase of both glutamate and kainate-induced currents, for both splice variants. Additionally, both GluA1(N) mutants exhibit a characteristic increase of the ratio of kainate-induced to glutamate-induced currents, upon co-expression with the TARP.

Co-application of TCM with glutamate significantly increased the extent of γ5 potentiation, while kainate + TCM induced responses were augmented for the flop variant only, when compared to kainate-elicited currents in the absence of TCM. This translated in the corresponding variation of the ratio of agonist + TCM-induced to agonist-induced currents.

Furthermore, glutamate-induced currents of GluA1(S)flip-L479Y were potentiated more robustly by γ5 than those of its desensitizing counterpart, as already
observed for the remaining analyzed mutants. However, currents induced by kainate were not increased by γ5, when compared to the desensitizing type. Thus once again, the expressive difference between the outcome prompted by the two agonists ultimately results in a reduction of the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant, by the TARP.

In agreement with the analysis of previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was considerably improved by γ5 also for GluA1(S)flip-L479Y.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(S) mutants are shown in Figures 3.58, 3.59 and 3.60. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.17.
Results

Figure 3.58 Current responses of GluA1(S)flip ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(S)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.59 Current responses of GluA1(S)flop ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(S)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application.

F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.60 Current responses of GluA1(S)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(S)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Table 3.17 Modulation of GluA1(S) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(S) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(S)flip</th>
<th>GluA1(S)flop</th>
<th>GluA1(S)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>33.7 ± 3.30</td>
<td>4.1 ± 0.26</td>
<td>257.8 ± 37.14</td>
</tr>
<tr>
<td>KA</td>
<td>121.4 ± 11.07</td>
<td>36.8 ± 6.51</td>
<td>30.9 ± 3.77</td>
</tr>
<tr>
<td>Glu + TCM</td>
<td>113.0 ± 16.21</td>
<td>8.7 ± 1.31</td>
<td>214.5 ± 23.15</td>
</tr>
<tr>
<td>KA + TCM</td>
<td>141.7 ± 19.56</td>
<td>84.0 ± 9.83</td>
<td>154.0 ± 18.92</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>4.0 ± 0.47</td>
<td>8.5 ± 1.25</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>4.6 ± 1.01</td>
<td>1.9 ± 0.15</td>
<td>0.9 ± 0.06</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>1.0 ± 0.15</td>
<td>1.4 ± 0.04</td>
<td>5.0 ± 0.21</td>
</tr>
</tbody>
</table>

3.1.5.3.4 GluA1(T)

As expected, given the high similarity between the two mutants, co-expression of γ5 with GluA1(T) resulted in a very similar response to GluA1(S), which involves a many fold potentiation of both agonist-induced currents, for both splice variants. Furthermore, co-expression with the TARP resulted in a dramatic increase of the ratio of kainate-induced to glutamate-induced currents, for both flip and flop variants.

Co-application of TCM with glutamate strongly increased the extent of γ5 potentiation, which translated into a high value for the ratio of glutamate + TCM-induced to glutamate-induced currents. The improvement of kainate-induced responses by TCM was more modest, although also significant.

Again, glutamate-induced currents of the non-desensitizing mutant were strongly regulated by γ5 and show a much higher potentiation factor than the desensitizing receptor type. Furthermore, kainate-induced responses were not as largely
increased by $\gamma_5$ as the ones from the single mutant. Therefore, unsurprisingly, the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant was considerably reduced by the TARP, also for GluA1(T)flip-L479Y.

Once more, in agreement with the analysis of previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was substantially increased by $\gamma_5$ also for GluA1(T)flip-L479Y.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(S) mutants are shown in Figures 3.61, 3.62 and 3.63. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.18.
Figure 3.61 Current responses of GluA1(T)flip ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(T)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

**Figure 3.62 Current responses of GluA1(T)flop ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(T)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.63 Current responses of GluA1(T)flip-L479Y ± γ5.**

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). D: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). E: Representative current traces of GluA1(T)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 μM glutamate + 600 μM TCM-induced currents to 300 μM glutamate-induced currents ratio (± SEM). H: Normalized 150 μM kainate + 600 μM TCM-induced currents to 150 μM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.18 Modulation of GluA1(T) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(T) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(T)flip</th>
<th>GluA1(T)flop</th>
<th>GluA1(T)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>31.2 ± 3.22</td>
<td>4.5 ± 0.62</td>
<td>557.3 ± 90.89</td>
</tr>
<tr>
<td>KA</td>
<td>258.6 ± 15.87</td>
<td>85.4 ± 16.79</td>
<td>164.6 ± 19.67</td>
</tr>
<tr>
<td>Glu + TCM</td>
<td>155.5 ± 10.94</td>
<td>18.1 ± 3.16</td>
<td>717.9 ± 101.4</td>
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<td>KA + TCM</td>
<td>441.8 ± 45.91</td>
<td>249.7 ± 38.07</td>
<td>1134 ± 129.2</td>
</tr>
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<td>KA/Glu</td>
<td>9.2 ± 0.72</td>
<td>15.3 ± 1.82</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>7.2 ± 1.19</td>
<td>2.6 ± 0.15</td>
<td>1.4 ± 0.04</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>1.8 ± 0.24</td>
<td>1.6 ± 0.04</td>
<td>7.1 ± 0.37</td>
</tr>
</tbody>
</table>

3.1.5.3.5 GluA1(C)

The last of the polar uncharged mutants to be analyzed was GluA1(C). Much alike all the remaining members of this group, with the remarkable exception of the wild-type GluA1(Q), co-expression of γ5 with GluA1(C) mutants resulted in potentiation of both glutamate- and kainate-induced currents, for both splice variants. Moreover, GluA1(C) mutants underwent the traditional increase of the ratio of kainate-induced to glutamate-induced currents, a characteristic consequence of γ5 co-expression.

Co-application of TCM with either agonist resulted in an unpretentious although significant reinforcement of γ5 potentiation, for flop variants. Nonetheless, for the other splice variant, the effect of TCM addition to either agonist did not significantly affect γ5 function. This translates into the values for the ratio of agonist + TCM-induced to agonist-induced currents displayed on Table 3.19.
Furthermore, glutamate-induced currents of GluA1(C)flip-L479Y were potentiated more robustly by γ5 than those of its desensitizing counterpart, as already observed for the remaining mutants. However, currents induced by kainate were not increased by γ5, when compared to the desensitizing type. Thus, a reduction of the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant by the TARP is once again in order.

In agreement with the analysis of previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was considerably improved by γ5 also for GluA1(C)flip-L479Y.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(S) mutants are shown in Figures 3.64, 3.65 and 3.66. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.19.
**Results**

**Figure 3.64 Current responses of GluA1(C)flip ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(C)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.65 Current responses of GluA1(C)flop ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(C)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.66 Current responses of GluA1(C)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(C)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.19 Modulation of GluA1(C) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(C) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(C)flip</th>
<th>GluA1(C)flop</th>
<th>GluA1(C)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>7.6 ± 1.37</td>
<td>2.7 ± 0.23</td>
<td>30.3 ± 2.89</td>
</tr>
<tr>
<td>KA</td>
<td>16.6 ± 2.70</td>
<td>7.6 ± 0.96</td>
<td>7.7 ± 1.43</td>
</tr>
<tr>
<td>Glu + TCM</td>
<td>6.9 ± 0.87</td>
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<td>41.6 ± 6.21</td>
</tr>
<tr>
<td>KA + TCM</td>
<td>7.9 ± 1.12</td>
<td>13.4 ± 0.62</td>
<td>70.6 ± 14.98</td>
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<tr>
<td>KA/Glu</td>
<td>2.3 ± 0.08</td>
<td>2.5 ± 0.22</td>
<td>0.2 ± 0.02</td>
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<tr>
<td>Glu + TCM/Glu</td>
<td>1.2 ± 0.15</td>
<td>1.3 ± 0.05</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>1.2 ± 0.13</td>
<td>1.4 ± 0.10</td>
<td>7.4 ± 0.33</td>
</tr>
</tbody>
</table>

3.1.5.3.6 Overview

Surprisingly, and in contrast to the results acquired with the wild type GluA1(Q), γ5 potentiated agonist-induced currents of all the remaining polar uncharged Q/R-site mutants. Additionally, the amplified TARP effect on glutamate-induced currents of non-desensitizing receptors was a constant for these mutants, as was their low ratios of kainate-induced to glutamate-induced current ratio. Furthermore, TCM consistently enhanced kainate-induced currents of non-desensitizing polar, uncharged mutants co-expressed with γ5.
3.1.5.4 Hydrophobic Q/R-site mutants

In this group are included the mutants GluA1(G), GluA1(A), GluA1(L), GluA1(M), GluA1(V), GluA1(I), and GluA1(P). For simplicity, the term “hydrophobic mutants” will be used here for reference to GluA1 mutants that have a hydrophobic amino acid at their Q/R editing site.

3.1.5.4.1 GluA1(G)

As observed for the Q variant, γ5 was able to decrease both glutamate and kainate-induced currents of GluA1(G) and to enhance the ratio of kainate-induced to glutamate-induced currents of the flip variant only. The high similarity in the response of both mutants to the TARP was highly unexpected, considering the different nature and size of glycine and glutamine side chains.

Exactly like for the GluA1(Q) flip variant, addition of TCM to the agonists completely abolished the inhibitory effect of γ5 on glutamate-induced responses while it inverted the inhibitory tendency on kainate-induced responses, which were in these circumstances promptly amplified by the TARP, although only slightly. This result differs from the one observed for the flop variant, whose current amplitudes were both reduced in co-expression with γ5, independently of TCM, like it was observed for GluA1(Q). Consequently, for the flip variant, the ratios of agonist + TCM-induced to agonist-induced currents were both enlarged by γ5. For the flop variant, only the kainate-related ratio was sizably elevated. The glutamate ratio was increased weakly, although significantly, by a meek factor of 1.1.

Consistently, and also in line with the observations made for GluA1(Q), glutamate-induced currents of the GluA1(G) double mutant were potentiated by γ5 in contrast to its desensitizing counterpart. This enhanced effect was stronger than that seen for the single mutants when desensitization was abolished with TCM. Contrariwise, currents induced by kainate were not significantly affected by γ5 for these mutants. Hence, the dissimilarity between the two agonist-induced responses, also here, largely impacted the ratio of kainate-induced to glutamate-induced currents of the double mutants, which was reduced by the TARP.
In agreement with the analysis of all previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was dramatically enlarged for GluA1(G)flip-L479Y by γ5.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(G) mutants are shown in Figures 3.67, 3.68 and 3.69. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.20.
Results

**Figure 3.67 Current responses of GluA1(G)flip ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(G)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

**Figure 3.68** Current responses of GluA1(G)flop ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(G)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.69 Current responses of GluA1(G)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(G)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Table 3.20 Modulation of GluA1(G) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(G) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(G)flip</th>
<th>GluA1(G)flop</th>
<th>GluA1(G)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.05</td>
<td>1.9 ± 0.19</td>
</tr>
<tr>
<td>KA</td>
<td>0.5 ± 0.03</td>
<td>0.4 ± 0.03</td>
<td>0.8 ± 0.07</td>
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<td>1.5 ± 0.06</td>
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<td>KA + TCM/KA</td>
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<td>4.2 ± 0.22</td>
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3.1.5.4.2 GluA1(A)

After the analysis of GluA1(G) and due to the high structural resemblance between alanine and glycine, it was expected that the GluA1(A) mutant would respond similarly to γ5. However, that was not the case. In fact, γ5 significantly potentiated all agonist-induced currents of both GluA1(A) splice variants in heavy contrast to what was observed for GluA1(G). Moreover, the ratio of kainate-induced to glutamate-induced currents was strongly potentiated by γ5 in both cases, more similarly to other positively modulated mutants than to GluA1(G) itself.

Furthermore, the addition of TCM to either agonist induced a drastic potentiation of the amplitude of the elicited currents by γ5, which was yet much higher than the potentiating effect observed upon solo agonist application, an effect that was more dramatic for the flip version. Such an observation translated into an enhancement
of the ratio of agonist + TCM-induced to agonist-induced currents by γ5, for both glutamate and kainate.

As seen for the other mutants, glutamate-induced currents of the GluA1(A) double mutant were potentiated more stoutly by γ5 than those of its desensitizing counterpart. This enhanced effect on the non-desensitizing receptor is consistent with the results obtained when desensitization was abolished by co-application of TCM. Contrariwise, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by γ5, when compared to the desensitizing mutant. Accordingly, also for GluA1(A)flip-L479Y ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant was harshly reduced by the TARP.

While in the presence of γ5, addition of TCM led only to a small, although significant, alteration of the double mutant glutamate-induced currents, TCM considerably potentiated kainate-induced currents of oocytes co-expressing γ5, which resulted in a substantial inflation of the kainate + TCM-induced currents to kainate-induced currents.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(A) mutants are shown in Figures 3.70, 3.71 and 3.72. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.21.
Figure 3.70 Current responses of GluA1(A)flip ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(A)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
**Figure 3.71 Current responses of GluA1(A)flop ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(A)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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Figure 3.72 Current responses of GluA1(A)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(A)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.21 Modulation of GluA1(A) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(A) mutants. Colored fonts specify the combinations that were significantly regulated.

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<th>GluA1(A)flip</th>
<th>GluA1(A)flop</th>
<th>GluA1(A)flip-L479Y</th>
</tr>
</thead>
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<td>Glu + TCM/Glu</td>
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<td>2.5 ± 0.26</td>
<td>1.4 ± 0.02</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>5.0 ± 0.39</td>
<td>4.4 ± 0.13</td>
<td>16.5 ± 0.65</td>
</tr>
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3.1.5.4.3 GluA1(L)

GluA1(L) response to γ5 resembles that seen for GluA1(G) and GluA1(Q). Co-expression with the TARP decreased glutamate- and kainate-induced currents significantly. Also for the L mutant, the ratio of kainate-induced to glutamate-induced currents was enhanced for the flip variant only.

Addition of TCM to the agonists completely reverted the inhibitory effect of γ5 on glutamate- and kainate-induced responses of the flip variant, which were under these circumstances amplified by the TARP, although more extensively for kainate. This result differs from the one observed for the flop variant, whose current amplitudes were both reduced in co-expression with γ5, independently of TCM, as already observed for GluA1(Q) and GluA1(G). Consequently, for the flip variant, the ratios of agonist + TCM-induced to agonist-induced currents were both enlarged by γ5. These ratios were not altered for the flop variant.
Consistently, and also in line with the data obtained from GluA1(Q) and GluA1(G), glutamate-induced currents of the double mutant were potentiated by \( \gamma_5 \), in contrast to its desensitizing counterpart. This result is consistent with the preceding observation, when desensitization was abolished with TCM. Contrariwise, currents induced by kainate were not significantly affected by \( \gamma_5 \) for these mutants. Hence, the dissimilarity between the two agonist-induced responses, also here, largely impacted ratio of kainate-induced to glutamate-induced currents of the double mutants, which was reduced by the TARP.

In agreement with the analysis of all previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was considerably enlarged for GluA1(L)flip-L479Y by \( \gamma_5 \).

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(L) mutants are shown in Figures 3.73, 3.74 and 3.75. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.22.
Results

**Figure 3.73 Current responses of GluA1(L)flip ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(L)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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**Figure 3.74 Current responses of GluA1(L)flop ± γ5.**

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). D: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). E: Representative current traces of GluA1(L)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 μM glutamate + 600 μM TCM-induced currents to 300 μM glutamate-induced currents ratio (± SEM). H: Normalized 150 μM kainate + 600 μM TCM-induced currents to 150 μM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.75 Current responses of GluA1(L)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(L)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1.5.4.4 GluA1(M)

The response of GluA1(M) to γ5 resembles that of GluA1(G), GluA1(L) and GluA1(Q). Co-expression of these mutants with the TARP induced a significant decrease of glutamate- and kainate-induced currents, for both splice variants. Furthermore, the ratio of kainate-induced to glutamate-induced currents was enhanced for the flip variant only, similarly to the remaining aforementioned receptors.

Addition of TCM to the agonists completely reverted the inhibitory effect of γ5 on kainate-induced responses of the flip variant, which were under these circumstances amplified by the TARP, and abolished the TARP effect on glutamate-induced currents, which were hence undistinguishable from those of the TARP-less receptor. The addition of TCM to the agonists did not change the inhibitory effect of the TARP on the flop variant. The same effects had already been observed on the aforesaid receptors. Consequently, for the flip variant, the ratios of agonist + TCM-induced to agonist-
Results

induced currents were both enlarged by γ5, while only the kainate-related ratio was potentiated for the flop variant.

Furthermore, glutamate-induced currents of GluA1(M)flip-L479Y were potentiated by γ5 in contrast to its desensitizing counterpart. This enhanced effect was stronger than that seen for the single mutants when desensitization was abolished with TCM. Contrariwise, currents induced by kainate were also inhibited by γ5 for these mutants. Hence, the dissimilarity between the two agonist-induced responses, once again, largely impacted the ratio of kainate-induced to glutamate-induced currents of the double mutants, which was considerably reduced by the TARP.

In agreement with the analysis of previous mutants, the ratio of kainate + TCM-induced to kainate-induced currents was dramatically enlarged for GluA1(M)flip-L479Y by γ5.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(M) mutants are shown in Figures 3.76, 3.77 and 3.78. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.23.
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![Graphs and traces]

**Figure 3.76 Current responses of GluA1(M)flip ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(M)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.77 Current responses of GluA1(M)flop ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(M)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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**Figure 3.78 Current responses of GluA1(M)flip-L479Y ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(M)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.23 Modulation of GluA1(M) by γ5. Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(M) mutants. Colored fonts specify the combinations that were significantly regulated.

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<th>GluA1(M)flip</th>
<th>GluA1(M)flop</th>
<th>GluA1(M)flip-L479Y</th>
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</thead>
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<td>Glu</td>
<td>0.4 ± 0.02</td>
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<td>KA</td>
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<td>1.5 ± 0.07</td>
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<td>Glu + TCM/Glu</td>
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<td>0.8 ± 0.02</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>2.3 ± 0.22</td>
<td>2.9 ± 0.19</td>
<td>7.1 ± 0.64</td>
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</table>

3.1.5.4.5 GluA1(V)

Type II-TARP γ5 significantly potentiated all agonist-induced currents of both GluA1(V) mutants. Moreover, the ratio of kainate-induced to glutamate-induced currents was strongly potentiated by γ5 in both cases.

Furthermore, for the flip variant, addition of TCM to either agonist induced a drastic potentiation of the amplitude of the elicited currents by γ5, which was yet much higher than the potentiating effect observed upon solo agonist application. Such an observation translated into an enhancement of the ratio of agonist + TCM-induced to agonist-induced currents by γ5, for both glutamate and kainate. This effect was not significant for the flop version.

As observed for all the other mutants, glutamate-induced currents of the GluA1(V) double mutant were potentiated more stoutly by γ5 than those of its
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

desensitizing counterpart. This enhanced effect on the non-desensitizing receptor is consistent with the results obtained when desensitization was abolished by co-application of TCM. Contrariwise, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by γ5, when compared to the desensitizing mutant. Accordingly, also for GluA1(V)flip-L479Y the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant was severely reduced by the TARP.

While in the presence of γ5, addition of TCM led only to a small change on the double mutant glutamate-induced currents, TCM considerably potentiated kainate-induced currents of oocytes co-expressing γ5, which resulted in a dramatic elevation of the ratio of kainate + TCM-induced to kainate-induced currents.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(V) mutants are shown in Figures 3.79, 3.80 and 3.81. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.24.
Figure 3.79 Current responses of GluA1(V)flip ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(V)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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**Figure 3.80 Current responses of GluA1(V)flop ± γ5.**
A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). D: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). E: Representative current traces of GluA1(V)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 μM glutamate + 600 μM TCM-induced currents to 300 μM glutamate-induced currents ratio (± SEM). H: Normalized 150 μM kainate + 600 μM TCM-induced currents to 150 μM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.8.1 Current responses of GluA1(V)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(V)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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Table 3.24 Modulation of GluA1(V) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(V) mutants. Colored fonts specify the combinations that were significantly regulated.

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<th>GluA1(V)flop</th>
<th>GluA1(V)flip-L479Y</th>
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<td>Glu</td>
<td>4.2 ± 0.46</td>
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<td>KA</td>
<td>37.5 ± 4.21</td>
<td>15.8 ± 3.30</td>
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<td>Glu + TCM</td>
<td>133.5 ± 10.82</td>
<td>2.5 ± 0.23</td>
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<td>212.7 ± 15.73</td>
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<th>GluA1(V)flip</th>
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</thead>
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<td>Glu + TCM/Glu</td>
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<td>KA + TCM/KA</td>
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<td>1.4 ± 0.04</td>
<td>8.3 ± 0.69</td>
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</table>

3.1.5.4.6 GluA1(I)

Neither glutamate nor kainate were able to induce any measurable currents for GluA1(I)flop, with or without γ5, unless applied with TCM. In the presence of TCM, there was only a small increase of the glutamate-induced currents of oocytes co-expressing GluA1(I)flop and γ5, when compared to the TARP-less receptor. However, co-expression with γ5 resulted in a strong potentiation of kainate + TCM-induced currents.

Contrary to the flop variant, both glutamate and kainate were able to produce measurable currents for GluA1(I)flip, which were in both cases potentiated by γ5. Moreover, γ5 significantly increased the ratio of kainate-induced to glutamate-induced currents of GluA1(I)flip.

Nonetheless, in strong contrast to GluA1(I)flop, whose glutamate + TCM-induced currents were only slightly affected by co-expression with γ5, for GluA1(I)flip
this agonist combination resulted in a drastic potentiation of the amplitude of the elicited currents by $\gamma$5, which was yet much higher than the potentiating effect observed upon solo glutamate application. Furthermore, for both splice variants co-expression with $\gamma$5 resulted in a significant potentiation of kainate + TCM-induced currents, although this effect was much higher for the flip variant, and again much higher than the potentiation observed when kainate was applied without TCM. The aforementioned observations translate into an enhancement of the ratio of agonist + TCM-induced to agonist-induced currents by $\gamma$5 for the flip variant, for both glutamate and kainate.

As was the case for the other positively charged mutants, glutamate-induced currents of the GluA1(I)flip-L479Y were potentiated more strongly by $\gamma$5 than those of its desensitizing counterpart. This enhanced effect on the non-desensitizing receptor is consistent with the results obtained when desensitization was abolished by co-application of TCM, to GluA1(I)flip. Conversely, and also analogously to the remaining positively charged mutants, for the double mutant, currents induced by the non-desensitizing partial agonist kainate were not increased as strongly by $\gamma$5, when compared to the desensitizing mutant, and therefore the perceived difference between the two agonists largely impacts the ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant, which was severely reduced by the TARP. The addition of TCM to kainate considerably potentiated kainate-induced currents of oocytes co-expressing the double mutant and $\gamma$5, which resulted in a considerable inflation of the ratio of kainate + TCM-induced to kainate-induced currents by $\gamma$5. These observations are consistent with what had previously been observed for the remaining receptors.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(I) mutants are shown in Figures 3.82, 3.83 and 3.84. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.25.
Figure 3.82 Current responses of GluA1(I)flip ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(I)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.83 Current responses of GluA1(I)flop ± γ5.
A: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). B: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). C: Representative current traces of GluA1(I)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.84 Current responses of GluA1(I)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM kainate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(I)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.25 Modulation of GluA1(I) by γ5.

Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(I) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(I)flip</th>
<th>GluA1(I)flop</th>
<th>GluA1(I)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>4.2 ± 0.46</td>
<td>_</td>
<td>114.6 ± 21.09</td>
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<tr>
<td>KA</td>
<td>37.5 ± 4.21</td>
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<td>23.1 ± 3.83</td>
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<tr>
<td>Glu + TCM</td>
<td>133.5 ± 10.82</td>
<td>1.4 ± 0.08</td>
<td>143.0 ± 25.54</td>
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<td>KA + TCM</td>
<td>212.7 ± 15.73</td>
<td>19.9 ± 1.95</td>
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<tr>
<td>KA/Glu</td>
<td>8.7 ± 0.63</td>
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<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>33.3 ± 4.81</td>
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<td>1.3 ± 0.02</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>5.4 ± 0.51</td>
<td>_</td>
<td>4.7 ± 0.43</td>
</tr>
</tbody>
</table>

3.1.5.4.7 GluA1(P)

Neither glutamate nor kainate were able to induce any measurable currents for either of the GluA1(P) splice variants, with or without γ5, unless the agonists were applied with TCM. However, in the presence of TCM, there was a significant increase of both glutamate- and kainate-induced currents of oocytes co-expressing the flip variant of GluA1(P) and γ5, when compared to the receptor expressed alone. Potentiation by γ5 was stronger for kainate + TCM-induced currents. There was no apparent modulation of GluA1(P)flop by γ5.

As neither glutamate nor kainate were able to induce any measurable currents for GluA1(P)flip in the absence of TCM, it is not possible to extensively compare it with the non-desensitizing mutant. Nevertheless, glutamate-induced responses of these double mutants are potentiated much stronger than those elicited by kainate, which resembles the tendency observed for the remaining non-desensitizing mutants. Also
resembling the preceding results is the significantly reduced ratio of kainate-induced to glutamate-induced currents of the non-desensitizing mutant.

The addition of TCM to glutamate led, as expected, to no significant increase of GluA1(P)flip-L479Y glutamate-induced currents, with or without γ5, and therefore the ratio of glutamate + TCM-induced to glutamate-induced currents, was not significantly altered.

However, as already observed for the remaining non-desensitizing mutants, although addition of TCM did not significantly alter kainate-induced currents of oocytes solo expressing the receptor, TCM drastically potentiated kainate-induced currents of oocytes co-expressing the mutant and γ5. Consequently, the resulting ratio of kainate + TCM-induced to kainate-induced currents was enlarged by γ5.

Glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(P) mutants are shown in Figures 3.85, 3.86 and 3.87. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.26.
Results

Figure 3.85 Current responses of GluA1(P)flip ± γ5.

A: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). B: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). C: Representative current traces of GluA1(P)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.86 Current responses of GluA1(P)flop ± γ5.
A: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). B: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). C: Representative current traces of GluA1(P)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Figure 3.87 Current responses of GluA1(P)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(P)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. 

F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). 

G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). 

H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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**Table 3.26 Modulation of GluA1(P) by γ5.**
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(P) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
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<th>GluA1(P)flip</th>
<th>GluA1(P)flop</th>
<th>GluA1(P)flip-L479Y</th>
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</thead>
<tbody>
<tr>
<td>Glu</td>
<td>_</td>
<td>_</td>
<td>20.7 ± 2.07</td>
</tr>
<tr>
<td>KA</td>
<td>_</td>
<td>_</td>
<td>1.5 ± 0.16</td>
</tr>
<tr>
<td>Glu + TCM</td>
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<td>1.2 ± 0.09</td>
<td>23.0 ± 2.16</td>
</tr>
<tr>
<td>KA + TCM</td>
<td>5.5 ± 0.62</td>
<td>1.2 ± 0.07</td>
<td>3.5 ± 0.37</td>
</tr>
<tr>
<td>KA/Glu</td>
<td>_</td>
<td>_</td>
<td>0.8 ± 0.02</td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>_</td>
<td>_</td>
<td>1.1 ± 0.07</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>_</td>
<td>_</td>
<td>2.1 ± 0.28</td>
</tr>
</tbody>
</table>

**3.1.5.4.8 Overview**

Hydrophobic mutants respond to γ5 very differently. While the TARP reduced agonist-induced currents of GluA1(G), GluA1(L), and GluA1(M), similar to the polar, uncharged wild-type, it potentiated agonist-induced currents of all the remaining hydrophobic mutants, resembling GluA1(R). The data shows how specific γ5 can be. It is truly extraordinary that such small differences between the amino acids that reside at the Q/R editing site of GluA1 can produce such a variety of responses as a result of co-expression with γ5.
3.1.5.5 Aromatic Q/R-site mutants

In this group are included the mutants GluA1(F), GluA1(Y), and GluA1(W). For simplicity, the term “aromatic mutants” will be used here for reference to GluA1 mutants that have an aromatic amino acid at their Q/R editing site.

3.1.5.5.1 GluA1(F)

Type II-TARP $\gamma$5 was able to potentiate both glutamate- and kainate-induced currents, as well as the ratio of kainate-induced to glutamate-induced currents of GluA1(F) mutants.

Moreover, the addition of TCM to both agonists significantly potentiated induced currents even further, which translates as an increase of the ratio of agonist + TCM-induced to agonist-induced currents.

Nonetheless, glutamate-induced currents of GluA1(F)flip-L479Y were not potentiated by $\gamma$5 much stronger than those of its desensitizing counterpart, distinguishing it from the remaining non-desensitizing mutants. However, currents induced by kainate were potentiated by $\gamma$5 to a much lesser extent than the desensitizing type. Hence, the ratio of kainate-induced to glutamate-induced currents was reduced by the TARP, also for GluA1(F)flip-L479Y.

The addition of TCM to glutamate led, as expected, to only minor effects on GluA1(F)flip-L479Y glutamate-induced currents, and therefore the ratio of glutamate + TCM-induced to glutamate-induced currents was only slightly higher. However, as already observed for all the remaining non-desensitizing mutants, although addition of TCM did not significantly alter kainate-induced currents of oocytes solo expressing the receptor, TCM potentiated kainate-induced currents of oocytes co-expressing any of the mutants with $\gamma$5. Consequently, the resulting the ratio of kainate + TCM-induced to kainate-induced currents was enlarged by $\gamma$5. Though, this effect was unmistakably the smallest amongst non-desensitizing mutants.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(F) mutants
are shown in Figures 3.88, 3.89 and 3.90. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.27.

Figure 3.88 Current responses of GluA1(F)flip ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(F)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

![Graphs and diagrams]

**Figure 3.89 Current responses of GluA1(F)flop ± γ5.**

A: Normalized 300 μM glutamate-induced currents (± SEM). B: Normalized 150 μM kainate-induced currents (± SEM). C: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). D: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). E: Representative current traces of GluA1(F)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application.

F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 μM glutamate + 600 μM TCM-induced currents to 300 μM glutamate-induced currents ratio (± SEM). H: Normalized 150 μM kainate + 600 μM TCM-induced currents to 150 μM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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**Figure 3.90 Current responses of GluA1(F)flip-L479Y ± γ5.**

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(F)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.27 Modulation of GluA1(F) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(F) mutants. Colored fonts specify the combinations that were significantly regulated.

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<thead>
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<th>GluA1(F)flip</th>
<th>GluA1(F)flop</th>
<th>GluA1(F)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>3.5 ± 0.42</td>
<td>4.5 ± 0.85</td>
<td>5.8 ± 0.76</td>
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<tr>
<td>KA</td>
<td>16.3 ± 2.45</td>
<td>7.4 ± 1.12</td>
<td>3.0 ± 0.43</td>
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<tr>
<td>Glu + TCM</td>
<td>11.5 ± 1.94</td>
<td>3.0 ± 0.37</td>
<td>6.5 ± 0.97</td>
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<td>KA + TCM</td>
<td>20.9 ± 3.76</td>
<td>9.3 ± 0.46</td>
<td>5.3 ± 0.85</td>
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<tr>
<td>KA/Glu</td>
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<td>0.6 ± 0.07</td>
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<td>Glu + TCM/Glu</td>
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<td>1.2 ± 0.05</td>
<td>1.7 ± 0.07</td>
<td>1.7 ± 0.09</td>
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3.1.5.5.2 GluA1(Y)

The results show a strong potentiation of both agonist-induced currents for the two splice variants of GluA1(Y) upon co-expression with γ5. Potentiation of kainate-induced responses was comparatively stronger and therefore the ratio of kainate-induced to glutamate-induced currents of GluA1(Y) mutants was considerably large.

Co-application of TCM with kainate did not significantly alter the extent of γ5 potentiation for the flop variant, but decreased the ratio of kainate + TCM-induced to kainate-induced currents for the flip variant, a rare effect that had been so far only observed for GluA1(N). Concurrently, the ratio of glutamate + TCM-induced to glutamate-induced currents was significantly increased for both variants.

Moreover, glutamate-induced currents of GluA1(Y)flip-L479Y were not potentiated by γ5 more strongly than those of its desensitizing counterpart, which is in agreement to the data obtained from the other aromatic mutant analyzed and
distinguishes them from all the remaining non-desensitizing mutants. Additionally, currents induced by kainate were potentiated by $\gamma_5$ to a much lesser extent than the desensitizing type. Hence, the ratio of kainate-induced to glutamate-induced currents was reduced by the TARP, also for GluA1(Y)flip-L479Y.

Addition of TCM to glutamate led, as expected, to only minor effects on GluA1(Y)flip-L479Y glutamate-induced currents, and therefore the ratio of glutamate + TCM-induced cto glutamate-induced currents, was only slightly higher in co-expression with $\gamma_5$. However, as already observed for all the remaining non-desensitizing mutants, TCM potentiated kainate-induced currents of oocytes co-expressing either of the mutants with $\gamma_5$, although addition of TCM did not significantly altered kainate-induced currents of oocytes solo expressing the receptor. Consequently, the resulting ratio of kainate + TCM-induced to kainate-induced currents was enlarged by $\gamma_5$. Though, this effect was much smaller if compared to the analyzed non-aromatic mutants, and comparable to the one registered for GluA1(F)flip-L479Y.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratios of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(Y) mutants are shown in Figures 3.91, 3.92 and 3.93. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.28.
Results

Figure 3.91 Current responses of GluA1(Y)flip ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(Y)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
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Figure 3.92 Current responses of GluA1(Y)flop ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(Y)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 3 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.93 Current responses of GluA1(Y)flip-L479Y ± γ5.

A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(Y)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Table 3.28 Modulation of GluA1(Y) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(Y) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(Y)flip</th>
<th>GluA1(Y)flop</th>
<th>GluA1(Y)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>35.9 ± 3.34</td>
<td>57.1 ± 4.67</td>
<td>18.9 ± 1.56</td>
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<tr>
<td>KA</td>
<td>278.6 ± 35.00</td>
<td>446.5 ± 36.26</td>
<td>14.3 ± 1.43</td>
</tr>
<tr>
<td>Glu + TCM</td>
<td>106.1 ± 14.23</td>
<td>88.2 ± 9.84</td>
<td>23.2 ± 1.87</td>
</tr>
<tr>
<td>KA + TCM</td>
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<td>615.8 ± 54.39</td>
<td>20.0 ± 1.65</td>
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<td>KA/Glu</td>
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<td>8.9 ± 0.80</td>
<td>0.8 ± 0.02</td>
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<tr>
<td>Glu + TCM/Glu</td>
<td>2.8 ± 0.28</td>
<td>1.4 ± 0.07</td>
<td>1.2 ± 0.04</td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>0.5 ± 0.02</td>
<td>0.9 ± 0.05</td>
<td>1.4 ± 0.03</td>
</tr>
</tbody>
</table>

3.1.5.5.3 GluA1(W)

Neither glutamate nor kainate were able to induce any measurable currents for any of the GluA1(W) splice variants, with or without γ5, unless the agonists were applied with TCM. In the presence of TCM, there was no significant change for agonist-induced currents of oocytes co-expressing GluA1(W)flop and γ5, when compared to the receptor expressed alone.

In the presence of TCM, distinguishing GluA1(W)flip from its flop counterpart, there was a significant potentiation of both agonist-induced currents for oocytes co-expressing the TARP.

As neither glutamate nor kainate were able to induce a response from GluA1(W)flip in the absence of TCM, it is not possible to thoroughly compare it to the non-desensitizing mutant.
Nevertheless, glutamate-induced responses of these double mutants were potentiated much stronger than those elicited by kainate, and therefore the ratio of kainate-induced to glutamate-induced currents was expressively reduced, which resembles the tendency observed for the remaining non-desensitizing mutants.

Additionally, as also observed for the remaining non-desensitizing mutants, TCM drastically potentiated kainate-induced currents of oocytes co-expressing the mutant and γ5. Consequently, the resulting ratio of kainate + TCM-induced to kainate-induced currents was enlarged by γ5, by a factor that resembles non-aromatic mutants more closely. On the other hand, addition of TCM to glutamate resulted on a small but significant decrease of GluA1(W)flip-L479Y glutamate-induced currents and therefore the ratio of glutamate + TCM-induced to glutamate-induced currents was slightly decreased.

The resulting glutamate- and kainate-induced responses, with or without TCM, as well as the calculated ratio of kainate-induced to glutamate-induced currents and the ratios between agonist-induced currents with and without TCM, for GluA1(W) mutants are shown in Figures 3.94, 3.95 and 3.96. In addition, representative current traces are shown in the same figures, while potentiation values are shown in detail on Table 3.29.
Figure 3.94 Current responses of GluA1(W)flip ± γ5.
A: Normalized 300 μM glutamate + 600 μM TCM-induced currents (± SEM). B: Normalized 150 μM kainate + 600 μM TCM-induced currents (± SEM). C: Representative current traces of GluA1(W)flip, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Results

Figure 3.95 Current responses of GluA1(W)flop ± γ5.

A: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). B: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). C: Representative current traces of GluA1(W)flop, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars indicate the duration of agonist application. Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
3.1 Investigation of type II-TARP modulation of AMPARs by TEVC

Figure 3.96 Current responses of GluA1(W)flip-L479Y ± γ5.
A: Normalized 300 µM glutamate-induced currents (± SEM). B: Normalized 150 µM kainate-induced currents (± SEM). C: Normalized 300 µM glutamate + 600 µM TCM-induced currents (± SEM). D: Normalized 150 µM kainate + 600 µM TCM-induced currents (± SEM). E: Representative current traces of GluA1(W)flip-L479Y, elicited by the agonist specified above the traces. Traces of the receptor alone are shown in black whereas colored traces show currents induced by co-expression with γ5. Grey bars describe indicate the duration of agonist application. F: Normalized kainate-induced current to glutamate-induced current ratios (± SEM). G: Normalized 300 µM glutamate + 600 µM TCM-induced currents to 300 µM glutamate-induced currents ratio (± SEM). H: Normalized 150 µM kainate + 600 µM TCM-induced currents to 150 µM kainate-induced currents ratio (± SEM). Each set of data was collected from a minimum of 2 batches. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001).
Table 3.29 Modulation of GluA1(W) by γ5.
Glutamate-induced currents and kainate-induced currents, in the presence or absence of TCM, potentiation of kainate-induced to glutamate-induced current ratios by γ5, and potentiation of ratios between agonist-induced currents in the presence and absence of TCM of both glutamate and kainate, by γ5, for GluA1(W) mutants. Colored fonts specify the combinations that were significantly regulated.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(W)flip</th>
<th>GluA1(W)flop</th>
<th>GluA1(W)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>_</td>
<td>_</td>
<td><strong>81.9 ± 4.29</strong></td>
</tr>
<tr>
<td>KA</td>
<td>_</td>
<td>_</td>
<td><strong>2.5 ± 0.28</strong></td>
</tr>
<tr>
<td>Glu + TCM</td>
<td><strong>3.6 ± 0.51</strong></td>
<td>1.1 ± 0.13</td>
<td><strong>64.6 ± 3.63</strong></td>
</tr>
<tr>
<td>KA + TCM</td>
<td><strong>8.0 ± 0.54</strong></td>
<td>1.0 ± 0.10</td>
<td><strong>27.1 ± 4.41</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GluA1(W)flip</th>
<th>GluA1(W)flop</th>
<th>GluA1(W)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA/Glu</td>
<td>_</td>
<td>_</td>
<td><strong>0.03 ± 0.003</strong></td>
</tr>
<tr>
<td>Glu + TCM/Glu</td>
<td>_</td>
<td>_</td>
<td><strong>0.8 ± 0.02</strong></td>
</tr>
<tr>
<td>KA + TCM/KA</td>
<td>_</td>
<td>_</td>
<td><strong>9.9 ± 1.02</strong></td>
</tr>
</tbody>
</table>

3.1.5.5.4 Overview

Once again, γ5 showed how superbly specific it can regulate AMPA receptors, discriminating between them merely based on the amino acid at the Q/R editing site. Aromatic mutants responded differently to the TARP, although, with the exception of GluA1(W)flop, all were positively modulated, albeit to different extents. Furthermore, the different responses of a receptor’s flip and flop variants to the TARP show that γ5 activity is to a certain degree also dependent on alternative splicing.
3.2 Patch clamp analysis

Fast receptor kinetics are not resolvable through TEVC in oocytes. Hence, the patch clamp method was applied in this study to take a more detailed look at receptor desensitization and deactivation in HEK293 cells. Cells transiently transfected with bicistronic vectors, simultaneously expressing the proteins of interest as well as fluorescent marker proteins were recorded using this technique in whole-cell mode, at a clamped potential of -70 mV. Successfully transfected cells expressed EGFP, DsRed, or both and could therefore be easily selected. The IRES sequence is cleaved after transcription and therefore the fluorescent proteins are separated from the AMPA receptor and TARP proteins.

Because cells have different sizes, capacitance was recorded for each cell as an indicator of its dimension. Subsequently, the measured currents were divided by the cell’s capacitance, and thus could be appropriately compared.

The influence of γ5 on homomeric GluA1(Q)flip, GluA1(Q)flip-L479Y, GluA1(R)flip, and GluA1(R)flip-L479Y receptors was analyzed, involving fast agonist application of 10 mM of glutamate, with and without TCM.

Patch clamp recordings were performed by Tobias Strasdeit (Department of Biochemistry I, Ruhr University, Bochum).

3.2.1 GluA1(Q)flip

In the absence of agonist, nearly all channels are at rest, and ions cannot pass through the membrane. As with patch clamp it is possible to perform fast agonist application, and HEK293 cells are relatively small when compared to oocytes, glutamate reaches all membrane receptors at virtually the same time. Thus, peak currents, which could not be recorded by TEVC, can now be effectively measured. These peak currents represent the maximum current that can be attained at a specific recording potential, due to the simultaneous activation of most of the ion channels that were hitherto in the deactivated state. The subsequent steady-state is the consequence of the equilibrium achieved amongst receptors at different states of activation.
Application of glutamate to cells expressing GluA1(Q)flip prompted a peak current of ~240 pA, which quickly declined to a steady-state current of ~7 pA (Figure 3.97). When glutamate was applied to cells co-expressing the receptor plus γ5, the resulting peak current of ~160 pA was only slightly smaller than the one elicited by the receptor, when expressed alone. However, the ensuing steady-state current was in this case much smaller (~0.5 pA), which is consistent with what had previously been observed in oocytes, using TEVC (see section 3.1.5.3.1).

It is well known that type I-TARPs decelerate both deactivation and desensitization rates of AMPARs. Furthermore, our previous results in oocytes suggest that γ5 might also have a role in modulating channel kinetics, more specifically desensitization. We have explored this hypothesis by comparing ion channel kinetics of cells expressing GluA1(Q)flip with and without the TARP.

However, the results indicate that there are only rather small differences in deactivation and desensitization rates of GluA1(Q)flip ion channels with and without γ5 (Figure 3.97).

Our previous results in oocytes had shown that γ5 increases glutamate current responses of GluA1 editing mutants when co-applied with TCM. More specifically, GluA1(Q)flip glutamate-induced currents were significantly decreased by γ5. Yet, when applied with TCM, glutamate-induced currents of oocytes expressing the TARP and GluA1 were virtually indistinguishable (see section 3.1.5.3.1). These results closely resemble the data obtained from HEK293 cells. Also in these cells, the drastic reduction in steady-state currents shown in Figure 3.97 by γ5 is abolished in the presence of TCM (Figure 3.98).

The results are also consistent with the previous finding (Mitchell & Fleck 2007) that TCM slows deactivation of GluA1, independently of desensitization. Accordingly, the data shows that deactivation times for GluA1 are more than three times higher (~14 ms vs. ~4 ms) when TCM is applied with glutamate. However, γ5 does not seem to have any impact on these values (Figure 3.98).
3.2 Patch clamp analysis

Figure 3.97 γ5 modulation of GluA1(Q)flip in HEK293 cells.

Peak currents (A) and steady-state currents (B) were induced by long application (500 ms) of 10 mM glutamate. C: steady-state to peak current ratios. Long application of glutamate (500 ms) was employed to determine desensitization half-life values (τ) (D) while short application pulses (1 ms) were employed to determine deactivation half-life values (τ) (E). Error bars, indicate SEM. The number at the bottom of each bar refers to the number of cells recorded for each combination. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001). F: Average current traces of GluA1(Q)flip in the presence and absence of γ5, elicited by 10 µM of glutamate (n ≥15).
Results

Figure 3.98 γ5 modulation of GluA1(Q)flip, in the presence of TCM.

Steady-state currents (A) were induced by long application (500 ms) of 10 mM glutamate while short application pulses (1 ms) were employed to determine deactivation half-life values (τ) (B). Error bars indicate SEM. The number at the bottom of each bar refers to the number of cells recorded for each combination. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001). C: Average current traces of GluA1(Q)flip in the presence and absence of γ5, elicited by 10 μM of glutamate + 500 μM TCM (n ≥15).

When measured in oocytes, GluA1(Q)flip-L479Y glutamate-induced currents were enhanced by γ5 (see section 3.1.5.3.1). However, in HEK293 cells, co-expression of the mutant with the TARP did not change the elicited steady-state currents, resembling the results obtained when desensitization had been abolished by TCM.

Additionally, deactivation half-life values (τ) for the mutant were much higher (~72 ms) than those of wild type GluA1 (~4 ms). Nonetheless, again, γ5 did not seem to impact these values (Figure 3.99).

In Table 3.30, γ5 potentiation of glutamate-induced peak and steady-state currents and kinetic values are shown in detail.

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3.2 Patch clamp analysis

Steady-state currents (A) were induced by long application (500 ms) of 10 mM glutamate while short application pulses (1 ms) were employed to determine deactivation half-life values (τ) (B). Error bars indicate SEM. The number at the bottom of each bar refers to the number of cells recorded for each combination. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001). C: Average current traces of GluA1(Q)flip-L479Y in the presence and absence of γ5, elicited by 10 µM of glutamate (n ≥15).

Figure 3.99 γ5 modulation of GluA1(Q)flip-L479Y, in HEK293 cells.
Table 3.30 Modulation of GluA1(Q)flip by γ5, in HEK293 cells.

Potentiation values (± SEM) of glutamate-induced currents in the presence or absence of TCM, potentiation values (± SEM) of glutamate-induced currents of GluA1(Q)flip-L479Y, and respective potentiation of desensitization and deactivation half-life values (τ) (± SEM) by γ5.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(Q)flip</th>
<th>GluA1(Q)flip (TCM)</th>
<th>GluA1(Q)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak $I_{\text{Glu}}$ (pA)</td>
<td>0.7 ± 0.22</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Steady-state $I_{\text{Glu}}$ (pA)</td>
<td>0.07 ± 0.025</td>
<td>1.0 ± 0.26</td>
<td>1.1 ± 0.27</td>
</tr>
<tr>
<td>$I_{\text{st}}/I_{\text{peak}}$ (%)</td>
<td>0.14 ± 0.032</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>$\tau_{\text{desensitization}}$ (ms)</td>
<td>0.8 ± 0.08</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>$\tau_{\text{deactivation}}$ (ms)</td>
<td>0.7 ± 0.05</td>
<td>0.8 ± 0.09</td>
<td>0.5 ± 0.06</td>
</tr>
</tbody>
</table>

Although peak currents of GluA1(Q)flip are only marginally reduced in HEK293 cells upon co-expression with γ5, steady-state currents are massively decreased, further supporting the findings gathered from the oocyte expression system. Additionally, in the presence of TCM, or upon insertion of the LY mutation, the inhibitory effect of γ5 is abolished, resembling also in this aspect the oocyte data. Paradoxically, the data do not directly support the idea that γ5 alters desensitization and deactivation kinetics, which were only slightly faster than for the TARP-less receptor.
3.2.2 GluA1(R)flip

Application of glutamate to cells expressing GluA1(R)flip prompted a peak current of ~11.9 pA, which quickly declined to a steady-state current of ~0.1 pA (Figure 3.100). When glutamate was applied to cells co-expressing the receptor plus γ5, the resulting peak current of ~70 pA was substantially larger than the one elicited by the receptor, when expressed alone. Likewise, the ensuing steady-state current was also considerably higher (~0.6 pA), which is consistent with what had already been observed in oocytes using TEVC (see section 3.1.5.1.1), and therefore confirms our previous findings on the editing-specific regulation of AMPARs by γ5.

The comparison of cells expressing GluA1(R)flip with and without γ5, indicates that there are virtually no alterations in ion channel kinetics of GluA1(R)flip when co-expressed with the TARP (Figure 3.100).
Results

Figure 3.100 γ5 modulation of GluA1(R)flip in HEK293 cells.

Peak currents (A) and steady-state currents (B) were induced by long application (500 ms) of 10 mM glutamate. C: steady-state to peak current ratios. Long application of glutamate (500 ms) was employed to determine desensitization half-life values (τ) (D) while short application pulses (1 ms) were employed to determine deactivation half-life values (τ) (E). Error bars, indicate SEM. The number at the bottom of each bar refers to the number of cells recorded for each combination. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001). F: Average current traces of GluA1(R)flip in the presence and absence of γ5, elicited by 10 µM of glutamate (n ≥15).

Our previous results in oocytes had shown that glutamate-induced currents of GluA1(R)flip were significantly potentiated by γ5. Furthermore, when applied with TCM, glutamate-induced currents of oocytes expressing the TARP and the receptor were potentiated even further (see section 3.1.5.3.1). These results closely resemble the data obtained from HEK293 cells. Also in these cells, the strong potentiation of the receptors steady-state currents by γ5 shown in Figure 3.101 is yet even more pronouncedly potentiated in the presence of TCM.
3.2 Patch clamp analysis

Additionally, our results show that deactivation half-life values (τ) for GluA1 are again much higher (~14.4 ms vs. ~3.2 ms) when TCM is applied with glutamate, as seen for the Q variant, although γ5 does not seem to have any impact on these values.

Figure 3.101 γ5 modulation of GluA1(R)flip, in the presence of TCM.
Steady-state currents (A) were induced by long application (500 ms) of 10 mM glutamate while short application pulses (1 ms) were employed to determine deactivation half-life values (τ) (B). Error bars indicate SEM. The number at the bottom of each bar refers to the number of cells recorded for each combination. An unpaired t-test was applied to determine significance (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001). C: Average current traces of GluA1(R)flip in the presence and absence of γ5, elicited by 10 µM of glutamate + 500 µM TCM (n ≥15).

When measured in oocytes, GluA1(R)flip-L479Y glutamate-induced currents were enhanced by γ5 (see section 3.1.5.1.1). Also in HEK293 cells, co-expression of the mutant with the TARP elicited larger steady-state currents, further resembling the results obtained when desensitization had been abolished by TCM.
Results

Additionally, half-life values ($\tau$) for the mutant were much higher (~24 ms) than those of wild type GluA1 (~3 ms). Nonetheless, $\gamma_5$ did not significantly impact these values (Figure 3.102).

In Table 3.31, $\gamma_5$ potentiation of glutamate-induced peak and steady-state currents and kinetic values are shown in detail.

![Figure 3.102 γ5 modulation of GluA1(R)flip-L479Y, in HEK293 cells.](image)

Steady-state currents (A) were induced by long application (500 ms) of 10 mM glutamate while short application pulses (1 ms) were employed to determine deactivation half-life values ($\tau$) (B). Error bars indicate SEM. The number at the bottom of each bar refers to the number of cells recorded for each combination. An unpaired t-test was applied to determine significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$). C: Average current traces of GluA1(R)flip-L479Y in the presence and absence of $\gamma_5$, elicited by 10 $\mu$M of glutamate ($n \geq 15$).
### Table 3.31 Modulation of GluA1(R)flip by γ5, in HEK293 cells.
Potentiation values (± SEM) of glutamate-induced currents in the presence or absence of TCM, potentiation values (± SEM) of glutamate-induced currents of GluA1(R)flip-L479Y, and respective potentiation of desensitization and deactivation half-life values (τ) (± SEM) by γ5.

<table>
<thead>
<tr>
<th></th>
<th>GluA1(R)flip (TCM)</th>
<th>GluA1(R)flip-L479Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I\textsubscript{Glu} (pA)</td>
<td>5.9 ± 1.73</td>
<td>_</td>
</tr>
<tr>
<td>Steady-state I\textsubscript{Glu} (pA)</td>
<td>5.2 ± 1.30</td>
<td>16.7 ± 0.26</td>
</tr>
<tr>
<td>I\textsubscript{ss}/I\textsubscript{peak} (%)</td>
<td>1.0 ± 0.13</td>
<td>_</td>
</tr>
<tr>
<td>τ\textsubscript{desensitization} (ms)</td>
<td>0.9 ± 0.05</td>
<td>_</td>
</tr>
<tr>
<td>τ\textsubscript{deactivation} (ms)</td>
<td>1.0 ± 0.05</td>
<td>0.9 ± 0.10</td>
</tr>
</tbody>
</table>

In HEK293 cells, γ5 potentiates current amplitudes of GluA1(R)flip in marked contrast to its modulatory effect on GluA1(Q)flip. The results are in agreement with the data obtained from oocytes. Furthermore, for GluA1(R)flip, both peak and steady-state currents were potentiated by γ5 to similar extents, which unarguably distinguishes the R variant in one more aspect from the Q variant. Addition of TCM amplifies the receptors response to the TARP, and the non-desensitizing mutant responds to γ5 more pronouncedly, as observed in oocytes. Remarkably, also for the R variant, desensitization and deactivation kinetics are not altered by γ5.
3.3 Protein biochemistry

To investigate whether γ5 editing-specific behavior is linked to an increase in protein expression and trafficking, we performed Western blotting to quantify membrane expression of all twenty flip-type GluA1 mutants, in oocytes. An antibody against the extracellular N-terminus of GluA1 was used to probe the nitrocellulose membrane and an anti-pan-cadherine antibody was used with every experiment, for both quality control and quantification purposes. Between 20 to 50 oocytes were used per sample. The final sample volume was adjusted according to the number of oocytes used in each probe. Results are shown in Figures 3.103 to 3.109.

The Western blot analysis indicates that there is no substantial variation in GluA1 membrane protein levels of oocytes expressing any of the twenty mutants when co-expressed with γ5 besides the typical variation related with the extensive sample preparation procedure. suggesting that the TARP effect on AMPARs is purely a consequence of channel properties modulation and not merely a function of altered protein levels.

![Western blot analysis](image)

Figure 3.103 Western blot analysis of membrane protein fractions of GluA1(Q)flip, GluA1(N)flip, and GluA1(P)flip ± γ5, in *X. laevis* oocytes.

Uninjected oocytes were used as a negative control. A: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). B: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.
Figure 3.104 Western blot analysis of membrane protein fractions of GluA1(A)flip, GluA1(C)flip, and GluA1(R)flip ± γ5, in X. laevis oocytes.
Uninjected oocytes were used as a negative control. A: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). B: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.

Figure 3.105 Western blot analysis of membrane protein fractions of GluA1(S)flip, GluA1(T)flip, and GluA1(V)flip ± γ5, in X. laevis oocytes.
Uninjected oocytes were used as a negative control. A: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). B: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.
Results

Figure 3.106 Western blot analysis of membrane protein fractions of GluA1(E)flip, GluA1(F)flip, and GluA1(G)flip ± γ5, in *X. laevis* oocytes.

Uninjected oocytes were used as a negative control. **A**: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). **B**: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.

Figure 3.107 Western blot analysis of membrane protein fractions of GluA1(K)flip, GluA1(L)flip, and GluA1(M)flip ± γ5, in *X. laevis* oocytes.

Uninjected oocytes were used as a negative control. **A**: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). **B**: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.
Figure 3.108 Western blot analysis of membrane protein fractions of GluA1(D)flip, GluA1(H)flip, and GluA1(I)flip ± γ5, in X. laevis oocytes.

Uninjected oocytes were used as a negative control. A: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). B: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.

Figure 3.109 Western blot analysis of membrane protein fractions of GluA1(W)flip, and GluA1(Y)flip ± γ5, in X. laevis oocytes.

Uninjected oocytes were used as a negative control. A: Western blot. The membrane was first probed for GluA1 (top) and subsequently for cadherins (bottom). B: Relative protein expression levels, normalized to cadherin expression levels and expressed relative to GluA1(Q)flip.
Results

Western Blot analyses suggest that γS does not significantly alter membrane expression of AMPARs, independently of the amino acid at the editing site. The electrophysiological changes prompted by the TARP and described in this study are therefore not merely a simple consequence of increased membrane protein levels but rather represent true modulation of the biophysical properties of the receptors investigated.
3.4 Permeability to calcium

Permeability to calcium ions is a property that is closely related to pore editing in AMPARs. The change from a glutamine to an arginine at the receptors’ Q/R editing sites render the receptors nearly calcium impermeable. Additionally, our data suggests that γ5 modulation of AMPARs strongly depends on the amino acid at this specific site, ultimately linking the TARP to ion channel permeability. Hence, it was of utmost importance to investigate the influence of γ5 on the regulation of calcium permeability of the various Q/R editing-site mutants.

Calcium permeability experiments were performed in oocytes, using an external buffer solution that consisted of calcium as the sole permeable cation. Furthermore, internal cellular calcium was removed by treatment with a calcium-quenching agent. Under those conditions, a shift in the recorded reversal potentials to less negative values is an indication of increased calcium permeability.

Every experiment was performed in parallel with two individual buffers that differed in calcium concentration (4 mM and 8 mM) but were otherwise identical, to assure consistency.

Some mutants produce inherently low agonist-induced currents when recorded in NFR. Naturally, these currents are even lower in the absence of monovalent ions such as sodium and potassium. Therefore, calcium permeability analyses were not in all cases possible, even in the presence of TCM. For all mutant with sufficient large current responses, however, reversal potentials were recorded and permeability values were estimated.

3.4.1 GluA1(Q)flip

The I/V curves recorded upon application of glutamate to cells expressing GluA1(Q)flip and γ5 clearly returned less negative reversal potentials when compared to the TARP-less receptor (Figure 3.110). It is therefore unsurprising that the calculated calcium permeability is considerably higher when the TARP is present. A similar outcome was obtained in the two buffers with different Ca\(^{2+}\) compositions, reassuring...
the reliability of the results. The data emphasizes the idea that the effect that \( \gamma_5 \) has on the receptors’ electrophysiological properties is in part due to a TARP-mediated manipulation of the cation permeabilities of the ion channels.

The measured reversal potentials and the respective calculated \( \text{Ca}^{2+} \) permeability values are shown in detail in Table 3.32.

![Normalized I/V relations recorded by application of 300 \( \mu \text{M} \) glutamate in 4 mM \( \text{Ca}^{2+} \)-Ringer in the absence (magenta) or in co-expression (blue) with \( \gamma_5 \). Error bars indicate SEM (8 \( \leq n \leq 9 \)].](image)

**Figure 3.110** Calcium permeability assay of GluA1(Q)flip ± \( \gamma_5 \), in *X. laevis* oocytes. A: Normalized I/V relations recorded by application of 300 \( \mu \text{M} \) glutamate in 4 mM \( \text{Ca}^{2+} \)-Ringer in the absence (magenta) or in co-expression (blue) with \( \gamma_5 \). Error bars indicate SEM (8 \( \leq n \leq 9 \)]. B: Calculated calcium permeability values assessed in 4 mM \( \text{Ca}^{2+} \)-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 \( \mu \text{M} \) glutamate in 8 mM \( \text{Ca}^{2+} \)-Ringer in the absence (magenta) or in co-expression (blue) with \( \gamma_5 \). Error bars indicate SEM (8 \( \leq n \leq 9 \)]. D: Calculated calcium permeability values assessed in 8 mM \( \text{Ca}^{2+} \)-Ringer. Error bars indicate SEM.
3.4 Permeability to calcium

Table 3.32 Reversal potentials and calcium permeability of GluA1(Q)flip ± γ5.

Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca\(^{2+}\) and 8 mM Ca\(^{2+}\) Ringer solutions. Colored numbers indicate statistical significance (8 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th>E(_{\text{rev}}) (mV)</th>
<th>GluA1(Q)flip</th>
<th>GluA1(Q)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>-33.2 ± 0.79</td>
<td>-21.1 ± 0.30</td>
<td>-</td>
</tr>
<tr>
<td>8 mM Ca(^{2+})</td>
<td>-19.6 ± 0.80</td>
<td>-7.5 ± 0.34</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P(<em>{\text{Ca}^{2+}})/P(</em>{\text{mono}})</th>
<th>4 mM Ca(^{2+})</th>
<th>8 mM Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 ± 0.10</td>
<td>4.6 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>2.8 ± 0.12</td>
<td>5.4 ± 0.11</td>
</tr>
</tbody>
</table>

3.4.2 GluA1(Q)flip/GluA2(R)flip

The characteristic low agonist-induced currents of AMPAR edited variants make it impossible to record I/V responses on homomeric combinations, especially in a setting where the only permeable cation available is calcium, for which they are known to have nearly no permeability. Hence, heteromeric combinations with GluA1(Q)flip were used in order to access the change in Ca\(^{2+}\) permeability induced by γ5 in receptors that have such an inherently low permeability to the cation. To avoid unwanted homomeric formation of the unedited receptor, the R variant was injected in large excess (1:5 molar ratio Q:R cRNAs). The linear shape of the I/V curves demonstrates the success of this approach.

The very low reversal potentials, below the -80 mV range, already suggest exceptionally low Ca\(^{2+}\) permeability rates for these receptors, as further confirmed by the actual permeability values (see Table 3.33). As observed for the Q variant, there was a discernible increase in the registered reversal potentials and the consequent calcium permeability in co-expression with the TARP (Figure 3.111). Similar results were obtained with both Ringer solutions.
Figure 3.111 Calcium permeability assay of GluA1(Q)flip/GluA2(R)flip ± γ5, in *X. laevis* oocytes.

**A:** Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca^{2+}-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). **B:** Calculated calcium permeability values assessed in 4 mM Ca^{2+}-Ringer. Error bars indicate SEM. **C:** Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca^{2+}-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). **D:** Calculated calcium permeability values assessed in 8 mM Ca^{2+}-Ringer. Error bars indicate SEM.
Table 3.33 Reversal potentials and calcium permeability of GluA1(Q)flip/GluA2(R)flip ± γ5.

Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 μM glutamate in 4 mM Ca²⁺ and 8 mM Ca²⁺ Ringer solutions. Colored numbers indicate statistical significance (8 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th></th>
<th>GluA1(Q)flip/ GluA2(R)flip</th>
<th>GluA1(Q)flip/ GluA2(R)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_{rev}(mV)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mM Ca²⁺</td>
<td>-96.6 ± 0.34</td>
<td>-89.6 ± 0.44</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca²⁺</td>
<td>-91.3 ± 0.71</td>
<td>-82.8 ± 1.31</td>
<td>_</td>
</tr>
<tr>
<td>P_{Ca²⁺}/P_{mono}</td>
<td>0.19 ± 0.003</td>
<td>0.26 ± 0.005</td>
<td>36.8</td>
</tr>
<tr>
<td>8 mM Ca²⁺</td>
<td>0.14 ± 0.004</td>
<td>0.19 ± 0.010</td>
<td>35.7</td>
</tr>
</tbody>
</table>

3.4.3 GluA1(Q)flip/GluA1(R)flip

To avoid unwanted formation of the homomeric unedited receptor, the R variant was injected in large excess (1:5 molar ratio Q:R cRNAs).

This homomeric combination yielded very low reversal potentials and subsequent Ca²⁺ permeability values, which were comparable to the ones observed for GluA1(Q)flip/GluA2(R)flip (Figure 3.112). Additionally, γ5 also here induced an increase in channel permeability, as observed for the other receptor combinations investigated so far. Although with different potentiation factors, the results obtained using both Ringer solutions showed the same tendency.

The measured reversal potentials and the respective calculated Ca²⁺ permeability values are shown in detail on Table 3.34.
Figure 3.112 Calcium permeability assay of GluA1(Q)flip/GluA1(R)flip ± γ5, in *X. laevis* oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (7 ≤ n ≤ 9). B: Calculated calcium permeability values assessed in 4 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (7 ≤ n ≤ 9). D: Calculated calcium permeability values assessed in 8 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM.
### Table 3.34 Reversal potentials and calcium permeability of GluA1(Q)flip/GluA1(R)flip $\pm \gamma_5$

Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca$^{2+}$ and 8 mM Ca$^{2+}$ Ringer solutions. Colored numbers indicate statistical significance ($7 \leq n \leq 9$).

<table>
<thead>
<tr>
<th>$\gamma_5$</th>
<th>GluA1(Q)flip/</th>
<th>GluA1(Q)flip/</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GluA1(R)flip</td>
<td>GluA1(R)flip $\pm \gamma_5$</td>
<td></td>
</tr>
<tr>
<td>4 mM Ca$^{2+}$</td>
<td>-90.7 ± 0.83</td>
<td>-85.4 ± 0.26</td>
<td>-</td>
</tr>
<tr>
<td>8 mM Ca$^{2+}$</td>
<td>-84.9 ± 1.40</td>
<td>-77.5 ± 0.54</td>
<td>-</td>
</tr>
<tr>
<td>4 mM Ca$^{2+}$</td>
<td>0.25 ± 0.008</td>
<td>0.30 ± 0.003</td>
<td>20.0</td>
</tr>
<tr>
<td>8 mM Ca$^{2+}$</td>
<td>0.18 ± 0.010</td>
<td>0.24 ± 0.005</td>
<td>33.3</td>
</tr>
</tbody>
</table>

### 3.4.4 GluA1(H)flip

GluA1(H)flip mutants showed a linear-shaped I/V response and yielded very low reversal potentials and subsequent Ca$^{2+}$ permeability values, which were increased by $\gamma_5$ (Figure 3.113). The results obtained with both Ringer solutions were consistent with each other.

The measured reversal potentials and the respective calculated Ca$^{2+}$ permeability values are shown in detail on Table 3.34.
Figure 3.113 Calcium permeability assay of GluA1(H)flip ± γ5, in *X. laevis* oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (n = 9). B: Calculated calcium permeability values assessed in 4 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (n = 9). D: Calculated calcium permeability values assessed in 8 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM.
Table 3.35 Reversal potentials and calcium permeability of GluA1(H)flip ± γ5.
Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca^{2+} and 8 mM Ca^{2+} Ringer solutions. Colored numbers indicate statistical significance (n = 8).

<table>
<thead>
<tr>
<th>E_{rev} (mV)</th>
<th>GluA1(H)flip</th>
<th>GluA1(H)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca^{2+}</td>
<td>-82.7 ± 1.51</td>
<td>-74.1 ± 0.44</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca^{2+}</td>
<td>-76.4 ± 1.95</td>
<td>-65.8 ± 0.26</td>
<td>_</td>
</tr>
</tbody>
</table>

3.4.5 GluA1(N)flip

GluA1(N)flip mutants showed a linear-shaped I/V response and yielded relatively high reversal potentials and subsequent Ca^{2+} permeability values, which were further increased by γ5 (Figure 3.114). Although to different extents, the results obtained with the two Ringer solutions both showed a potentiating influence of the TARP.

The measured reversal potentials and the respective calculated Ca^{2+} permeability values are shown in detail on Table 3.36.
Figure 3.114 Calcium permeability assay of GluA1(N)flip ± γ5, in *X. laevis* oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca$^{2+}$-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). B: Calculated calcium permeability values assessed in 4 mM Ca$^{2+}$-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca$^{2+}$-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). D: Calculated calcium permeability values assessed in 8 mM Ca$^{2+}$-Ringer. Error bars indicate SEM.
Table 3.36 Reversal potentials and calcium permeability of GluA1(N)flip ± γ5.

Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca\(^{2+}\) and 8 mM Ca\(^{2+}\) Ringer solutions. Colored numbers indicate statistical significance (8 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th>E(_{\text{rev}}) (mV)</th>
<th>GluA1(N)flip</th>
<th>GluA1(N)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>-41.0 ± 0.29</td>
<td>-31.4 ± 0.71</td>
<td>–</td>
</tr>
<tr>
<td>8 mM Ca(^{2+})</td>
<td>-27.1 ± 0.71</td>
<td>-24.1 ± 0.48</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P(<em>{\text{Ca}^{2+}})/P(</em>{\text{mono}})</th>
<th>4 mM Ca(^{2+})</th>
<th>8 mM Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.1 ± 0.03</td>
<td>2.4 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>3.3 ± 0.12</td>
<td>2.8 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>57.1</td>
<td>16.7</td>
</tr>
</tbody>
</table>

3.4.6 GluA1(S)flip

GluA1(S)flip mutants showed a linear-shaped I/V response. The corresponding potentials and subsequent Ca\(^{2+}\) permeability values were consistently potentiated by γ5 in both Ringer solutions Figure 3.115.

The measured reversal potentials and the respective calculated Ca\(^{2+}\) permeability values are shown in detail on Table 3.37.
Results

Figure 3.115 Calcium permeability assay of GluA1(S)flip ± γ5, in X. laevis oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca$^{2+}$-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (n = 9).

B: Calculated calcium permeability values assessed in 4 mM Ca$^{2+}$-Ringer. Error bars indicate SEM.

C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca$^{2+}$-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (n = 9).

D: Calculated calcium permeability values assessed in 8 mM Ca$^{2+}$-Ringer. Error bars indicate SEM.
Table 3.37 Reversal potentials and calcium permeability of GluA1(S)flip ± γ5.
Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca\textsuperscript{2+} and 8 mM Ca\textsuperscript{2+} Ringer solutions. Colored numbers indicate statistical significance (n = 9).

<table>
<thead>
<tr>
<th></th>
<th>GluA1(S)flip</th>
<th>GluA1(S)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca\textsuperscript{2+}</td>
<td>-48.1 ± 2.21</td>
<td>-32.1 ± 0.25</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca\textsuperscript{2+}</td>
<td>-36.8 ± 1.12</td>
<td>-23.7 ± 0.80</td>
<td>_</td>
</tr>
</tbody>
</table>

3.4.7 GluA1(C)flip

GluA1(C)flip mutants showed an inwardly rectifying I/V response. Rectification was strongly attenuated by γ5 in both Ca\textsuperscript{2+}-Ringer solutions. The high reversal potentials and subsequent Ca\textsuperscript{2+} permeability values were substantially increased by the TARP in both Ringer solutions, although the calculated potentiation was much higher in the 8 mM Ca\textsuperscript{2+}-Ringer (Figure 3.116). Although there is some discrepancy between the values obtained in both Ringer solutions, they both suggest the same role of the TARP in upregulating the receptor’s Ca\textsuperscript{2+} permeability.

The measured reversal potentials and the respective calculated Ca\textsuperscript{2+} permeability values are shown in detail on Table 3.38.
Figure 3.116 Calcium permeability assay of GluA1(C)flip ± γ5, in *X. laevis* oocytes.

A: Normalized I/V relations recorded by application of 300 μM glutamate in 4 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). B: Calculated calcium permeability values assessed in 4 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 μM glutamate in 8 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). D: Calculated calcium permeability values assessed in 8 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM.
3.4 Permeability to calcium

Table 3.38 Reversal potentials and calcium permeability of GluA1(C)flip ± γ5.
Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca\(^{2+}\) and 8 mM Ca\(^{2+}\) Ringer solutions. Colored numbers indicate statistical significance (8 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th>E(_{\text{rev}}) (mV)</th>
<th>GluA1(C)flip</th>
<th>GluA1(C)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>-32.6 ± 0.90</td>
<td>-23.1 ± 0.43</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca(^{2+})</td>
<td>-28.3 ± 1.43</td>
<td>-10.9 ± 0.60</td>
<td>_</td>
</tr>
<tr>
<td>P(<em>{\text{Ca}^{2+}/\text{P}</em>{\text{mono}}})</td>
<td>4 mM Ca(^{2+})</td>
<td>3.2 ± 0.14</td>
<td>5.1 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>8 mM Ca(^{2+})</td>
<td>2.3 ± 0.17</td>
<td>5.7 ± 0.19</td>
</tr>
</tbody>
</table>

3.4.8 GluA1(G)flip

GluA1(G)flip mutants showed a linear shaped I/V response. Recordings performed in the 4 mM Ca\(^{2+}\)-Ringer showed that the measured reversal potentials and subsequent Ca\(^{2+}\) permeability values were significantly potentiated by γ5, which is in line with the results already obtained for the previously analyzed receptors. However, the recordings performed in the 8 mM Ca\(^{2+}\)-Ringer challenge these results, since they showed no significant alteration in Ca\(^{2+}\) permeability by the TARP (see Figure 3.117). Considering the results obtained for the other receptors analyzed, a part of the data seems more plausible than the other, however, reliable conclusions can only be safely drawn after repetition of the experiment.

The measured reversal potentials and the respective calculated Ca\(^{2+}\) permeability values are shown in detail on Table 3.39.
Results

Figure 3.117 Calcium permeability assay of GluA1(G)flip ± γ5, in X. laevis oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca\textsuperscript{2+}-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). B: Calculated calcium permeability values assessed in 4 mM Ca\textsuperscript{2+}-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca\textsuperscript{2+}-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). D: Calculated calcium permeability values assessed in 8 mM Ca\textsuperscript{2+}-Ringer. Error bars indicate SEM.
Table 3.39 Reversal potentials and calcium permeability of GluA1(G)flip ± γ5.

Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca²⁺ and 8 mM Ca²⁺ Ringer solutions. Colored numbers indicate statistical significance (6 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th>E_{rev} (mV)</th>
<th>GluA1(G)flip</th>
<th>GluA1(G)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca²⁺</td>
<td>-48.7 ± 0.75</td>
<td>-38.3 ± 1.08</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca²⁺</td>
<td>-42.9 ± 0.37</td>
<td>-41.4 ± 0.89</td>
<td>_</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P_{Ca²⁺/P_{mono}}</th>
<th>4 mM Ca²⁺</th>
<th>8 mM Ca²⁺</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.6 ± 0.06</td>
<td>2.7 ± 0.14</td>
<td>68.8</td>
</tr>
<tr>
<td></td>
<td>1.2 ± 0.02</td>
<td>1.3 ± 0.06</td>
<td>0.8</td>
</tr>
</tbody>
</table>

3.4.9 GluA1(L)flip

GluA1(L)flip mutants showed an inwardly rectifying I/V response. Rectification was strongly attenuated by γ5 in both Ca²⁺-Ringer solutions. Reversal potentials and subsequent Ca²⁺ permeability values were substantially increased by the TARPs in both Ringer solutions, although the calculated potentiation was much higher in the 8mM Ca²⁺-Ringer (Figure 3.118). Although there is some discrepancy between the values obtained in both Ringer solutions, they both advocate for the same role of the TARPs in up regulating the receptors Ca²⁺ permeability.

The measured reversal potentials and the respective calculated Ca²⁺ permeability values are shown in detail on Table 3.40.
Figure 3.118 Calcium permeability assay of GluA1(L)flip ± γ5, in *X. laevis* oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). B: Calculated calcium permeability values assessed in 4 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (8 ≤ n ≤ 9). D: Calculated calcium permeability values assessed in 8 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM.
Table 3.40 Reversal potentials and calcium permeability of GluA1(L)flip ± γ5.
Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca\(^{2+}\) and 8 mM Ca\(^{2+}\) Ringer solutions. Colored numbers indicate statistical significance (8 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th>E(_{\text{rev}}) (mV)</th>
<th>GluA1(L)flip</th>
<th>GluA1(L)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>-45.4 ± 0.83</td>
<td>-39.6 ± 0.56</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca(^{2+})</td>
<td>-38.1 ± 2.17</td>
<td>-27.3 ± 0.66</td>
<td>_</td>
</tr>
</tbody>
</table>

\(P_{\text{Ca}^{2+}}/P_{\text{mono}}\) values:

<table>
<thead>
<tr>
<th></th>
<th>GluA1(L)flip</th>
<th>GluA1(L)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>1.7 ± 0.07</td>
<td>2.2 ± 0.06</td>
<td>29.4</td>
</tr>
<tr>
<td>8 mM Ca(^{2+})</td>
<td>1.4 ± 0.13</td>
<td>2.3 ± 0.08</td>
<td>64.3</td>
</tr>
</tbody>
</table>

3.4.10 GluA1(F)flip

GluA1(F)flip mutants showed an elusively rectifying I/V response. Reversal potentials and subsequent Ca\(^{2+}\) permeability values were consistently potentiated by γ5 in both Ringer solutions (Figure 3.119).

The measured reversal potentials and the respective calculated Ca\(^{2+}\) permeability values are shown in detail on Table 3.41.
Results

Figure 3.119 Calcium permeability assay of GluA1(F)flip ± γ5, in X. laevis oocytes.

A: Normalized I/V relations recorded by application of 300 µM glutamate in 4 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (6 ≤ n ≤ 9). B: Calculated calcium permeability values assessed in 4 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM. C: Normalized I/V relations recorded by application of 300 µM glutamate in 8 mM Ca\(^{2+}\)-Ringer in the absence (magenta) or in co-expression (blue) with γ5. Error bars indicate SEM (6 ≤ n ≤ 9). D: Calculated calcium permeability values assessed in 8 mM Ca\(^{2+}\)-Ringer. Error bars indicate SEM.
3.4 Permeability to calcium

Table 3.41 Reversal potentials and calcium permeability of GluA1(F)flip ± γ5.
Reversal potential (± SEM) and corresponding estimated calcium permeability values (± SEM) were assessed by application of 300 µM glutamate in 4 mM Ca\(^{2+}\) and 8 mM Ca\(^{2+}\) Ringer solutions. Colored numbers indicate statistical significance (6 ≤ n ≤ 9).

<table>
<thead>
<tr>
<th>(E_{\text{rev}}) (mV)</th>
<th>GluA1(F)flip</th>
<th>GluA1(F)flip + γ5</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mM Ca(^{2+})</td>
<td>-43.8 ± 0.50</td>
<td>-34.3 ± 0.41</td>
<td>_</td>
</tr>
<tr>
<td>8 mM Ca(^{2+})</td>
<td>-36.2 ± 1.01</td>
<td>-26.2 ± 0.36</td>
<td>_</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(P_{Ca^{2+}}/P_{mono})</th>
<th>4 mM Ca(^{2+})</th>
<th>8 mM Ca(^{2+})</th>
<th>Potentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 ± 0.05</td>
<td>1.7 ± 0.08</td>
<td>60.0</td>
</tr>
</tbody>
</table>

3.4.11 Overview

Through the analysis of the different receptor combinations it became evident that permeability of the ion channel to calcium is one of the aspects through which γ5 performs its fine-regulation of AMPARs. Albeit to different extents, the TARP γ5 appears to increase permeability to calcium, of all the receptor variants and mutants alike, independently of the amino acid at the Q/R editing site.
4 Discussion

The discovery of transmembrane AMPA receptor regulatory proteins (TARPs) was a major breakthrough in AMPAR-related research and has irreversibly changed the field ever since. A lot of progress has been made since this discovery, especially regarding the function of stargazin and its type I-TARP relatives, which have broad and well-documented control over every aspect of AMPAR performance. Due to the enormous influence of these regulatory proteins on the receptors, the more modest type II-TARPs have fallen by the wayside. However, the data presented in this thesis are proof that although these truly remarkable proteins do not affect AMPAR agonist-induced response as efficiently as type I-TARPs, they are certainly unrivaled in terms of versatility and plasticity.

4.1 The non-TARP status of γ6

Although γ6 was found to be expressed very scarcely throughout both rat and human brains, it has also been found to be expressed in several other organs, and most prominently in skeletal and cardiac muscles (Burgess et al 2001, Chu et al 2001, Fukaya et al 2005). Furthermore, γ6 is more closely phylogenetically related to γ1, a voltage-dependent calcium channel subunit that shows no TARP-like activity, than to the other six remaining γ subunits.

The non-TARP γ6, as it is currently classified, has been electrophysiologically characterized in detail in the course of this thesis (see sections 3.1.1 to 3.1.3). It has been co-expressed not only with all existing homomeric AMPA receptors but also with different heteromeric subunit combinations, and additionally with a few variants of KA receptors.

Confirming its non-TARP classification, γ6 did not change the current amplitudes of any of the receptors analyzed, with the single exception of GluA1(Q)flop, whose agonist-induced responses were only very slightly decreased. Given the very small inhibitory effect on this specific receptor, and the lack of effects on any other
receptor, there is no basis to assign \( \gamma_6 \) any TARP activity. The results are consistent with an earlier study (Kato et al 2007), which had already found no signs of a TARP effect of \( \gamma_6 \) on GluA1(Q)flip in HEK293 cells.

Furthermore, \( \gamma_6 \) did not induce any change to the shape of the I/V curves of any of the receptor combinations analyzed, which were virtually indistinguishable from the I/V responses elicited by the receptors alone.

Given our results, the high homology of \( \gamma_6 \) to \( \gamma_1 \), and the expression pattern of \( \gamma_6 \), it is not unreasonable to assume that \( \gamma_6 \) is functionally closer to \( \gamma_1 \) than to the other \( \gamma \) subunits that have shown inherent TARP function.

Nevertheless, even though \( \gamma_6 \) has shown no major impact on the electrophysiological properties of AMPARs, the possibility of interaction with the receptors cannot be automatically excluded. In fact, using BiFC, the bimolecular fluorescence complementation approach (see section 3.1.4), we made the surprising observation that the fluorescence level observed with NGFP-tagged \( \gamma_6 \) in co-expression with the \( \text{Ca}^{2+} \) permeable GluA1(Q)flip-C-GFP or GluA1(Q)flip-C-GFP/GluA2(Q)flip, was comparable to that of NGFP-tagged type II-TARPs. This suggests that \( \gamma_6 \) has a similar binding affinity to these receptor combinations as \( \gamma \) subunits classified as type II-TARPs. Furthermore, fluorescence induced by co-expression of \( \gamma_6 \)-C-NGFP with \( \text{Ca}^{2+} \) impermeable receptors, such as GluA2(R)flip-C-CGFP and GluA1(Q)flip-C-CGFP/GluA2(R)flip was at the same level as the one induced by co-expression of the receptor with \( \gamma_2 \)-C-NGFP and surpassed both \( \gamma_5 \)-C-NGFP and \( \gamma_7 \)-C-NGFP-induced fluorescence.

Taken together, these results suggest that, despite its apparent lack of functional modulation, not only is \( \gamma_6 \) able to interact with AMPARs, but it also does so more efficiently with \( \text{Ca}^{2+} \) impermeable receptors, although the reason behind this preferential interaction is unknown.

### 4.2 The type I-TARP-like behavior of \( \gamma_7 \)

It was not until recently that \( \gamma_7 \) has been described as a member of the TARP family (Kato et al 2008, Kato et al 2007). This TARP had earlier been shown to nearly
abolish expression of certain calcium channels (Moss et al 2002). Furthermore, the observation that type II-TARPs lack a typical PDZ-binding domain and are apparently not involved in AMPA receptor trafficking, have largely contributed to this premature declassification.

However, γ7 is abundantly expressed in many regions of human and rodent brains, most prominently in the cerebellum and olfactory bulb, while it is absent or only scarcely expressed in other organs (see Figure 4.1). Moreover, it has been shown to selectively bind to AMPARs and even to PSD-95 (Burgess et al 2001, Chu et al 2001, Fukaya et al 2005, Kato et al 2007), which clearly suggests a role as an AMPA receptor modulator. PSD-95 has been reported to cluster AMPARs but not NMDA receptors at the synapse, although AMPARs cannot bind to PSD-95 on their own (El-Husseini et al 2000, Stein et al 2003). It has also been shown that γ7 and γ2 co-immunoprecipitate, suggesting that both TARPs might act cooperatively (Kato et al 2007) and that the TARP is able to further increase AMPAR single channel conductance on its own (Studniarczyk et al 2013).

A previous study in HEK293 cells has shown that γ7 potentiates steady-state currents of the flip variants of GluA1(Q), GluA2(R), GluA2(Q), and more modestly of GluA3(Q), while it does not significantly alter those of GluA4(Q) (Kato et al 2008). Our oocyte data partially supports such findings. However, our investigation of γ7 modulation of the different AMPARs found the same effects independently of the AMPA receptor analyzed. The TARP was able to enhance agonist-induced responses of all the receptors alike, with the only exception being glutamate-induced currents of the flop variant of GluA4(Q) (see section 3.1.1). Additionally, our data demonstrates that the γ7 TARP effect is considerably higher for the edited variants in every case.

Furthermore, γ7 significantly potentiated glutamate-induced responses of all the heteromeric combinations analyzed, with the sole exception of the flop splice variant of the non-physiological combination GluA1(R)/GluA2(Q) (see section 3.1.2). However, also for these receptors, kainate-induced currents were amplified by the TARP. The extent of the potentiation of heteromeric Ca²⁺ impermeable receptors induced by γ7 was in every case more closely related to the potentiation seen for the homomeric Q variants, suggesting a surprising regulatory preference by the TARP γ7 for heteromeric subunit combinations containing unedited variants.
The ability to potentiate all receptor types indiscriminately, albeit with different efficiencies, is not the only characteristic of γ7 that resembles type I-TARPs. Also, the measured ratios of kainate- to glutamate induced currents underwent changes similar to those induced by γ2. These ratios are altered by type I-TARPs as a direct consequence of reduced receptor desensitization and increased agonist efficacy (Kott et al. 2007, Levchenko-Lambert et al. 2011, Turetsky et al. 2005). It has been proposed that the desensitization rate and the kainate efficacy are not altered as strongly by γ7 as they are by γ2. Yet, glutamate affinity, which is increased by γ2, is not changed by γ7 (Kato et al. 2010). In consequence, it is not surprising that both TARPs induced comparable changes to the kainate- to glutamate-induced current ratios (see sections 3.1.1 and 3.1.2).

Additionally, γ7 has been reported to selectively enhance membrane expression of Ca\(^{2+}\) permeable AMPARs, in cooperation with stargazin (Studniarczyk et al. 2013, Yamazaki et al. 2010).

Modulation of the analyzed heteromeric flip-variant receptors by γ7 was virtually indistinguishable from modulation of their flop-variant counterparts (see sections 3.1.1 and 3.12). This suggests that alternative splicing is not particularly determinant for modulation. The observed differences between splice variants do not seem to be consistent amongst the different receptors, but are rather minor variations that may have occurred due to the use of different oocyte batches.

Lastly, γ7 induced the same relief of rectification of Ca\(^{2+}\) permeable receptor I/V responses as type I-TARPs; but also in this aspect, the effects of the type I-TARPs were substantially stronger (see sections 3.1.1 and 3.1.2).
4.2 The type I-TARP-like behavior of γ7

For clarity, a reference section has been added on the right side of the picture to help to readily identify the brain regions expressing γ7. Pictures were downloaded from the Allen Brain Atlas website (http://www.brain-map.org).

4.3 The editing-specific behavior of γ5

The type II-TARP γ5, similar to γ7, was not classified as such until recently (Kato et al 2008). The classification as a type II-TARP relies on the fact that γ5 does not possess a typical PDZ-binding domain characteristic of type I-TARPs and is not involved in trafficking. The atypical PDZ-binding domain further implies that type II-TARPs have different interaction partners, which likely accounts for some of the functional differences observed between the members of the two TARP subfamilies and affects receptor clustering and trafficking.

The TARP γ5 was found to be expressed in human and rodent brains, more specifically in the cerebellum and olfactory bulb (see Figure 4.2), and also to a lesser extent in some other organs, such as lung, kidney, and testis (Burgess et al 2001, Chu et al 2005, Fukaya et al 2005, Kato et al 2007).

It has been suggested that γ5 modulation of AMPARs is strictly dependent on editing, exclusively modulating Ca\(^{2+}\) impermeable receptors (Kato et al 2008). Nonetheless, it has also been proposed by a different group that γ5 specifically modulates receptor combinations composed by long-form AMPAR subunits which are mainly Ca\(^{2+}\) permeable (Soto et al 2009), partially contradicting the abovementioned report.
Our data strongly supports the conclusion by Kato et al. that state that γ5 modulation is dependent on editing. However, we also found that γ5 does not only regulate Ca\(^{2+}\) impermeable receptors, as proposed by the authors, but all the analyzed AMPARs, albeit in very distinctive ways (see sections 3.1.1 and 3.1.2). In particular, our data shows that γ5 strongly potentiates agonist-induced currents of all the analyzed homomeric Ca\(^{2+}\) impermeable receptors, while it drastically reduces current amplitudes of homomeric Ca\(^{2+}\) permeable receptors (see section 3.1.1). However, the inhibitory effect of the TARP is not restricted to Ca\(^{2+}\) permeable receptors. In fact, heteromers composed of both edited and unedited subunits, which are therefore Ca\(^{2+}\) impermeable, showed a similar response as their homomeric Ca\(^{2+}\) permeable counterparts (see section 3.1.2). The data suggests that the unedited (Q) forms of the receptors are determinants for γ5 modulation in heteromeric Ca\(^{2+}\) impermeable receptor combinations.

Our experiments on HEK293 cells further support the findings made in the oocyte expression system (see section 3.2). We have observed that co-expression of γ5 with GluA1(Q)flip resulted in a severe decrease of the elicited steady-state currents despite a much smaller decline of the peak current. In contrast, co-expression of the TARP with GluA1(R)flip resulted in a considerable potentiation of both peak and steady state currents. Both results are in perfect agreement with the data obtained from oocytes.

Figure 4.2 Expression pattern of γ5 in the mouse brain.
For clarity, a reference section has been added on the right side of the picture to help to readily identify the brain regions expressing γ5. Pictures were downloaded from the Allen Brain Atlas website (http://www.brain-map.org).
4.3. Differential γ5 modulation of GluA1 editing mutants

The finding that γ5 regulates identical receptors so specifically that it distinguishes receptors that vary only by a single amino acid at the Q/R editing site (see sections 3.1.1 and 3.1.2), is both surprising and exciting. Therefore, we set out to investigate this matter further by introducing different point mutations at this specific site of GluA1 and co-expressing the mutants with γ5 in oocytes. The data shows that γ5 modulation of AMPARs is indeed highly dependent on the editing site amino acid. The response of the different mutants to the TARP is varied and very specific (see section 3.1.5).

Since ionotropic glutamate receptors are cation-selective ion channels, the positive nature of arginine, lysine, and histidine is responsible for the receptors low ion permeability, particularly to calcium. Accordingly, in vivo, editing of GluA2 receptors from a glutamine to an arginine, at the ion pore Q/R editing site, is accountable for the characteristic calcium impermeable nature of AMPA receptor combinations that contain this subunit. Furthermore, editing dictates many other channel biophysical properties, such as receptor kinetics, single-channel conductance and block by endogenous polyamines, leading to rectification of the I/V curve (Isaac et al 2007). Hence, electrophysiological recordings of these positively charged mutants are associated with very low current amplitudes.

The three positively charged mutants showed different responses to agonists (see section 3.1.5.1), with GluA1(H) mutants having considerably higher current amplitudes than the other two mutants, probably due to the combination of a more compact side chain and a comparatively weaker positive charge.

Co-expression of these mutants with γ5 resulted in a significant potentiation of their agonist-induced responses, with the exception of GluA1(K)flop, the currents of which were below the limit of detection permitted by the system. However, the TARP succeeded in rescuing its kainate-induced currents to small but detectable levels.

The low agonist-induced currents observed for both glutamate and aspartate mutants (see section 3.1.5.2) are likely associated with the negative charge at their side chain, which could potentially retain positively charged ions at the pore region, which would ultimately block the ion channel.
Both flop variants of GluA1(D) did not show any measurable currents in response to either glutamate or kainate. However, co-expression with γ5 rescued kainate-induced currents of GluA1(D)flip. Furthermore, γ5 dramatically increased current responses of both GluA1(E) splice variants.

In marked contrast to the results obtained for the wild type GluA1(Q), co-expression of γ5 with the remaining polar, uncharged mutants resulted in the potentiation of both agonist-induced responses, as observed for the quite unrelated R mutant (see section 3.1.5.3). These results were highly unexpected, due to the high similarity between glutamine and the other polar uncharged amino acids. This is especially true for asparagine, whose side chain differs from glutamine by a single methylene bridge. Yet, despite the high structural resemblance between the two amino acids, the response of GluA1(Q) and GluA1(N) to the TARP was extremely different.

Also, hydrophobic mutants responded differently to γ5 (see section 3.1.5.4). While the TARP reduced agonist-induced currents of GluA1(G), GluA1(L), and GluA1(M), resembling the wild type Q variant, it amplified the agonist-mediated responses of all the remaining hydrophobic mutants, similar to GluA1(R). Again, the high similarity between glycine and alanine, or between leucine and isoleucine or valine, made it hard to predict this outcome based on general hydrophobicity.

Finally, kainate- and glutamate-induced currents of the aromatic mutants were positively modulated, with the exception of GluA1(W), which did not yield any measurable currents in response to any of the agonists (see section 3.1.5.5).

Taken together, these results show how versatile and specific γ5 is in the regulation of AMPARs. The TARP efficiently discriminates between the receptors merely via the amino acid at the Q/R editing site, producing very dissimilar outcomes that manifest themselves in a variety of biophysical properties as well as agonist potency. The molecular reasons behind this intimate relationship between the TARP and the Q/R editing site are difficult to understand. The differences between amino acids that cause such big variations in the response to the TARP are often only minimal, and make it hard to find a logical link that is able to consistently explain the data.
4.3.2 The role of γ5 in desensitization

Studies performed in γ5-expressing hippocampal CA2 neurons show that, in these cells, AMPARs desensitize faster and more completely than in CA3 neurons that do not express the TARP (Kato et al 2008). These results are supported by the experiments of the same authors, which show that transfection of rat cerebral cortices with γ5 produces the same outcome. They further propose that all the observed effects of γ5 require editing of GluA2. Our data on GluA1 mutants partly supports the findings of these authors.

The first hint that γ5 might regulate desensitization kinetics of the receptor arises from the observation that the TARP effect on kainate-induced currents was stronger than on glutamate-induced currents, and consequently, the ratio between the two agonist-induced currents was consistently increased by γ5 (see sections 3.1.1 and 3.1.2). The partial agonist kainate elicits responses that are virtually desensitization-independent and is therefore commonly described as a non-desensitizing agonist for AMPARs, which in essence links the TARP effect to desensitization (Levchenko-Lambert et al 2011). This observation alone, however, is not sufficient to prove such a claim, as the observed effect could instead be related to an increase in kainate efficacy, a decrease in the affinity for glutamate, or a combination of both.

Addition of TCM to the agonists, however, resulted in most cases in a significant amplification of the TARP effect (see section 3.1.5). TCM is known to stabilize the receptor's dimer interface, abolishing desensitization in the process. Additionally, the factor for glutamate-mediated current potentiation driven by γ5 was, with the exception of aromatic mutants, much higher for every single non-desensitizing mutant, when compared to their desensitizing counterpart (see section 3.1.5). Furthermore, for editing mutants whose responses were inhibited by γ5, the implementation of the desensitization-inhibiting L479Y mutation completely reversed the TARPs inhibitory effect on glutamate-induced currents. Additionally, the differences observed between flip and flop splice variants further reflect the distinct kinetic properties characteristic of each variant. The flop variant is known to have faster desensitization kinetics (Mosbacher et al 1994, Partin et al 1994, Sommer et al 1990). Thus, it is not surprising that flip and flop variants respond differently to the TARP, which is in agreement with the premise that γ5 regulates receptor desensitization kinetics.
Together, the data suggests that $\gamma_5$ exerts its TARP effect through multiple mechanisms, one of which being the alteration of the receptors' desensitization kinetics, although not only for GluA2 edited variants, as previously suggested. Hence, given the presented data, it would be reasonable to assume that $\gamma_5$ either accelerates the rate or increases the extent of desensitization of GluA1 mutants. Therefore, to confirm the assumption that $\gamma_5$ indeed alters desensitization kinetics of GluA1 editing mutants, GluA1(Q)flip and GluA1(R)flip were further investigated by patch-clamping in HEK293 cells (see section 3.2). Surprisingly though, these experiments did not confirm the premise. Instead, there was no significant change in desensitization rates of either mutant, although co-application of TCM or addition of the L479Y mutation intriguingly had the same consequences on both receptors' agonist-induced currents in HEK293 cells as it had in oocytes. The results suggest that $\gamma_5$ acts on AMPARs through a wide range of different, intricate mechanisms.

### 4.3.3 Influence of $\gamma_5$ on other channel properties

The present data shows that the electrophysiological effects of $\gamma_5$ on the different receptors analyzed is complex and it seems to be related to different pharmacological and biophysical channel properties, such as agonist efficacy, single channel conductance, channel open probability, and pore size, as well as kinetic properties such as deactivation and desensitization rates.

#### 4.3.3.1 Modulation of deactivation kinetics by $\gamma_5$

The decrease in glutamate-induced currents of some mutants by $\gamma_5$, and the general increase in the kainate- to glutamate-induced currents of the analyzed mutants (see section 3.1.5) can be partially explained by a reduction in glutamate affinity, as already described for edited GluA2 receptors (Kato et al 2008). However, it does not explain the decrease in kainate-induced currents of the same mutants or the increased agonist-induced currents of the remaining mutants.
As a consequence of a reduction of the receptors affinity for glutamate, it is reasonable that the receptor binds glutamate more weakly and for shorter periods of time, which translates into an acceleration of its deactivation kinetics. GluA2 deactivation kinetics have further been shown to be altered by γ5 in HEK293 cells (Kato et al 2008). Furthermore, an earlier study had shown that transfection of hippocampal neurons with a stargazin chimera that contains the N-terminal domain of γ5 resulted in the acceleration of the decay kinetics of EPSCs (Tomita et al 2005a).

However, our own experiments in HEK293 cells again do not support the notion that γ5 significantly alters desensitization kinetics of either edited or unedited receptors (see section 3.2). Nevertheless, there was a small but not significant acceleration of the deactivation kinetics of GluA1(Q)flip, which was further decreased by introduction of the L479Y mutation, and also a slight reduction of the deactivation rate of the edited non-desensitizing mutant. The apparent incompatibility between our results and the data of Tomita et al. (2005) could be attributed to a possible GluA2(R)-specific effect of γ5 on deactivation, which ought to be further investigated.

### 4.3.3.2 Modulation of channel biophysical properties by γ5

Biophysical channel properties, such as pore diameter, open time, and open probability, are potential targets for TARP modulation, and result in altered single channel conductance.

Inhibition and potentiation by γ5 of agonist-induced currents of the vast majority of the analyzed receptors and GluA1 mutants can be partially explained by an altered open channel probability and channel conductance. An increase in the single channel conductance of GluA1(Q), GluA4(Q), and the long form of GluA2(Q) homomeric receptors despite the decrease in the open probability for GluA1(Q) and GluA4(Q), but not GluA2(Q) homomeric receptors, by γ5 has been previously reported (Soto et al 2009).

Furthermore, our data clearly shows that there is a consistent increase in channel permeability to calcium ions upon co-expression with γ5 (see section 3.4). Although the extent of modulation of this specific property is variable between the different mutants, calcium permeability is consistently increased by the TARP, independently of the
Discussion

amino acid at the Q/R editing site. As an augmented calcium permeability contributes to a higher channel conductance, it is to be expected that the up-regulation of this channel property is also a staple of the different receptors. Therefore, the editing-dependency of the modulation by γ5 may rather be due to the modulation of other channel properties that are not indiscriminately increased across the board for all receptor variants.

The described effect of γ5 on the channels' open probabilities (Soto et al 2009) is variable and depends on the receptor analyzed. Therefore, it is reasonable to assume that the observed inhibitory effect of γ5 on GluA1(Q) and GluA4(Q) agonist-induced currents (see section 3.1.1), in particular, and by extension its effect on all the other AMPARs, which was in some cases inhibitory, in others potentiating, may be the result of an interplay between a variable channel open probability and simultaneous modulation of other channel properties.

Although, it appears that the augmented ion flux rate induced by the type I-TARP, stargazin, occurs without an apparent alteration of the pore size (Soto et al 2014), an eventual contribute of γ5 in the regulation of this property can not be discarded and remains to be investigated.

4.3.4 AMPA receptor trafficking and type II-TARPs

It has already been established that type I-TARPs differ from type II-TARPs by their ability to cluster and traffic AMPA receptors to the membrane and thus aid in correct synaptic delivery (Haering et al 2014, Sumioka 2013). This is primarily due to the fact that type II-TARPs do not have a typical PDZ-binding domain at their C-termini, which is crucial for interaction with PSD-95 and other scaffolding proteins that cannot bind AMPARs on their own. However, it has been show that γ7 enhances membrane expression of AMPARs, although only slightly (Kato et al 2007).

Nine distinct phosphorylation sites have been found to be well conserved in type I-TARPs (Tomita et al 2005b). Phosphorylation of stargazin has been shown to be essential for AMPAR trafficking and synaptic plasticity (Sumioka et al 2010, Tomita et al 2005b). Synaptic activity induces phosphorylation of stargazin by CaMKII and PKC. Phosphorylated stargazin then binds to PDZ domain-containing proteins, such as PSD-
95, enabling stargazin-mediated AMPAR trafficking. In the hippocampus, phosphorylated stargazin is involved in LTP, while dephosphorylation of the TARP induces LTD (Tomita et al 2005b). Additionally, dephosphorylated stargazin interacts electrostatically with the negatively charged lipid bilayer, further preventing its interaction with PSD-95 and trafficking (Sumioka et al 2010).

In addition to the aforementioned phosphorylation sites, it has also been proposed that type I-TARPs are additionally phosphorylated by PKA at position T321, in their PDZ-binding domain, and that phosphorylation at this specific location regulates both their interaction with members of the PSD-95 family and their stability at the synaptic membrane (Choi et al 2002).

Alignment of type II-TARP C-termini with stargazin shows that γ5 and γ7 only contain two out of the nine phosphorylation sites identified in stargazin (for details see Figure 4.3), further reinforcing the perception that type II-TARPs are not involved in trafficking.

In summary, our results show that γ5 was not able to substantially alter membrane expression of any of the mutants analyzed, independently of the amino acid at the Q/R editing site (see section 3.3). Therefore, the data supports the idea that the regulatory effect of γ5 on AMPARs is directed purely at their biophysical properties and does not affect the receptors' trafficking.

Figure 4.3 Conserved phosphorylation sites of stargazin.

Partial amino acid sequence of the C-terminal domains of type I- and type II-TARPs in alignment with γ6 and γ1. Purple boxes show identified phosphoserines. TARP-typical (dark green) and atypical (turquoise) PDZ-binding domains are represented at the end of amino acid sequence. The alignment was performed with the software MegAlign, DNASTAR Lasergene 8.
4.4 AMPAR rectification and type II-TARPs

Although none of the type II-TARPs nor \(\gamma 6\) were able to convert either the linear or the rectifying shape of the I/V curves of homomeric or heteromeric receptors, all TARPs except for \(\gamma 6\) attenuated rectification of Ca\(^{2+}\) permeable receptors (see sections 3.1.1 and 3.1.2), just like stargazin and the remaining type I-TARPs (Kato et al 2010, Soto et al 2009). The effect on rectification was not as obvious for the type II-TARPs as for stargazin, and \(\gamma 7\) had a stronger effect on the shape of the curves than \(\gamma 5\). The inwardly rectifying nature of the I/V curves of Ca\(^{2+}\) permeable receptors is related to the receptors interaction with intracellular, positively charged polyamines that migrate to the cell membrane at less negative potentials and, due to their relatively large size, block the ion pore, preventing ions from crossing the channel. Type II-TARPs, similarly to type I-TARPs, were shown to be able to decrease the receptors' affinity for the polyamines and therefore reduce rectification.

Furthermore, the different responses of a receptor's flip and flop variants to the TARP shows that \(\gamma 5\) activity is, to a certain degree, also dependent on alternative splicing.

4.5 Interaction of type II-TARPs with other proteins

Although the exact stoichiometry between AMPARs and TARPs remains controversial, it has been reported that the number of TARP molecules bound to a given receptor can determine the type and strength of modulation, and can vary anywhere from zero to four TARP units per receptor (Hastie et al 2013, Kim et al 2010, Shi et al 2009, Zhao et al 2016). Additionally, an eventual interaction of different TARPs with the same receptor could further allow for differential regulation of the various receptors and fine-tune the AMPAR response upon agonist binding.

It has been further proposed that TARPs and AMPARs interact hydrophobically through a large interface between the receptors transmembrane domains A and C, which includes the pore helix, and transmembrane domains III and IV of the TARP, while TARP transmembrane domains I and II face the lipid layer and therefore do not interact with the receptor (Twomey et al 2016). However, a different group has reported that the
receptor interacts with TARPs through all transmembrane domains except for TMD B (Zhao et al 2016). Our data shows that γ5 TARPs effects are highly dependent on Q/R editing (see section 3.1.5); therefore, the findings of both groups that TARPs are able to directly interact via their transmembrane domains with the inner pore region of AMPARs provide a direct link between the AMPAR/TARP interaction profile and the Q/R editing site dependence of γ5, and are thus in agreement with our findings.

Furthermore, the high number of conserved negatively charged residues of type I-TARPs, located at the ß4-TMD II loop, has been reported to interact with positively charged amino acids in those parts of the S1 and S2 domains that form the lower lobe of the receptor's LBD, stabilizing the receptor in the open state and therefore decelerating desensitization and deactivation kinetics (Twomey et al 2016, Zhao et al 2016). Accordingly, mutation to aspartate of the positive residues in the well-conserved KGK motif at this specific location greatly decreased stargazin's TARP effect on GluA2 (Dawe et al 2016), although full deletion of the amino-terminal domain of the receptor did not affect the ability of stargazin to effectively traffic the receptor (Cais et al 2014).

However, type II-TARPs lack most of these type I-TARP-conserved residues (see Figure 4.4). While γ7 has four negatively charged residues in this region, γ5 has only one. This could explain the more modest effect of γ7 and the reverse effect of γ5 on desensitization and deactivation kinetics, when compared to type I-TARPs. In fact, substitution of the full extracellular loop between TMD I and TMD II of stargazin by that of γ5 has been shown to nearly abolish the TARP-mediated effects of stargazin on AMPARs (Tomita et al 2005a).

Moreover, it has been reported that the flip/flop domains of AMPARs differentially affect the stability of AMPAR interaction with the TARPs (Cais et al 2014). This alternatively spliced domain is strategically located at the end of the S2 domain, only a few amino acids downstream of the aforementioned conserved positive residues of AMPARs. This change in TARP affinity would further explain the differences observed between the two splice variants during the electrophysiological characterization of wild type receptors and GluA1 mutants alike.

Together, these observations suggest that TARP modulation of AMPARs depends on several distinct interaction sites, which is not surprising given the multitude of reported TARP effects on these receptors.
In the CNS, AMPA receptors mediate most of fast excitatory neurotransmission and are directly involved in the processes of memory and learning. AMPAR malfunction is thus associated with different developmental and neurodegenerative disorders. Hence, in order to comprehend the molecular mechanisms behind brain function and disease, it is essential to also understand AMPARs, both structurally and functionally. However, it is impossible to fully understand AMPARs without considering the contribution of their most intimate partners, the auxiliary TARP subunits. It is therefore not surprising that these proteins have received a lot of attention since they were first discovered. However, only much later it became clear that not all TARPs are equal. Initially, only four proteins were classified as TARPs, \( \gamma_2, \gamma_3, \gamma_4, \) and \( \gamma_8 \). These four subunits, which are now classified as type I- TARPs, were found to regulate nearly every aspect of AMPAR trafficking, pharmacology, and gating. Their impact on AMPAR performance is as variable as it is critical, as agonist-induced responses elicited after co-expression of these proteins with any of the AMPAR subtypes, homomeric and heteromeric alike, are heavily modulated (see sections 3.1.1 and 3.1.2).
Type II-TARPs are a set of homologous proteins that have a much more discrete role than type I-TARPs and therefore received much less attention. In fact, it was not until recently that they were classified as true TARPs (Kato et al. 2008, Kato et al. 2007). These TARPs differ from the first group mainly due to the absence of any trafficking capability. Their C-termini lack the typical PDZ-binding domain that is conserved amongst type I-TARPs. Hence, their interaction with PSD-95 and other scaffolding proteins is limited and, consequently, they fail in driving AMPAR trafficking, clustering, and synaptic deliver. However, as shown here, they are not at all trivial and fulfill a unique role in AMPAR modulation. They bring diversity into play. These unique TARPs have proven to be more specialized and capable of distinguishing among receptor variants based on small structural differences than any of the type I-TARPs. In this thesis I have addressed different aspects related to the modulation of AMPARs by type II-TARPs. The data acquired in the course of this thesis project and described in detail in the Results section, have been assembled with the findings reported by other authors and summarized in Table 4.1. The well-established Type I-TARPs are included in the table for comparison.

The non-TARP status of γ6 has been confirmed. This subunit did not alter the electrophysiological profile of AMPARs (see sections 3.1.1 and 3.1.2), despite a clear interaction with all of the analyzed receptors, in particular with those containing edited subunits (see section 3.1.4). The reason behind this selective interaction remains unclear, but it is possible that γ6 serves a suppressive purpose and interacts with AMPARs only to block their binding with functional TARPs. An eventual competition between γ6 and TARPs remains to be investigated.

The investigation of a potential role of type II-TARPs or γ6 as kainate receptor modulators returned in every case a negative result (see section 3.1.3). The receptor combinations analyzed were not modulated by any of the proteins. Because KARs are the ionotropic glutamate receptors most closely related to AMPARs, no other ionotropic glutamate receptor types were investigated. However, although improbable, we cannot exclude the possibility of interaction between γ6 or type II-TARPs with other receptors that were not considered in this thesis and remain to be analyzed.

The electrophysiological characterization of γ7 by TEVC revealed a TARP behavior that resembles that of type I-TARPs, as this subunit was capable of strongly
potentiating current amplitudes of all the receptor combinations analyzed, except for the flop variant of the non-physiological heteromeric combination assembled from GluA1(R) and GluA2(Q) (see sections 3.1.1 and 3.1.2). Although γ7 appears to affect gating in the same fashion as its type I relatives, its regulatory effect is somewhat weaker and its effect on trafficking negligible. If γ7 affects other receptor properties such as channel permeability to calcium ions and open probability, or if it has an influence on the ion pore diameter, to the best of our knowledge is still unknown.

However, γ5 has presented itself to be the most complex and intriguing TARP. This TARP has shown such versatility that it is able to either potentiate or inhibit different receptors and receptor variants, in some cases distinguishing them only by the amino acid at the Q/R editing site (see sections 3.1.1, 3.1.2 and 3.1.5). γ5 is the only TARP that has the ability to reduce agonist-induced currents of selected receptors. This unique suppressive ability of γ5 when acting on Ca²⁺ permeable homomeric receptors and, in addition, on every heteromeric subunit combination, independently of its permeability to calcium, might be vital for both neurons and glial cells. It is possible that γ5 and other TARPs cooperate in vivo in order to achieve an optimized agonist-induced response. Furthermore, as a characteristic type II-TARP feature, γ5 has a comparatively short C-terminal domain that includes only two out of nine phosphorylation sites when compared to type I-TARPs and ends in an atypical PDZ-binding domain. The phosphorylation sites have been implied in the trafficking of AMPARs (Tomita et al 2005b) and might explain why γ5 fails in this particular task, independently of the receptor's editing status, as shown hitherto (see section 3.3). Moreover, the unusual PDZ-binding domain hints at interaction partners that might be considerably different from those interacting with type I-TARPs. Therefore, it is imperative to discover such interacting proteins, which would help to better understand these distinctive regulatory features of γ5.
Table 4.1 Different aspects of TARP and γ6 modulation of AMPARs.
When relevant, distinction between Q and R editing variants is made. Blue arrows: positive modulation; magenta arrows: negative modulation; grey arrows: no modulation; ?: conflicting or inconclusive evidence; n.d.: not determined.

<table>
<thead>
<tr>
<th>Type I-TARPs</th>
<th>γ5 Q</th>
<th>R</th>
<th>γ7 Q</th>
<th>R</th>
<th>γ6 Q</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>↑↑</td>
<td>a,b</td>
<td>↑*</td>
<td>q,r</td>
<td>↑*</td>
<td>q,r</td>
</tr>
<tr>
<td>Trafficking</td>
<td>↑↑↑</td>
<td>c,d,e</td>
<td>←</td>
<td>q</td>
<td>←</td>
<td>q</td>
</tr>
<tr>
<td>Glutamate affinity</td>
<td>↑↑↑  f,g,h</td>
<td>n.d.</td>
<td>↓</td>
<td>q,s</td>
<td>←</td>
<td>q,s,t</td>
</tr>
<tr>
<td>Kainate efficacy</td>
<td>↑↑↑  f,g,i,j</td>
<td>n.d.</td>
<td>←</td>
<td>q</td>
<td>←</td>
<td>t,u</td>
</tr>
<tr>
<td>Steady-state currents</td>
<td>↑↑↑  i,j,k</td>
<td>↓↓</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>*</td>
</tr>
<tr>
<td>Peak currents</td>
<td>↑↑↑  g,l</td>
<td>↓*</td>
<td>q,r</td>
<td>↑*</td>
<td>q,r</td>
<td>↑*</td>
</tr>
<tr>
<td>Desensitization</td>
<td>↓↓    c,f,g,h,m</td>
<td>?*</td>
<td>q</td>
<td>?*</td>
<td>q</td>
<td>↑*</td>
</tr>
<tr>
<td>Deactivation</td>
<td>↓↓    c,f,g,h,m</td>
<td>?*</td>
<td>q</td>
<td>?*</td>
<td>q</td>
<td>↑*</td>
</tr>
<tr>
<td>Rectification</td>
<td>↑↑↑  i,n,o</td>
<td>↑*</td>
<td>q,r</td>
<td>↑*</td>
<td>q,r</td>
<td>↑*</td>
</tr>
<tr>
<td>Ca2+ permeability</td>
<td>↑↑↑  i</td>
<td>↑↑*</td>
<td>↑↑*</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Channel conductance</td>
<td>↑↑↑  f,l,n,p</td>
<td>↑*</td>
<td>q,r</td>
<td>↑*</td>
<td>q,r</td>
<td>↑*</td>
</tr>
<tr>
<td>Open probability</td>
<td>↑↑↑  p</td>
<td>↓*</td>
<td>r</td>
<td>↓*</td>
<td>r</td>
<td>n.d.</td>
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A lot more still needs to be done in order to understand this unique TARP. We now know that its modulation of AMPARs is highly dependent on receptor editing, but the exact molecular mechanisms behind this behavior still elude us. Furthermore, the $\gamma_5$ effect on channel kinetics was inconclusive and needs to be further investigated. In addition, a better understanding of the TARP modulation of the exact biophysical properties of the ion channel contributing to the macroscopically observed alterations in receptor currents would greatly improve our understanding of $\gamma_5$ regulatory mechanisms.
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Curriculum Vitae

Sandra da Conceição Pereira Lemos

Curriculum Vitae

sandra.lemos@rub.de

Ruhr University Bochum, Faculty of Chemistry and Biochemistry
Department of Biochemistry I, 44780 Bochum, Germany

Personal information

Date of Birth 08.12.1983
Place of Birth Meda/Guarda, Portugal
Nationality Portuguese

Languages

<table>
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<th>Language</th>
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<tr>
<td>Portuguese</td>
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</tr>
<tr>
<td>German</td>
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Education and training
Curriculum Vitae

2011-current date
PhD, Ruhr University Bochum, Germany

09/2010- 11/2010
Internship, Laboratory of Quality Control, Medice, Arzneimittel Pütter GmbH & Co. KG, Iserlohn, Germany

2008- 2009
2nd MSc in Biochemistry according to the Bologna process. University of Beira Interior, Portugal/Lund University, Sweden with the financial support of the Novo Scholarship Programme Novo Nordisk, Denmark

2001-2008
“Licenciatura” (equivalent to a MsC) in Biochemistry, University of Beira Interior, Portugal. Final thesis under the Socrates-Erasmus programme at Lund University, Sweden

Laboratory rotations

04/2014
Boehringer Ingelheim, Biberach, Germany

06/2013
Institute Curie, Paris, France

06/2012
Institute Curie, Paris, France

Teaching experience

02/2015
Supervisor of the course “Heterologous Expression of Neurotransmitter Receptors in Frog Oocytes”, Ruhr University Bochum, Germany

01/2014
Supervisor of the course “Heterologous Expression of Neurotransmitter Receptors in Frog Oocytes”, Ruhr University Bochum, Germany

03/2013
Supervisor of the course “Heterologous Expression of Neurotransmitter Receptors in Frog Oocytes”, Ruhr University Bochum, Germany
Curriculum Vitae

01/2012         Supervisor of the course “Heterologous Expression of Neurotransmitter Receptors in Frog Oocytes”, Ruhr University Bochum, Germany


02/2010 - 03/2010 Supervisor of the course “Cell Biology “, Lund University, Sweden

10/2009         Supervisor of the course “Principles of Mass Spectrometric Protein Characterization”, Lund University, Sweden

Courses and soft-skills

SS 2016         Molecular Oncology, Ruhr University Bochum, Germany

WS 2012/13      Stem Cell Physiology, Ruhr University Bochum, Germany

31/05/2013      Career planing and job hunting, Pultusk, Poland

28/01/2013 – 30/01/2013 Scientific Writting, Ruhr University Bochum, Germany

14/11/2012 – 16/11/2012 Scientific Presentation, Ruhr University Bochum, Germany

Meetings and conferences


25/05/2013 – 30/05/2013 ESF-EMBO Conference on Molecular Perspectives On Protein-Protein Interactions, , Pultusk, Poland – poster presentation
Curriculum Vitae

10/10/2012 – 12/10/2012 ITN-Midterm Meeting, Feldafing, Germany – poster and oral presentation

13/09/2012 – 15/09/2012 Perspectives in Molecular Neurobiology, Bochum, Germany – poster presentation

01/08/2012 – 03/08/2012 Marie Curie Initial Training Network TRANSPOL Workshop, Valetta, Malta - poster and oral presentation


21/01/2010 Novo Scholarship Symposium, Bagsværd, Denmark – poster presentation

09/03/2008- 13/03/2008 Symposium on MicroScale Bio separations (msb), Berlin, Germany – poster presentation

Organizational skills

- Organization of the Marie Curie Initial Training Network TRANSPOL Summer School, May 2013, Pultusk, Poland
- Active participation in the meeting “Perpectives in Molecular Neurobiology”, September 2012, Bochum, Germany
- Organization of the Marie Curie Initial Training Network TRANSPOL Workshop, August 2012, Valletta, Malta
- Participation at Tekniska Mässan (International Technical Fair), 2009, Stockholm, Sweden, promoting LTH

Scientific papers

Declaration

I certify herewith that the dissertation at hand was completed and written independently and without outside assistance. The “Guidelines for Good Scientific Practice” according to § 9, Sec. 3 were adhered to. This work has never been submitted in this or a similar from at this or any other domestic or foreign institution of higher education as a dissertation.

Sandra Lemos
Bochum, the 12th of January, 2017