Assembly and Intracellular Trafficking of Ionotropic Glutamate Receptors

Doctoral Dissertation

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To mama and papa
Declaration

This dissertation is based on the experimental work carried out at the department of Receptor Biochemistry, Ruhr University Bochum, Germany, and has been written independently with no other sources and aids than required. The "Guidelines for Good Scientific Practice" (Leitlinien guter wissenschaftlicher Praxis und Grundsätze für das Verfahren bei vermutetem wissenschaftlichen Fehlverhaltens) according to § 9, Sec. 3 of the Promotionsordnung der International Graduate School of Neuroscience der Ruhr-Universität Bochum were followed. Furthermore, the thesis has never been submitted in this or a similar form to any other institution of higher learning as a dissertation.

Zhanlu Ma

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Contents

Abstract ................................................................................................................................ 1

1 Introduction ...................................................................................................................... 4
  1.1 AMPA receptor assembly and trafficking ................................................................. 6
  1.2 Kainate receptors ......................................................................................................... 13
  1.3 Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate
      receptor complexes............................................................................................................. 17

2 Aims of the study ............................................................................................................ 19

3 Materials and methods ................................................................................................... 21
  3.1 Molecular cloning......................................................................................................... 21
    3.1.1 Polymerase chain reaction (PCR) ................................................................. 21
    3.1.2 Restriction ........................................................................................................... 22
    3.1.3 Purification of DNA fragments ............................................................................. 23
    3.1.4 Ligation ............................................................................................................ 23
    3.1.5 Transformation ..................................................................................................... 23
    3.1.6 Preparation of plasmid DNA ............................................................................ 24
      3.1.6.1 Mini DNA preparation .................................................................................. 24
      3.1.6.2 Midi DNA preparation .................................................................................. 24
    3.1.7 Vectors and plasmids .......................................................................................... 24
      3.1.7.1 N-terminally fluorescent protein vectors ..................................................... 24
      3.1.7.2 Subcellular localization vectors ..................................................................... 25
      3.1.7.3 Other mammalian expression vectors ....................................................... 25
  3.2 Cell culture ................................................................................................................. 26
    3.2.1 HEK 293 cell culture .......................................................................................... 26
      3.2.1.1 Maintenance of HEK 293 cells ................................................................. 26
      3.2.1.2 Revival of HEK 293 cells .......................................................................... 27
      3.2.1.3 Passaging of HEK 293 cells ....................................................................... 27
      3.2.1.4 Preparation of HEK 293 frozen stocks ....................................................... 27
      3.2.1.5 Transient transfection of HEK 293 cells (modified calcium phosphate method) ................................................................................................................................................. 28
      3.2.1.6 Transient transfection of HEK 293 cells (by Metafectene Pro) .................... 28
      3.2.1.7 Transient transfection of HEK 293 cells (by PolyFect) .................................. 29
      3.2.1.8 Transient transfection of HEK 293 cells (biolistic transfection method) ....... 29
    3.2.2 Primary culture of hippocampal and cortical neurons ........................................ 29
      3.2.2.1 Preparation of mixed glial cell culture, microglia culture and neuron culture .... 29
      3.2.2.2 Maintenance and passaging of microglia culture ........................................ 30
3.2.2.3 Transient transfection of hippocampal and cortical neurons (nucleofection method) ..............................................................................................................................31
3.2.2.4 Transient transfection of hippocampal and cortical neurons (calcium phosphate method) ..............................................................................................................................31

3.3 Cell counting for estimation of expression efficiency and coexpression efficiency ........32

3.4 Patch clamp recording ...................................................................................................... 32

3.5 Protein biochemistry ....................................................................................................... .....32
3.5.1 Protein extraction from HEK 293 cells ........................................................................32
3.5.2 Western blot analysis ..................................................................................................... 33
3.5.2.1 SDS-PAGE ............................................................................................................33
3.5.2.2 Wet transfer ............................................................................................................33
3.5.2.3 Immunodetection ...................................................................................................34
3.5.2.3.1 Immunodetection with anti-GFP monoclonal antibody .........................................34
3.5.2.3.2 Immunodetection with anti-DsRed monoclonal antibody ....................................34
3.5.2.3.3 Immunodetection with anti-FLAG M2 monoclonal antibody ...............................34

3.6 Immunoprecipitation ...........................................................................................................35

3.7 Immunocytochemistry .........................................................................................................35

3.8 Digital image acquisition, processing, and analysis ..........................................................35
3.8.1 Live cell confocal imaging .............................................................................................35
3.8.2 Colocalization analysis by confocal microscopy ...........................................................36
3.8.3 Fluorescence Resonance Energy Transfer (FRET) by confocal microscopy ..............37
3.8.4 Dynamic FRET measurement by fluorescence microscopy .........................................38

3.9 Instruments ................................................................................................................ ...........39

3.10 Disposable materials .......................................................................................................41

3.11 Chemicals and enzymes .....................................................................................................42

4 Results ..................................................................................................................................43

4.1 Construction of recombinant proteins .............................................................................43
4.1.1 C-terminally fluorescently-labeled GluR1 constructs ........................................................................43
4.1.2 C-terminally fluorescently-labeled GluR2(R) constructs ................................................44
4.1.3 C-terminally fluorescently-labeled GluR2(Q) constructs ................................................45
4.1.4 C-terminally fluorescently-labeled GluR6(Q) constructs ................................................45
4.1.5 C-terminally fluorescently-labeled GluR6(R) constructs ................................................45
4.1.6 C-terminally fluorescently-labeled KA2 constructs .........................................................46
4.1.7 C-terminally fluorescently-labeled stargazin constructs ................................................46
4.1.8 N-terminally fluorescently-labeled GluR1 constructs .....................................................46
4.1.9 Attempt to engineer N-terminally fluorescently-labeled GluR2(R) constructs ...............47
4.1.10 N-terminally fluorescently-labeled stargazin constructs ..............................................49
4.2 Monitoring assembly and intracellular trafficking of AMPA receptors

4.2.1 GluR1 fusion proteins are localized at the ER as well as plasma membrane of live cells.

4.2.2 Retention of GluR2(R) fusion proteins in the ER.

4.2.3 GluR2(Q) subunits are predominantly retained in the ER, with a small amount present at the cell surface.

4.2.4 Surface expression of AMPA receptors is regulated by auxiliary subunits.

4.2.4.1 Cell surface expression of N- and C-terminally fluorescently-labeled stargazin fusion proteins.

4.2.4.2 Stargazin enhances surface expression of AMPA receptor subunits in live HEK 293 cells.

4.2.4.3 Stargazin interacts with AMPA receptors at the cell surface as demonstrated by FRET analysis.

4.2.4.4 Dynamics of in vivo stargazin-AMPAR association upon L-glutamate application.

4.2.5 The role of the endoplasmic reticulum and the Golgi apparatus in intracellular trafficking of AMPA receptors.

4.3 Monitoring assembly and intracellular trafficking of kainate receptors

4.3.1 Monitoring intracellular trafficking of GluR6(Q) in live mammalian cells.

4.3.1.1 Functional GluR6(Q) fusion proteins express mainly at the plasma membrane.

4.3.1.2 GluR6(Q) fusion proteins localize at a cytosolic network-like compartment and a peri-nuclear region before their transport to the plasma membrane.

4.3.1.3 GluR6(Q) fusion proteins colocalize with intracellular membrane compartments.

4.3.1.4 Colocalization analysis demonstrates an ER–Golgi apparatus–plasma membrane trafficking pathway for GluR6(Q) subunit.

4.3.2 Monitoring intracellular trafficking of GluR6(R) in live mammalian cells.

4.3.2.1 GluR6(R) fusion proteins form functional homomeric receptors at the plasma membrane.
4.3.2.2 An ER – Golgi apparatus – plasma membrane trafficking pathway for GluR6(R) subunit..............................................................................................................................116
4.3.3 Retention of KA2 fusion proteins in the ER............................................................119
4.3.4 Monitoring intracellular trafficking of GluR6/KA2 heteromeric complexes in live mammalian cells ...................................................................................................................125
  4.3.4.1 Colocalization of GluR6 and KA2 subunits in the ER ........................................125
  4.3.4.2 A significant delay of plasma membrane-targeted GluR6 trafficking by coexpression with KA2.................................................................................................................126
  4.3.4.3 KA2 subunits are transported to the plasma membrane by coexpression with GluR6 while a significant amount of the subunits are retained in the ER......................130
4.3.5 Assembly and intracellular trafficking of kainate receptors analyzed by FRET in live cells ..................................................................................................................135
  4.3.5.1 FRET analysis indicates subunit interactions at the plasma membrane of live cells.................................................................................................................................136
  4.3.5.2 FRET measurements show that subunits assemble in the ER .........................139
  4.3.5.3 The role of the Golgi apparatus in receptor assembly and trafficking demonstrated by FRET analysis.................................................................................................142
  4.3.5.4 Summary of FRET analysis with kainate receptor subunits..........................145
4.4 Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes..............................................................................................................148
  4.4.1 Cumulative transfection efficiency is not dependent on the total amount of DNA, and varies with the proteins to be expressed........................................................................149
  4.4.2 Coexpression efficiency is influenced by several factors ....................................152
  4.4.3 Evaluation of the marker protein cotransfection method commonly used in electrophysiological studies..............................................................................................154
  4.4.4 Coexpression efficiencies of two glutamate receptor subunits are well below 100% ........................................................................................................................................157
  4.4.5 Various alternative transfection methods also result in a variable range of coexpression efficiencies.............................................................................................................163
5 Discussion ..........................................................................................................................165
5.1 Assembly and intracellular trafficking of AMPA receptors ....................................165
  5.1.1 Distinct intracellular distribution but similar intracellular tetramerization of GluR1, GluR2(R), and GluR2(Q) subunits ..................................................................................165
  5.1.2 Stargazin regulates AMPAR surface expression and interacts with AMPAR at the plasma membrane.................................................................................................................168
  5.1.3 Dynamics of dissociation of surface AMPAR/stargazin complexes ....................172
5.2 Assembly and intracellular trafficking of kainate receptors .................................176
5.2.1 GluR6 surface expression is not significantly regulated by Q/R editing..............176
5.2.2 KA2 homomeric assemblies are retained in the ER, fail to deliver to the cell surface, and undergo a dynamic retrograde recycling from the Golgi apparatus to the ER..........177
5.2.3 Assembly of kainate receptor subunits occurs as early as in the ER.....................180
5.2.4 An endoplasmic reticulum – Golgi apparatus – plasma membrane trafficking pathway for oligomeric kainate receptor complexes .........................................................182
5.2.5 Modulation of surface expression of GluR6/KA2 heteromeric receptor complexes by both constituent subunits...........................................................................................................184

5.3 Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes..............................................................................................................187

References ..................................................................................................................194
Acknowledgment ..................................................................................................... 205
Curriculum Vitae.......................................................................................................207
Publication list ..........................................................................................................208
Poster presentations and published abstracts..........................................................210
Abstract

Ionotropic glutamate receptor trafficking is most likely one of the key factors regulating synaptic strength and synaptic plasticity in the central nervous system. The intracellular trafficking and membrane delivery of the receptors presumably are under tight control so that only fully assembled functional receptors can be expressed at the plasma membrane.

Selected AMPA and kainate receptor subunits, as well as stargazin, a protein regulating surface delivery and electrophysiological function of AMPA receptors, were engineered to generate in-frame fusion proteins with various fluorescent proteins at the N or C terminus of the subunits. The recombinant proteins were transfected into HEK 293 cells, and expression was confirmed by Western blot. Most of these recombinant proteins have been verified for electrophysiological function by patch clamp recording. The C-terminal fusion proteins were utilized throughout the present study. Confocal microscopy techniques, including live cell imaging, multicolor imaging (up to four channels without UV excitation), digital colocalization analysis (up to three channels), and FRET analysis, were established and utilized in the present study.

Monitoring of fluorescent protein expression and observation of FRET in live cells strongly suggests a direct protein-protein interaction between GluR2 subunits in the ER. The data suggest that the assembly of GluR2 homomers (i.e., at least homodimers) occurs before subunits exit the ER. In addition, by simultaneously labelling ER or the plasma membrane in live cells, it was shown that labeled GluR2(Q) expresses at the plasma membrane, whereas the edited variant, GluR2(R) does not. However, in the presence of stargazin, tetramerization of both un-edited and edited isoforms occured in HEK 293 cells.

When coexpressed with stargazin, both GluR1 and GluR2 are highly expressed at the HEK293 cell membrane. Analyzed by colocalization software, AMPAR subunits and stargazin are found to be colocalized at the plasma membrane. Interestingly, before they arrive at the plasma membrane, GluR1 (or GluR2) and stargazin were found to colocalize intracellularly as well, which may
indicate an interaction of the two proteins before their transport to the plasma membrane occurs. This perinuclear region, which is perhaps the Golgi apparatus, may play a critical role in stargazin-AMPAR association before surface expression of the complexes.

Furthermore, for the first time, the present study demonstrated a direct protein-protein interaction between stargazin and AMPA receptors at the plasma membrane of live mammalian cells by FRET analysis, which may indicate a role for stargazin in membrane-targeting of AMPA receptors in non-neuronal cells, and which is perhaps the first step in stargazin-associated AMPA receptor assembly and synaptic trafficking.

Conflicting evidence has been provided whether plasma membrane-resident AMPA receptors are released from associated stargazin upon agonist application. In the present study, utilizing confocal dual-channel four-dimensional imaging and digital colocalization analysis carried out on live cells, dynamic changes of cell surface stargazin/AMPAR complexes upon L-glutamate application were demonstrated.

Assembly and intracellular trafficking of kainate receptors are largely unknown. When labeled kainate receptor subunits were expressed in HEK293 cells, differential localization of high affinity (KA2 subunit) and low affinity (GluR6 subunit) kainate receptor subunits was observed. The role of the amino acid at the Q/R editing site of GluR6 is discussed. In addition, it was demonstrated that not only GluR6 but also KA2 plays a critical role in surface delivery of heteromeric kainate receptors, which might suggest possible stoichiometries of these receptor complexes which were not previously considered.

In the present study, FRET analysis has also been analyzed to study the assembly of kainate receptor complexes at the plasma membrane as well as intracellular compartments. Assembly of the receptor complexes (i.e., at least dimers) was demonstrated to occur as early as in the ER. The role of ER and the Golgi apparatus in receptor assembly and trafficking is discussed.
In a separate, more technical study, an evaluation of cotransfection efficiencies in heterologous expression systems for the electrophysiological study of heteromeric receptor complexes was performed. Analyzing the expression of multiple fusion proteins by confocal microscopy, the coexpression efficiencies for various glutamate receptor cDNA combinations, cDNA amounts, and cDNA ratios, were evaluated. Several factors were found to influence the individual, cumulative, and cotransfection efficiencies, including the cDNA ratio, the nature of the expressed protein, and the specific combination of cotransfected cDNAs. Following simultaneous transfection with equal amounts of several cDNAs it was demonstrated that several distinct populations of cells that express different receptor subunit combinations are generated consistently. The evidence present in this study suggests that cotransfected cells should always be independently tested for the expression of all target subunits before picking cells for the analysis of specific heteromeric receptor assemblies. This set of data has already been published (Ma et al., 2007).
1. Introduction

The ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate the vast majority of fast excitatory neurotransmission in the mammalian central nervous system (CNS). They are traditionally classified into three major subtypes, AMPA, kainate and NMDA receptors, originally named after selective agonists, and defined by pharmacological and electrophysiological properties (Hollmann and Heinemann, 1994). In addition, two other subfamilies, two orphan receptor subunits named delta subunits and the kainate binding proteins (KBPs) form non-functional homo- or heteromeric channels; the latter subunits are found exclusively in non-mammalian vertebrates (Dingledine et al., 1999; Hollmann, 1999).

Functional receptors are most likely homo- or heterotetramers consisting of subunits exclusively from a given receptor subfamily. The hypothesis is that the assembly of the receptor complex proceeds in two steps: subunit dimerization and dimer-dimer association forming dimers of dimers (Ayalon and Stern-Bach, 2001; Laube et al., 1998; Rosenmund et al., 1998; Schorge and Colquhoun, 2003).

Though several molecular mechanisms, such as multiple genes, alternative splice variants, and RNA editing, generate subunit diversity, all receptor subunits share a common transmembrane topology, possess three hydrophobic regions within the central portion of the sequence (Bennett and Dingledine, 1995; Hollmann et al., 1994) (Figure 1.1). Each subunit has a large extracellular N-terminal domain, three transmembrane domains, a pore domain, and an intracellular C-terminal domain. The N-terminal domain is believed to mediate dimer formation at the first step of receptor complex assembly, whereas the C-terminal domain is highly divergent, interacts with cytoplasmic proteins and is crucial for subunit-specific regulation of receptor function. The S1 domain of the amino-terminal domain, and the S2 domain of the extracellular loop 3 together form the ligand binding site, consisting of two lobes separated by a ligand binding cleft where the degree of the cleft closure determines the extent of receptor activation upon agonist binding (Armstrong and Gouaux, 2000; Armstrong et al., 1998).
Although AMPA, kainate and NMDA receptors all bind and respond to presynaptically released glutamate, they play distinct roles in brain function. AMPA receptors mediate the majority of fast excitatory synaptic transmission. Kainate receptors are found present at both postsynaptic and presynaptic regions where they may play a key role in modulating synaptic networks. Whereas postsynaptic calcium influx via NMDA receptor activation is crucial for the induction of specific forms of synaptic plasticity. According to sequence homology, AMPA and kainate receptors are categorized as non-NMDA receptors and their assembly and intracellular trafficking are the major topics of the present study.

Figure 1.1. Schematic membrane topology of iGluR subunits (adapted from Daniel Tapken). The N-terminal domain (NTD) consists of a bacterial leucine-isoleucine-valine binding protein (LIVBP) homology domain, and a bacterial lysine-arginine-ornithine binding protein (LAOBP) homology domain called the S1 domain. Another LAOBP homology domain, the extracellular S2 domain between transmembrane domains (TMD) B and C, forms a ligand binding site together with the S1 domain. The region between TMD A and B forms a hairpin loop, which is the pore region of the subunit. The Q/R editing site resides in the pore region as indicated. The flip/flop region of alternative splicing is located at the TMD C-proximal segment of the extracellular loop 3 (L3).
1.1 AMPA receptor assembly and trafficking

It has been suggested that nearly all forms of experience-dependent plasticity, including learning and memory, involve long-lasting activity-dependent alterations in the strength of synaptic communication between neurons. Long-term potentiation (LTP) and long-term depression (LTD) generated at excitatory synapses on hippocampal CA1 pyramidal cells are the most widely accepted models suggesting cellular mechanisms of such synaptic plasticity in the central nervous system. Decades after the discovery of LTP, excitatory synaptic plasticity has only recently been shown to be associated with the sensitivity as well as the number of postsynaptic AMPA receptors, a finding that focused research on the regulation of AMPA receptors.

AMPA receptors are present at virtually all excitatory synapses and mediate the majority of fast excitatory synaptic transmission. They are homomeric and heteromeric assemblies composed of GluR1 – GluR4 subunits in heterologous expression systems. The four receptor subunits share 68 – 73% amino acid identity which is distributed such across the protein that the extracellular and transmembrane domains are very similar whereas the cytoplasmic carboxyl domains are distinct. Native AMPA receptors at mature hippocampal excitatory synapses assemble mainly as GluR1/GluR2 heteromers, in some cases also forming GluR2/GluR3 heteromers (Wenthold et al., 1996). GluR4 was found in immature hippocampus as well as some mature brain regions, and forms receptor complexes with GluR2 (Zhu et al., 2000).

In addition, more than 99% of native GluR2 subunits are present as an ‘edited’ form, Q586R, in which a genomically encoded glutamine residue in the pore region is altered to an arginine residue by a posttranscriptional process of RNA editing (Seeburg et al., 1998; Sommer et al., 1991). The edited GluR2 subunit, GluR2(R), dominates electrophysiological properties of GluR2-containing AMPA receptors, determining Ca\(^{2+}\) permeability, desensitization, and the current-voltage relationship (Burnashev et al., 1992; Higuchi et al., 1993; Hollmann et al., 1991; Hume et al., 1991; Verdoorn et al., 1991). Moreover, Q/R editing has been shown to regulate AMPA receptor plasma membrane expression by controlling receptor exit from the endoplasmic reticulum (ER) (Greger et al., 2002), and was proposed to mediate AMPA receptor tetramerization (Greger et al., 2003).
Evidence has been provided that LTP is associated with a rapid increase in the postsynaptic response upon AMPA application or CaMKII activation (Lledo et al., 1995; Montgomery et al., 2001), an increase in AMPA binding due to increased numbers of AMPA binding sites (Maren et al., 1993), and an increase in the single-channel conductance of AMPA receptors (Benke et al., 1998). Furthermore, in adult GluR1-/- mice, associative LTP was found to be absent in CA3 to CA1 synapses, suggesting that CA1 hippocampal LTP is controlled by the number or subunit composition of AMPA receptors (Zamanillo et al., 1999). Expression of GFP-tagged GluR1 in GluR1-/- mice restored hippocampus-dependent spatial working memory, and rescued CA3-CA1 LTP (Schmitt et al., 2005). Finally, the discovery of silent synapses (Kullmann, 1994), a population of synapses that lack functional AMPA receptors but contain functional NMDA receptors, and the indication that these synapses can be switched on by LTP-inducing protocol (Isaac et al., 1995; Liao et al., 1995), support the hypothesis that LTP involves the activity-dependent rapid recruitment of synaptic AMPA receptors. Indeed, AMPA receptors were recently shown to be transported from recycling endosomes to the plasma membrane during NMDAR-LTP (Lu et al., 2001; Park et al., 2004).

Activation of NMDA receptors causes not only LTP but also LTD, a long-lasting and presumably stable form of synaptic depression. Activation of NMDA receptors thus can also cause a loss of synaptic AMPA receptors (but not NMDA receptors) via a signalling mechanism required for the induction of LTD. More than one mechanism may be involved in AMPA receptor internalization: a selective activity-dependent receptor endocytosis (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000; Lin et al., 2000; Zhou et al., 2001), and a direct internalization as a consequence of agonist binding to surface receptors (Ehlers, 2000; Lin et al., 2000; Lissin et al., 1999). AMPA receptors internalized via distinct endocytosis mechanisms then undergo divergent cellular pathways (Ehlers, 2000; Lee et al., 2004). The receptors are sorted in early endosomes and are directed towards recycling and degradation (Ehlers, 2000). It is likely that GluR1 is internalized independently of synaptic activity, whereas GluR2 determines endosomal sorting of internalized GluR2-containing AMPA receptors such that NMDA receptor activation diverts GluR2-containing receptors to late endosomes/lysosomes (Holman et al., 2007; Lee et al., 2004).
However, contrary evidence has been provided in an earlier study, perhaps due to technical differences (Ehlers, 2000). GluR3 is targeted to lysosomes regardless of NMDA receptor activation (Lee et al., 2004).

As discussed above, trafficking of AMPA receptors to and from synapses plays a critical role in regulating synaptic strength during LTP and LTD. Investigations of the distinct mechanisms of AMPA receptor exocytosis as well as endocytosis, that have been suggested as the cellular mechanisms of AMPA receptor insertion and removal from the neuronal surface, are thus of great interest in the study of synaptic plasticity.

Intracellular distribution of AMPA receptors in neurons seems to be divergent according to the receptor composition. The majority of AMPA receptors are extrasynaptic. By functional mapping of glutamate receptors with two-photon uncaging of glutamate, AMPA receptors were found abundant in large mushroom-shaped spines but were moderately expressed in smaller spines, where the latter may be serving as the structural substrates of silent synapses (Matsuzaki et al., 2001). It has been shown that exogenous GluR1 subunits forming homomeric recombinant receptors are mainly distributed to intracellular compartments within dendrites of CA1 pyramidal neurons and are targeted to the dendritic spines following NMDA receptor activation (Shi et al., 1999). By contrast, exogenous GluR2 homomeric recombinant receptors were found to reside in dendritic spines participating in synaptic transmission but not delivered to silent synapses (Shi et al., 2001). The length of the cytoplasmic carboxyterminal tails appears to regulate subunit trafficking such that subunits with long tails (GluR1 and the predominant splice form of GluR4) may insert into extrasynaptic neuronal plasma membrane and diffuse laterally within the plasma membrane to postsynaptic active zones in an activity-dependent manner, whereas the subunits with short tails (GluR3 and the predominant splice form of GluR2) may directly insert into the postsynaptic density and recycle continuously in an activity-independent manner (Passafaro et al., 2001). Interestingly, this regulation of receptor surface/synaptic trafficking is probably controlled by receptor stoichiometry and dominated by GluR1 (or any subunit with long carboxyl tail) in the complexes, and involves PDZ domain interaction via the tail (Hayashi et al., 2000; Passafaro et al.,
Therefore, synaptic targeting of the predominant native receptors, such as GluR1/GluR2 heteromeric complexes, are dominated by the GluR1 subunit and initially accumulate at extrasynaptic sites, whereas GluR2/GluR3 heteromeric complexes readily insert into the synapse. By contrast, recycling and degradation of AMPA receptors after endocytosis is determined by the GluR2 subunit (Lee et al., 2004). For the common GluR1/GluR2 heteromeric complexes, the composite subunits GluR1 and GluR2 may therefore determine regulated redistribution to the synaptic membrane and to intracellular compartments, respectively.

Individual C-terminal domains of individual AMPA receptor subunits regulate receptor trafficking via interactions with specific intracellular proteins, such as scaffold and cytoskeletal proteins. GluR1 interacts with the second PDZ (PSD-95/SAP-90, Disc-large, ZO-1 homologous) domain of SAP97 (Leonard et al., 1998), a synapse-associated protein belonging to MAGUK (membrane-associated guanylate kinase) protein family that contains several interactors of NMDA receptor subunit NR2B. This interaction is determined by an ATGL motif at the GluR1 C-terminus and a proximal SSG sequence (Cai et al., 2002), and may occur early in the biosynthetic pathway when the receptors are in the ER or cis-Golgi (Sans et al., 2001). It might play a role in activity- or CaMKII-dependent synaptic delivery of AMPA receptors (Hayashi et al., 2000; Rumbaugh et al., 2003).

The C-terminal region of GluR1 also interacts with non-PDZ protein, such as proteins 4.1G and 4.1N, homologs of the erythrocyte membrane cytoskeletal protein 4.1 (Shen et al., 2000). The interaction of the GluR1 subunit with protein 4.1N may link surface AMPA receptors to the cortical actin cytoskeleton network underneath the synaptic plasma membrane and PSD, and therefore stabilize the receptors.

GluR1/SAP97 interaction is a type I PDZ domain interaction, while GluR2, GluR3 and the short form of GluR4 (GluR4c) terminate with motif SVKI, a type II PDZ binding site. Several PDZ proteins bind to the C-termini of these short-tail subunits, namely GRIP/ABP (glutamate receptor interacting protein, also known as AMPA receptor binding protein) (Dong et al., 1997; Srivastava et al., 1998) and PICK1 (protein interacting with C kinase) (Xia et al., 1999). Both variants of GRIP (GRIP1 and GRIP2) interact with AMPA receptors and appear to be critical for the synaptic
accumulation of the receptor (Dong et al., 1999; Liu and Cull-Candy, 2005; Osten et al., 2000), while GRIP1 may also be involved in the transport of GluR2 to dendrites (Setou et al., 2002). Interestingly, phosphorylation of S880 in the SVK1 PDZ binding motif of GluR2 prevents GluR2/GRIP interaction but promotes GluR2/PICK1 interaction (Chung et al., 2000; Matsuda et al., 1999; Perez et al., 2001). Recently evidence has been provided for a critical role of GluR2/PICK1 interaction and GluR2 phosphorylation at S880 site in regulating activity-dependent GluR2 internalization and LTD expression in cerebellum (Chung et al., 2003; Gardner et al., 2005; Hanley and Henley, 2005; Liu and Cull-Candy, 2005; Perez et al., 2001; Steinberg et al., 2006). Moreover, protein interaction also occurs between GRIP/ABP and PICK1, and therefore regulates constitutive surface GluR2 expression, activity-dependent endocytosis of GluR2, and recycling of internalized GluR2 (Lu and Ziff, 2005).

The intracellular C-termini of GluR2 also interact with non-PDZ proteins, namely NSF (N-ethylmaleimide sensitive factor) and AP2. A 10-amino-acid membrane-proximal segment of GluR2 tail interacts with NSF, a hexameric ATPase involved generally in membrane fusion events (Hay and Scheller, 1997). GluR2/NSF interactions is essential for both basal and dynamical maintenance of synaptic AMPA receptors but not directly required for LTD (Luthi et al., 1999; Nishimune et al., 1998; Noel et al., 1999; Osten et al., 1998; Song et al., 1998), and this synaptic stabilization of AMPA receptors involves disruption of GluR2/PICK1 interactions (Hanley, 2007; Hanley et al., 2002). Remarkably, the NSF binding site of GluR2 also binds to AP2, a clathrin adaptor complex important for endocytosis. This interaction may be involved in activity-dependent, but not ligand-dependent, AMPA receptor endocytosis (Lee et al., 2002).

While AMPA receptor trafficking is regulated by their divergent intracellular carboxyl tails, studies of stargazin uncovered additional regulatory mechanism of AMPA receptor synaptic targeting and channel function.

Stargazin is the mutated protein of an ataxic and epileptic mutant mouse called Stargazer, which lacks functional AMPA receptors on cerebellar granule cells (Chen et al., 1999; Hashimoto et al., 1999). Studies on stargazin-mediated AMPA receptor trafficking revealed that stargazin is
required for AMPA receptor synaptic targeting via two steps: recruiting AMPARs to the plasma membrane, then directing them AMPARs to postsynaptic sites and anchoring them in the active zone (Chen et al., 2000; Chen et al., 2003).

Stargazin may play a role in ER processing of AMPARs (Vandenberghe et al., 2005a; Vandenberghe et al., 2005b). GluR2 in Stargazer cerebellum has an immature ER-type glycosylation (Tomita et al., 2003), and stargazin-deficient neurons show suppressed residual AMPAR currents by inhibition of the unfolded protein response (UPR), a cellular stress response unique to the endoplasmic reticulum (Vandenberghe et al., 2005a). It has also been proposed that stargazin associates exclusively with AMPAR tetramers, but not monomers or dimmers, possibly via a binding site formed by the AMPAR dimer-dimer interface (Vandenberghe et al., 2005b). However, the role of stargazin in the AMPAR biosynthetic pathway remains largely unknown.

The intracellular carboxyl tail of stargazin appears to be critical for regulating AMPA receptor trafficking (Tomita et al., 2005a; Tomita et al., 2004). The interaction between stargazin and PSD-95 (postsynaptic density protein) may stabilize AMPA receptors at postsynaptic densities (Schnell et al., 2002). Phosphorylation of stargazin plays a critical role in stargazin/PSD-95 interaction. Phosphorylation of T321 in the PDZ binding domain (RRTTPV) of the stargazin intracellular carboxyl tail disrupts stargazin interaction and clustering with PSD-95. As a consequence, stargazin clustering at synaptic spines is eliminated and synaptic AMPAR function is downregulated (Chetkovich et al., 2002). Moreover, synaptic stargazin is heavily phosphorylated at a set of conserved serine residues in its intracellular carboxyl tail, a process that is regulated by synaptic activity such that LTP and LTD require stargazin phosphorylation and dephosphorylation, respectively (Tomita et al., 2005b). Furthermore, palmitate cycling of PSD-95 regulates surface and synaptic AMPA receptor clustering (El-Husseini et al., 2002).

Interestingly, stargazin not only regulates AMPAR synaptic trafficking but also modulates their electrophysiological properties, such as receptor desensitization, deactivation, and agonist efficacy, determined by the first extracellular and second transmembrane domains of stargazin (Priel et al., 2005; Tomita et al., 2005a). The domains of AMPA receptors responsible for stargazin-AMPAR interaction, however, remain unclear. The transmembrane domains of AMPA receptors have been
shown to be associated with stargazin by single particle electron microscopy (Nakagawa et al., 2005; Nakagawa et al., 2006). Evidence has been provided that the intracellular C-terminal domain of AMPA receptors might directly interact with stargazin, and that deletion of this domain reduces plasma membrane trafficking but has no effect on desensitization of AMPA receptors (Bedoukian et al., 2006). Allosterical change of the extracellular ligand binding site, formed by the S1 and S2 domains of AMPA receptors may be modulated by stargazin upon agonist binding (Nishimune et al., 1998; Tomita et al., 2005a; Turetsky et al., 2005). It has been most recently proposed that the Q/R editing site of AMPA receptors may regulate the interaction of AMPA receptors and stargazin via a conformational change, as stargazin-mediated channel gating was found to be dependent on the amino acid at the Q/R site, whereas the stargazin-mediated increase in trafficking toward the plasma membrane remained independent of this amino acid (Körber et al., 2007).

Thus, stargazin plays critical roles in AMPA receptor trafficking and channel gating, and has even been proposed to represent the auxiliary subunit of AMPA receptors (Vandenberghe et al., 2005b). In addition to stargazin (γ2), several homologs have been defined as a family of proteins, transmembrane AMPA receptor regulatory proteins (TARPs), including the γ3, γ4, γ7, and γ8 subunits of voltage-dependent calcium channels (Kato et al., 2007; Tomita et al., 2003). Immunoblotting shows distinct distributions of TARP members in the adult brain: stargazin (γ2) mainly in cerebellum, γ3 in cerebral cortex, γ4 in olfactory bulb, γ7 in cerebellum, and γ8 in hippocampus (Kato et al., 2007; Tomita et al., 2003). Stargazin-like TARPs may play a similar role in AMPA receptor trafficking that is not TARP subunit-specific (Rouach et al., 2005; Tomita et al., 2003; Tomita et al., 2004). However, recent evidence suggests that the composition of TARP/AMPA R complexes mainly determines the electrophysiological properties of AMPA receptors (Kott et al., 2007; Tomita et al., 2005a; Turetsky et al., 2005).
1.2 Kainate receptors

After the cloning of kainate receptor subunits in the early 1990s (Bettler et al., 1990; Egebjerg et al., 1991; Hollmann and Heinemann, 1994; Lomeli et al., 1992; Sommer et al., 1992), the discovery of the selective AMPAR antagonist GYKI 53655 (also known as LY300168, (Lerma et al., 2001; Paternain et al., 1995; Wilding and Huettner, 1995)) allowed the characterization of kainate receptors with respect to both their pharmacological and electrophysiological properties.

Kainate receptors are tetrameric cationic channels assembled from the five subunits GluR5, GluR6, GluR7, KA1 and KA2. The low kainate binding affinity (50 – 100 nM) subunits GluR5, GluR6 and GluR7 can form functional homomeric and heteromeric ion channels in recombinant systems. The high affinity (5 – 15 nM) subunits KA1 and KA2, while not able to form functional homomeric cationic channels, can coassemble with low affinity subunits, thereby forming functional heteromeric channels with alternative pharmacological properties. There is 75 – 80% homology between GluR5, GluR6, and GluR7 subunits, and 70% between KA1 and KA2 subunits. By contrast, the low affinity kainate receptor subunits share only 43% amino acid identity with high affinity kainate receptor subunits, and 37% with AMPA receptor subunits (Hollmann and Heinemann, 1994).

Similar to AMPA receptors, posttranslational modifications such as RNA editing and alternative splicing generate diversity of low affinity kainate receptors. The amino acid at the Q/R editing site in the pore region of GluR5 and GluR6 (Sommer et al., 1992), similar to the AMPA receptor subunit GluR2, determines Ca\(^{2+}\) permeability and the current-voltage relationship of receptors containing this subunit (Bahring et al., 1997; Bowie and Mayer, 1995; Burnashev et al., 1995). In addition, two other edited codons in the first transmembrane domain (I/V and Y/C) were found in the GluR6 subunit, and cause fully edited GluR6 to exhibit higher impermeability to Ca\(^{2+}\) (Burnashev et al., 1995; Köhler et al., 1993) whereas fully un-edited GluR6 exhibits higher unitary conductance (Howe, 1996; Swanson et al., 1996). However, unlike the situation at AMPA receptors, where virtually all native GluR2 subunits (> 99%) are present in the edited form, native low affinity kainate receptor subunits in most tissues are present in both edited and un-edited forms (Hollmann, 1999). Furthermore, alternative splice variants exist for GluR5 which differ at their
extracellular LIVBP domains and their intracellular C-terminal domains. In addition, GluR6 and GluR7 differ also at their intracellular C-terminal domains (Bettler et al., 1990; Gregor et al., 1993; Schiffer et al., 1997; Sommer et al., 1992).

*In situ* hybridization experiments showed that GluR5, GluR6, GluR7, and KA2 subunits are distributed throughout the central nervous system including cortex, striatum, hippocampus, and cerebellum, whereas KA1 is strongly expressed in hippocampal CA3 and dentate granule neurons (Huettner, 2003). Unlike the extensively investigated AMPA receptors, which are closely related to kainate receptors and categorized together with them as non-NMDA receptors, the physiological role of kainate receptors remains largely unknown. Recent work focused on directed trafficking of kainate receptors and its impact on functional properties. Such studies indicated that these receptors play a key role in synaptic transmission and are modulating synaptic networks depending on their subunit composition, subcellular localization (presynaptic, postsynaptic, and extrasynaptic domains), and membrane delivery.

Similar to AMPA and NMDA receptors, postsynaptic kainate receptors respond to synaptically released glutamate: repetitive activation of the hippocampal mossy fibre pathway generates slow EPSCs (excitatory postsynaptic currents) in hippocampal CA3 neurons (Castillo et al., 1997; Vignes and Collingridge, 1997), and similar EPSCs were also reported for hippocampal CA1 interneurons (Cossart et al., 1998; Frerking et al., 1998) as well as for other types of neuronal cells in neocortex, spinal cord, retina, and amygdala (Pinheiro and Mulle, 2006). In addition to synaptic excitation, postsynaptic kainate receptors are also proposed to contribute to the regulation of neuronal excitability (Pinheiro and Mulle, 2006). This noncanonical pathway is proposed to involve kainate receptor subunits in metabotropic activities triggering G-protein-coupled second messenger cascades (Melyan et al., 2004; Melyan et al., 2002; Rozas et al., 2003; Ruiz et al., 2005).

Evidence has been provided for the existence of presynaptic kainate receptors, especially in hippocampal mossy fibres, the axons of dentate granule cells (Nicoll and Schmitz, 2005). Presynaptic kainate receptors can be activated by endogenous glutamate released from both mossy fibre synapses and the neighbouring associational/commissural synapses (Kamiya and Ozawa,
Both autoreceptor and heteroreceptor functions have been suggested for presynaptic kainate receptors (Pinheiro and Mulle, 2006) and are related to receptor composition (Contractor et al., 2003; Contractor et al., 2001; Pinheiro and Mulle, 2006; Pinheiro et al., 2007). These receptors may exhibit biphasic effects in both facilitating and inhibiting neurotransmitter release in glutamatergic (excitatory) and GABAergic (inhibitory) synapses (Huettner, 2003; Nicoll and Schmitz, 2005; Pinheiro and Mulle, 2006). In addition, presynaptic kainate receptors may play a role in hippocampal mossy fibre LTP, a widely studied model of NMDAR-independent presynaptic LTP, although they are not essential for its induction (Lauri et al., 2003; Schmitz et al., 2003).

Finally, extrasynaptic somatodendritic kainate receptors have been proposed to modulate inhibitory synaptic transmission in some brain regions including hippocampus (Huettner, 2003; Pinheiro and Mulle, 2006).

Directed trafficking of kainate receptors to axons and dendrites is strongly related to receptor function; however, the rules governing this trafficking remain unknown. Recent studies revealed that the subunit composition of the receptor as well as several key domains of the receptor subunits play critical roles in regulating kainate receptor trafficking to plasma membrane.

The intracellular carboxyl domain of kainate receptor subunits plays a central role in determining ER retention/retrieval or ER exit of the receptor, and therefore regulates plasma membrane delivery of the receptors. An ER retention/retrieval motif (RXR) of the KA2 subunit and a forward trafficking motif (CQRRLKHK) of GluR6a and GluR7a subunits have been characterized to be crucial for receptor ER retention and exit, respectively (Jaskolski et al., 2004; Jaskolski et al., 2005; Ren et al., 2003a; Yan et al., 2004). The arginine-rich ER retention/retrieval motif of KA2 (RXR) interacts with COPI (coatamer protein complex I), which plays a critical role in misfolded/unassembled protein retrograde trafficking from the Golgi apparatus to the ER (Vivithanaporn et al., 2006). In addition, two dileucine motifs in the C-terminal domain of the KA2 subunit which may be involved in clathrin-dependent endocytosis, may serve as an endocytotic signal (Ren et al., 2003a).
As for the low affinity kainate receptor subunits, an intracellular C-terminal key region regulates the distinct surface expression of splice variants. The splice variants of GluR6 and GluR7 subunits containing the forward trafficking motif (CQRRLKHK) are predominantly expressed at the plasma membrane, whereas variants lacking this motif are not (Jaskolski et al., 2005; Yan et al., 2004). Interestingly, similar to KA2, there is an RXR motif in the carboxyl tail of GluR5c that prevents the surface expression of this isoform of the GluR5 subunit (Jaskolski et al., 2004; Ren et al., 2003a). An additional ER retention motif consisting of a critical arginine (R896) and its surrounding amino acids was identified for GluR5b, explaining the poor surface expression of this isoform. However, a homologous motif in GluR6 and GluR7 appears to be functionally disabled (Ren et al., 2003b).

In addition, a very recent report demonstrated that the intracellular loop between the pore and the second transmembrane domains of KA2 determines receptor ER retention and thus plays a role in KA2 surface expression as well (Nasu-Nishimura et al., 2006). However, when KA2 is associated with either of the low affinity kainate receptor subunits GluR5-7, the ER retention/retrieval motifs in the carboxy-terminal region of KA2 may be masked and the plasma membrane expression of KA2 is facilitated (Gallyas Jr et al., 2003; Ren et al., 2003a). Subunit composition of heteromeric kainate receptors hence critically regulates plasma membrane delivery of kainate receptors (Gallyas Jr et al., 2003; Jaskolski et al., 2004; Nasu-Nishimura et al., 2006; Ren et al., 2003a).
1.3 Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes

Since the cloning of the first functional subunit of ionotropic glutamate receptors (Hollmann et al., 1989), many different expression systems have been used to study the functional properties of these ligand-gated ion channel proteins. Among the most frequently used heterologous expression systems are cultured mammalian and insect cells, and neuronal primary cell cultures. To use these cells in investigations of cell biological, biochemical, structural, and electrophysiological properties of glutamate receptors, transient transfection methods commonly are employed.

As functional glutamate receptor complexes are assembled from four identical or different subunits (Hollmann, 1999; Rosenmund et al., 1998), the successful coexpression in a single cell of all target subunits of an intended heteromer obviously is a key prerequisite. To achieve this, transient cotransfection of various combinations of glutamate receptor subunit-encoding cDNAs in cultured cells is routinely being used. Yet, this method is based on the ‘all-or-none’ assumption that any given cell expresses all subunits present during transfection, an assumption that rarely is verified experimentally. In one such study, Vicini and colleagues (Vicini et al., 1998) tried to address this issue with immunocytochemistry by double staining of NR2A and NR2B subunits in HEK 293 cells triple-transfected with NR2A, NR2B, and NR1a cDNAs. While showing ‘most’ cells were stained by both NR2A and NR2B antibodies following cotransfection at various cDNA ratios, the authors pointed out the fact that the relative proportion of cells with electrophysiological properties of the ternary combination was rather small. For visualizing transfected cells in electrophysiological experiments, cotransfection of a fluorescent protein-encoding ‘transfection marker’ cDNA together with one or more receptor subunits is frequently applied (Coussen et al., 2005; Fleck et al., 2003; Marshall et al., 1995; Schwarz et al., 2001; Yan et al., 2004). This approach carries the implicit assumption that, in any cell transfected with the transfection marker, the target subunits are also expressed. A further assumption is that heteromeric receptor complexes assemble according to the ratio between the amounts of subunit cDNAs present during transfection. This is an important issue especially in studies aimed at the analysis of the stoichiometric assembly
of receptors in general, and glutamate receptors in particular (Mansour et al., 2001; Robert et al., 2001), which are non-symmetrical tetramers (Ayalon and Stern-Bach, 2001; Laube et al., 1998; Schorge and Colquhoun, 2003).

The purpose of the present study was to critically analyze these assumptions and test their validity. The efficiency of the commonly used calcium phosphate transfection method for transiently introducing exogenous cDNAs into HEK 293 cells (Chen and Okayama, 1987; Graham et al., 1977) was investigated. A calcium phosphate transfection procedure was used without chemical shock which yields higher transfection efficiencies compared to the original method (Chen and Okayama, 1987). By generating fluorescently-labeled fusion proteins with different fluorophores we took advantage of easy visualization and excellent separation of fluorescence signals of the fluorescently-tagged receptor subunits, and evaluated the coexpression efficiencies under various conditions. The most important question addressed in this study is whether multiple populations of transfected cells carrying different receptor subunit combinations are being formed after transient cotransfection with multiple cDNAs. It was found that when cotransfected with a fluorescent marker protein-encoding cDNA, expression of a receptor subunit is not necessarily concomitant with the expression of the fluorescent marker, and vice versa. Moreover, when two receptor subunit cDNAs are transfected simultaneously, there will be a significant population of transfected cells expressing only a single subunit. In addition, cotransfections of triple and quadruple combinations of receptor subunit cDNAs were tested, and a similar pattern was demonstrated as observed for double transfections.
2. Aims of the study

The intracellular trafficking and surface delivery of the receptors are probably under tight control so that only fully assembled functional receptors can be expressed at the plasma membrane. In order to monitor homomeric and heteromeric receptor complexes, various fluorescent proteins, namely ECFP, EGFP, EYFP, and DsRed2, were used to label selected non-NMDA glutamate receptor subunits and the encoding recombinant cDNAs were transiently transfected into cultured mammalian cells. Establishment of one of the most challenging techniques in modern biological research, live cell confocal imaging, is thus a prerequisite for the project.

By expression of fluorescent protein-fused ionotropic glutamate receptor subunits in HEK 293 cells and digital imaging with confocal microscopy, attempting high temporal and lateral resolution, visualization, and digital image analysis (staining or labeling of intracellular organelles, digital colocalization analysis, and FRET measurement in live cells, etc.) of live trafficking of glutamate receptor subunits and complexes, may help identify the rules governing assembly of functional ion channels. Specially, the following experiments are to be conducted:

-- Monitor the formation of homomeric and heteromeric receptor complexes;
-- Test hypotheses of regulatory mechanisms of the assembly progress;
-- Study possible modulation of assembly and trafficking of receptor complexes by RNA editing at the Q/R site within the pore region;
-- Answer the question whether receptor complexes assemble before subunit exit from the ER;
-- Find out the role of the Golgi apparatus in assembly and trafficking of glutamate receptors;
-- Investigate the role of stargazin in regulating AMPA and/or kainate receptor plasma membrane delivery;
-- Explore the unique trafficking mechanism for kainate receptors which has been proposed.
Furthermore, receptor trafficking is likely to be a key factor in regulating synaptic strength. Thus, monitoring receptor retention and trafficking in subcellular compartments of neurons will help to understand the dynamic surface membrane insertion and distribution of the glutamate receptors, hence, the dynamic organization of synapses. Preliminary experiments will be carried out with primary neuronal cultures.

Transient transfection of cultured mammalian cells is widely employed in the study of ionotropic glutamate receptors. Heteromeric expression is usually achieved by simultaneous transfection of various combinations of glutamate receptor subunit-encoding cDNAs. A side project evaluating cotransfection efficiencies in heterologous expression systems for the electrophysiological study of heteromeric receptor complexes is to be performed. The aim is to check the validity of the following assumptions for ionotropic glutamate receptors as model transmembrane receptors:

-- An ‘all-or-none’ assumption, rarely verified experimentally, that any given cell expresses all subunits present during transfection;

-- A similar assumption implicitly is made when cotransfection of a cDNA encoding a fluorescent marker protein is applied to distinguish transfected from untransfected cells;

-- A further frequent assumption alleges that the ratio between cDNAs used in cotransfection experiments governs the assembly of receptor complexes in heterologous expression systems.
3. Materials and Methods

3.1 Molecular cloning

3.1.1 Polymerase chain reaction (PCR)

In principle, a PCR reaction contains: 1 × PCR buffer with the desired concentration of Mg$^{2+}$, 0.2 mM dNTPs, 1 μM of each primer, template DNA, and polymerase. The cycling reactions were started after a short denaturation (94°C or 98°C for 1 to 5 min, depending on the polymerase used) and usually consisted of three major steps which were repeated for 20 to 30 cycles:

- **Denaturation**: 94°C or 98°C for 45 sec to 1 min, depending on the polymerase;
- **Annealing**: 50°C to 65°C for 30 sec to 1 min, depending on the annealing temperature of the primers as well as the polymerase;
- **Extension**: 72°C for 30 sec to 2 min, depending on the length of expected DNA fragment to be amplified.

The reactions were then held at 4°C after a final extension step at 72°C for 10 min.

In this work, several PCR methods were utilized in order to construct cDNA encoding recombinant proteins. These methods included mutagenesis PCR and overlap extension PCR to introduce point mutations and to fuse two or more DNA fragments. PCR strategies for specific constructs can be found in Chapter 4.1. The primers designed for cloning purposes are listed below:

- **P267**: 5’- GTT CTT CAG GAG ATC TAA AAT CGC TGT GT -3’
- **M310**: 5’- CTG TCT GCT CCG TCG ACA ATC CTG TGG CT -3’
- **P268**: 5’- TTT CAG GAG ATC TAA AAT CGC AGT G -3’
- **M311**: 5’- CTC AAG GTC ATC TCG AGA ATT TTA ACA CTC T -3’
- **P314**: 5’- TGG TTT TTC ACA ATC ATC ATT TCT TC -3’
- **M335a**: 5’- CAG ACC GGT GCC ATG GTT TCT TTA C -3’
- **P345**: 5’- CCA CAG GCG CCT CAA TTG CAA CCT CA -3’
- **M355**: 5’- CCC CGT CGT CGA CCA TTC ATG CTC AGT -3’
- **TP90**: 5’- GAA GAG ATC TCC ATG TAC ACC CTC AGT AG -3’
Materials and methods

M339: 5’- GCA TGT CGA CAC GGG CGT GGT C -3’
P433: 5’- TTA ATT CGA GCT CGG TAC CCA -3’
TP142: 5’- CAT TGG ATC CGT CGA CCA GGA TGA TGA CAT CTG -3’
TP143: 5’- CAT TGG ATC CGT CGA CAC GGG CGT GGT CCG GCG -3’
P511: 5’- GCT AGC GCT ACC GGA CTC AGA TCT CGA -3’
M453: 5’- GGG TCT TT G CTC AGC TTG GAC TGG GTG C -3’
M454: 5’- GGG GTC TTT GCT CAG CTT GGA CTG GTA G -3’
P542: 5’- CGA CTC ACT ATA CGG AGA CC -3’
TP226: 5’- CCT CGC CCT TGC TCA CCA TGG CAC CCA AAT TCC CCA ACA ATA TCC -3’
TP227: 5’- GCA TGG TCG AGC TGT ACA AGG CCA ATT TCC CCA ACA ATA TCC -3’
P543: 5’- GAG AAC TGG GAA CAG AAA CGG -3’
TP225: 5’- GGT GCG GTT GTG GGT GCC ATG GTG AGC AAG GCC GAG G -3’
TP228: 5’- GGA TAT TGT TGG GGA AAT TGG CCT TGT ACA GCT CGT CCA TGC -3’
TP230: 5’- CCT CGC CCT TGC TCA CCA TGA CAC CAA AAA TCA GTC CCC A -3’
TP231: 5’- GCA TGG ACG AGC TGT ACA AGG TCT CTT CTA ACA GCA TAC AGA -3’
P544: 5’- TCC AGA GAC ATT TGC TCC TCC -3’
TP229: 5’- TGG GGA CTG ATT TTT GGT GTC ATG GTG AGC AAG GCC GAG G -3’
TP232: 5’- TCT GTA TGC TGT TAG AAG AGA CCT TGT ACA GCT CGT CCA TGC -3’
TP233: 5’- GCA TGG ACG AGC TGT ACA AGA TGG GCC TGT TTG ATC GAG GT -3’
P546: 5’- GTCC TGC ACA GAC CCT TGA AGT -3’
P545: 5’- GGG AGG TCT ATG AAA TAA GCA GAG -3’
TP234: 5’- ACC TGG TAT TCT AAA CAG CCC CAT CTT GTA CAG CTC GTC CAT GC -3’

3.1.2 Restriction

Purified from various bacterial strains, the restriction endonucleases recognize particular palindromic DNA sequences and cleave at specific sites. A typical restriction reaction containing a desired amount of the target DNA, 1 × restriction buffer, 1 × BSA if essential, and one or two of
the restriction enzymes (1 – 3 units each / μg DNA) was carried out at the desired reaction temperature of the enzyme (37°C if not specified otherwise) for 1 – 2 hours. Special restriction methods, for example partial digestions were performed according to the most favourable condition after optimization.

3.1.3 Purification of DNA fragments

The DNA band of the desired size was sliced from an agarose gel (0.8 – 2%) under long wave UV light, and purified with one of the following kits according to the manufacturer’s handbook: QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and JETSORB Gel Extraction Kit (Genomed, Löhne, Germany).

3.1.4 Ligation

After purification of the DNA fragment and the vector to be used for ligation, usually the linearized vector was dephosphorylated with Shrimp Alkaline Phosphatase (SAP, Amersham, Munich, Germany) (~ 0.5 unit / 5 μg DNA) for 30 min at 37 ºC, followed by deactivation for 10 min at 70 ºC. The insert fragment and vector was then incubated at a proper ratio, together with 1× ligation buffer and T4 DNA ligase (0.5 – 1 unit) (Promega, Mannheim, Germany) at 4 ºC overnight. Special ligation methods, for example, three fragment ligations (= two insert fragments and vector simultaneously) were used if required by particular cloning strategy (Chapter 4.1).

3.1.5 Transformation

Competent cells of various E.coli strains, namely DH5α, TG-1, and TOP10, were prepared according to the standard protocol for the production of chemically competent cells by the CaCl2 method (Sambrook and Russell, 2001). The competent cells were added to the ligation mixture and incubated for 1 hour on ice. A proper amount of the mixture was then plated on LB plates (with desired antibiotics for selection) and incubated at 37 ºC overnight.
3.1.6 Preparation of plasmid DNA

3.1.6.1 Mini DNA preparation

A rapid DNA mini preparation method was performed according to the standard protocol of alkaline lysis method (Sambrook and Russell, 2001).

3.1.6.2 Midi DNA preparation

For purification of plasmid DNA in the range of 50 – 200 μg, a midi preparation was carried out with one of the following kits according to the manufacturer’s handbook: QIAGEN Plasmid Midi Kit (QIAGEN), and JETSTAR 2.0 Plasmid Purification Kit (Genomed).

3.1.7 Vectors and plasmids

3.1.7.1 N-terminally fluorescent protein vectors

The vectors pECFP-N1, pEGFP-N1, pEYFP-N1, and pDsRed2-N1 (BD Clontech, Heidelberg, Germany) were used to generate fusion proteins with the gene of interest inserted and fused to the N-terminus of the fluorescent protein (Figure 3.1). The fusion proteins were to be expressed in mammalian cells under the control of the CMV IE promoter (human cytomegalovirus immediate early promoter). The mutations introduced to shift the excitation and emission peaks compared to wildtype GFP or DsRed, or to enhance the brightness and solubility of the proteins, are listed in Table 3.1. In addition, there are more than 190 silent base pair changes resulting in human codon optimization for a high expression in mammalian systems.

![Figure 3.1. Vector map of the N-terminal fluorescent protein vectors](adapted from www.clontech.com)
**3.1.7.2 Subcellular localization vectors**

pECFP-ER and pDsRed2-ER (BD Clontech) were used for labeling the endoplasmic reticulum (ER) in live mammalian cells. The plasmids encode fusion proteins consisting of ECFP or DsRed2, with the ER targeting sequence of calreticulin at the N-terminus, and the ER retention sequence KDEL at the C-terminus of the fluorescent protein (Fliegel et al., 1989; Munro and Pelham, 1987). The fusion proteins are soluble proteins localizing in the lumen of the ER in expressing cells.

pECFP-Golgi (BD Clontech) was used for labeling the Golgi apparatus in live mammalian cells. The plasmid encodes fusion proteins consisting of ECFP and a sequence encoding the N-terminal 81 amino acids of human beta-1,4-galactosyltransferase (GT). This region contains the membrane-anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus (Llopis et al., 1998; Watzele and Berger, 1990; Yamaguchi and Fukuda, 1995).

**Table 3.1 Some spectral variants of GFP (1 – 3) and DsRed (4)**

<table>
<thead>
<tr>
<th></th>
<th>Excitation Maxima (nm)</th>
<th>Emission Maxima (nm)</th>
<th>Chromophore Mutations</th>
<th>Additional Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ECFP</td>
<td>433 &amp; 453</td>
<td>475 &amp; 501</td>
<td>Tyr-66 to Trp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Phe-64 to Leu, Ser-65 to Thr, Asn-146 to Ile, Met-153 to Thr, and Val-163 to Ala</td>
</tr>
<tr>
<td>2</td>
<td>EGFP</td>
<td>488</td>
<td>507</td>
<td>His-231 to Leu</td>
</tr>
<tr>
<td>3</td>
<td>EYFP</td>
<td>513</td>
<td>527</td>
<td>Ser-65 to Gly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Val-68 to Leu, Ser-72 to Ala, and Thr-203 to Tyr</td>
</tr>
<tr>
<td>4</td>
<td>DsRed2</td>
<td>558</td>
<td>583</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arg-2 to Ala, Lys-5 to Glu, Lys-9 to Tyr, Ala-105 to Val, Ile-161 to Tyr, and Ser-197 to Ala</td>
</tr>
</tbody>
</table>

**3.1.7.3 Other mammalian expression vectors**

pcDNA3 (Invitrogen, Karlsruhe, Germany) is a commonly used mammalian expression vector. The gene of interest is controled by a CMV promoter as shown in Figure 3.2.
pcDNA4-TO-FLAG is a vector based on pcDNA4/TO/myc-His (Invitrogen), with the myc epitope exchanged by the FLAG epitope (DYKDDDDK) utilizing Xba I and Age I sites. The plasmid encodes fusion proteins carrying FLAG and 6× His tags where the gene of interest is fused to the N-terminus of the FLAG epitope.

3.2 Cell culture

3.2.1 HEK 293 cell culture

3.2.1.1 Maintenance of HEK 293 cells

Human embryonic kidney (HEK) 293 cells (Acc 305, DSMZ, Braunschweig, Germany) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma D5523, Taufkirchen, Germany) (with Earle’s BSS, 4 mM L-glutamine, 1 mM sodium pyruvate, low glucose, 3.7 g/L NaHCO₃, 100 μg/ml streptomycin, and 100 units/ml penicillin) supplemented with 10% heat-inactivated FBS (Invitrogen / GIBCO 10270-106), 1× non-essential amino acids (Invitrogen / GIBCO 11140-035), at 37°C, 8% CO₂ with humidity control.

Single layer HEK 293 cells can also be maintained in Minimum Essential Medium (Eagle) (MEM, Sigma M0268) (with Earle’s BSS, 2 mM L-glutamine, 2.2 g/L NaHCO₃, 100 μg/ml streptomycin, and 100 units/ml penicillin) supplemented with 10% FBS, 1× non-essential amino
acids, and 1 mM sodium pyruvate (Invitrogen / GIBCO 11360-039) at 37°C, 5% CO₂ with humidity control.

3.2.1.2 Revival of HEK 293 cells

HEK 293 frozen stock was quickly thawed in a 37°C water bath, and dropped slowly into 10 ml pre-warmed DMEM (without antibiotics) containing 20% FBS. The cells were pelleted by centrifugation at 900 rpm for 5 min at room temperature, and resuspended carefully in 10 ml medium. The centrifugation and resuspension was repeated once. The cells were then seeded into T-75 flasks (Nunc, Nunclon Surface, Wiesbaden, Germany) at the desired density (1 – 5×10⁶). One day after revival, the medium was changed to complete medium containing 20% FBS.

3.2.1.3 Passaging of HEK 293 cells

The doubling time for HEK 293 cells was around two days. Confluent cells were washed with 5 ml pre-warmed PBS (for T-75 flasks), and treated with 2 ml trypsin-EDTA (Invitrogen / GIBCO 25300-054) for one min at 37°C. 2 ml pre-warmed complete medium was added to the flask to stop trypsinization. The detached cells were collected into a Falcon tube containing 8 ml complete medium, pelleted by centrifugation at 900 rpm for 5 min at room temperature, resuspended in 10 ml medium, and then seeded into T-75 flasks at the desired density.

3.2.1.4 Preparation of HEK 293 frozen stocks

Confluent cells were collected by trypsin-EDTA treatment as described above. The cells were resuspended carefully and maintained in 70% medium, 20% FBS, and 10% DMSO (Sigma D2650), stored at -80 °C overnight. On the next day the frozen vials were transferred into a nitrogen tank for long term storage at -196 °C.
3.2.1.5 Transient transfection of HEK 293 cells (modified calcium phosphate method)

Single layer HEK 293 cells were seeded at a density of $5 \times 10^4$ on polyornithine-coated (or poly-D-lysine- (Sigma P6407) coated) 35 mm glass bottom dishes (WillCo Wells, Amsterdam, Netherlands) 48 hours before transfection. About 2 hours before transfection, the cells were washed with pre-warmed PBS and incubated in 500 µl DMEM (without antibiotics) at 3% CO$_2$. In brief, compared to the classical method (Chen and Okayama, 1987), a DNA-calcium chloride mixture was prepared at first and this mixture was incubated at room temperature for 20 min. The DNA-calcium chloride mixture contained the desired amount of DNA and 10 µl 2.5 M CaCl$_2$, adjusted to 100 µl volume with Millipore water. 100 µl 2×HBS buffer (280 mM NaCl, 1.5 mM Na$_2$HPO$_4$, 40 mM HEPES, pH 7.1 – experimentally found to be optimal) was then added drop-wise to the above mixture, and the mixture was incubated in the dark for at least another 20 min at room temperature. 500 µl pre-warmed medium (without antibiotics) was dropped into the DNA-calcium precipitate with gentle mixing. The precipitate was then added drop-wise to the cells covering the whole surface of the culture dish. The cells were incubated with the precipitate for at least 15 hours at 37°C, 3% CO$_2$.

For patch clamp recording, cells were seeded in 35 mm plastic dishes (Nunc), and were split 2 days after transfection.

For Western blots, cells were seeded at a density of $1 \times 10^6$ in 90 mm plastic dishes (Nunc).

3.2.1.6 Transient transfection of HEK 293 cells (by Metafectene Pro)

The transfection was carried out according to the manufacturer’s handbook (Metafectene Pro, Biontex, Martinsried/Planegg, Germany). The combination of $2 + 2$ µg of GluR6-ECFP and GluR6-EYFP cDNAs, respectively, was used as well as the single cDNAs. The ratio of DNA to Metafectene Pro was optimized at 1:3 for the experiments.
3.2.1.7 Transient transfection of HEK 293 cells (by PolyFect)

The transfection was carried out according to the manufacturer’s handbook (PolyFect, QIAGEN, Hilden, Germany). The combination of 2 + 2 µg of GluR6-ECFP and GluR6-EYFP cDNAs, respectively, was used as well as the single cDNAs.

3.2.1.8 Transient transfection of HEK 293 cells (biolistic transfection method)

The experiments were carried out with 10 + 10 µg of GluR6-ECFP and GluR6-EYFP, respectively. cDNA coating of the gold particles and preparation of the cartridges were performed as described previously (Wirth and Wahle, 2003). A hand-held Helios gene gun (Biorad, Munich, Germany) was used directly on 35 mm polyornithine-coated glass bottom dishes, with the optimal Helium pressure set to 60 – 120 psi.

3.2.2 Primary culture of hippocampal and cortical neurons

3.2.2.1 Preparation of mixed glial cell culture, microglia culture and neuron culture

Under sterile conditions, the hippocampi or cortices were dissected on ice from early postnatal rats (P0 – P2), and incubated in 1 ml (for two hippocampi or one cortex) HBS (Invitrogen / GIBCO, Karlsruhe, Germany) containing DNase and trypsin at 37°C for 8 – 18 min. The supernatant from each vial was transferred into a Falcon tube containing 2 ml DMEM supplemented with 10% FBS. 900 µl pre-cooled HBS was added to each dissociation mixture for a careful trituration. After a brief incubation on ice, the supernatant was also transferred to the above Falcon tube. 450 µl pre-cooled HBS was then added again to each dissociation mixture and the supernatant was transferred as described above. The cells were centrifuged at 1000 rpm for 10 min at 4 °C and resuspended with 5 ml DMEM.

The hippocampal and cortical cell resuspensions were seeded into 90 mm plastic dishes separately, and incubated for 1 hour at 37°C, 5% CO2. After the pre-plating procedure, the neuron suspension from the dishes was collected and re-suspended in pre-warmed DMEM. The dissociated hippocampal or cortical neurons were then seeded into either 35 mm glass bottom
Materials and methods

dishes (coated with poly-D-lysine and laminin (20 μg/μl in Q, Invitrogen / GIBCO 23017-015), or
for co-culture on top of passaged microglia culture (2×10^5 hippocampal neurons / 35 mm dish) at
the desired density in DMEM at 37°C, 5% CO2 with humidity control. One day after dissociation,
the medium was exchanged to Neurobasal-A medium (Invitrogen / GIBCO 10888-022)
supplemented with 0.02 mg/ml insulin, 0.02 mg/ml transferrin, 5% FBS, 1× B27 supplement
(Invitrogen / GIBCO 17504-044), 1× GlutaMAX I (Invitrogen / GIBCO 35050-038), 1 μg/ml
gentamycin (Invitrogen / GIBCO 15750-037). Two days after dissociation, 5 μM cytosine
arabinoside was added to the medium.

If transient transfection by the nucleofactor method was to be performed (see Chapter 3.2.2.3),
the neurons were seeded after the nucleofection.

The pre-plated mixed hippocampal cultures were discarded, while the mixed cortical cultures
were maintained in DMEM for microglia culture.

3.2.2.2 Maintenance and passaging of microglia culture

A mixed cortical culture was obtained from P0 – P2 rat cortical dissociation as described above.
The microglia culture was maintained in DMEM containing 10% FBS at 37°C, 5% CO₂ with
humidity control.

Confluent cells (approximately 1×10^6 / dish, usually four weeks in culture after dissociation)
were washed with 5 ml pre-warmed PBS (for 90 mm dish), treated with 1.5 ml trpsin-EDTA for 20 –
45 min at 37°C. 1 ml pre-warmed complete medium was added to the flask to stop
trypsinization. The detached cells were collected into a Falcon tube containing 2 ml complete
medium, pelleted by centrifugation at 900 rpm for 9 min at room temperature, resuspended in 3 ml
medium, and then seeded in 35 mm glass bottom dishes at the desired density (5×10^4 – 1×10^5). 1 –
4 weeks after passaging, the culture was used as a supporting layer for primary neurons.
3.2.2.3 Transient transfection of hippocampal and cortical neurons (nucleofection method)

The nucleofection was carried out on the same day as the dissociation of the microculture, following the protocol of the Rat Neuron Nucleofector Kit (amaxa, Köln, Germany). In brief, 300 µl DMEM was incubated in pre-coated glass bottom dishes at 37°C, 5% CO₂ before transfection. The neurons were resuspended in room temperature Rat Neuron Nucleofector Solution to a final concentration of 4 – 5×10⁶ cells / 100 µl. The desired amount of DNA (1 – 3 µg) was mixed with the cell suspension and transferred into a cuvette for performance of nucleofection. Program 0-03 was selected for the transfection. The cuvette was removed immediately after the program was finished. 500 µl pre-warmed DMEM was added to the cell suspension, and the mixture was transferred into the prepared dishes by a plastic pipette. The cells were incubated with the DNA mixture for 2 – 4 hours at 37°C, 5% CO₂. 24 hours after transfection, the medium was replaced with fresh Neurobasal-A medium supplemented with 0.02 mg/ml insulin, 0.02 mg/ml transferrin, 5% FBS, 1× B27 supplement, 1× GlutaMAX I, 1 µg/ml gentamycin, and 5 µM cytosine arabinoside.

3.2.2.4 Transient transfection of hippocampal and cortical neurons (calcium phosphate method)

The calcium phosphate transfection was carried out with hippocampal or cortical neurons 8 – 9 days in vitro (DIV). In brief, a DNA-calcium chloride mixture was prepared and incubated at room temperature for 20 min. The DNA-calcium chloride mixture contained the desired amount of DNA and 3.6 µl 2.5 M CaCl₂, adjusted to 40 µl volume with Millipore water. 40 µl 2×BBS buffer (280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM BES, pH 7.06) was then added drop-wise to the above mixture, and the mixture was incubated in the dark for at least another 20 min at room temperature. 800 µl conditioned medium was dropped into the DNA-calcium precipitate with gentle mixing. The precipitate was then added drop-wise to the cells covering the whole surface of the culture dish. The cells were incubated with the precipitate for 3 hours at 37°C, 5% CO₂.
3.3 Cell counting for estimation of expression efficiency and coexpression efficiency

HEK 293 cells were assayed for expression of fluorescent proteins at day 3 or day 4 after transfection to allow efficient detection of all fluorophores, even the possibly more slowly folding DsRed2. For each individual combination of constructs used for co-transfection, 6 - 16 different areas of $5.6 \times 10^4 \mu m^2$ each were counted from 3 – 5 independent transfections (see Table 4.11, Chapter 4.4). All images were acquired on a Leica TCS SP2 AOBS confocal microscope (Leica, Mannheim, Germany). To prevent cross talk from distorting the data in dual and multiple color imaging, a sequential scanning method was employed (see Chapter 3.8.1). All the data are presented as mean ± SEM if not specified otherwise. Significance was calculated using an unpaired t-test implemented in the program Origin 6.0.

3.4 Patch clamp recording

Whole cell recordings of HEK 293 cells were performed with a HEKA EPC-9 amplifier controlled by Pulse 8.7 software (HEKA, Lambrecht, Germany) 3 days after transfection. Currents were digitalized with a sampling rate of 10 kHz and filtered at 3 kHz. (Bessel-low pass filter). The external solution contained 10 mM HEPES, 140 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, and 1 mM MgCl$_2$ (pH 7.3, adjusted with NaOH at room temperature). The internal solution contained 10 mM HEPES, 130 mM CsF, 33 mM KOH, 4 mM NaCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 11 mM EGTA, and 10 mM HEPES (pH 7.3, adjusted with KOH). The holding potential was -60 mV.

3.5 Protein biochemistry

3.5.1 Protein extraction from HEK 293 cells

HEK 293 cells were washed with pre-cooled PBS 3 days after transfection. 400 µl lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 40 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM Na$_3$VO$_4$, 1% SDS, 1% Triton X-100, 0.1% sodium desoxycholate) containing proteinase inhibitor (Proteinase inhibitor cocktail, Roche, Mannheim, Germany) was added to the cells and incubated for 1 hour on ice while shaking. The cells were scraped (cell scraper, A.Hartenstein, Würzburg,
Germany) from the dish and the suspension was homogenized on ice and centrifuged for 15 min at 14000 rpm 4°C. The supernatant was transferred to a new 1.5 ml tube. To achieve a highly concentrated protein sample, 4vols. pre-cooled acetone was added to the above supernatant and incubated at -70°C overnight. After centrifugation for 15 min at 14000 rpm 4°C, the protein precipitate was dried in a vacuum centrifuge for 5 min and redissolved in 10 µl ddH₂O and 10 µl 2× Laemmli buffer (125mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.006% bromophenol blue, 10% β-mercaptoethanol), or in 10 µl ddH₂O and 10 µl 2× loading buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol). The protein samples were stored at -20°C.

3.5.2 Western blot analysis

3.5.2.1 SDS-PAGE

The denatured cell lysate samples (15 – 25 µg total protein per lane) were loaded on an SDS-PAGE (7.5% resolving gel and 3% stacking gel were used for most of the GluR fusion proteins with molecular weight of around 130 KD). The gel electrophoresis was carried out in 1× TG buffer (25mM Tris, 190 mM glycine) containing 0.1% SDS at 20 mA for around 2 – 3 hours.

3.5.2.2 Wet transfer

The proteins were transferred from the gel to an nitrocellulose membrane (Hybond-ECL nitrocellulose membrane, RPN2020D, Amersham) in transfer buffer (25mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS) at 1 W constant power or equivalent (30 mA constant current) overnight at 4 °C. Optionally, the transfer efficiency was demonstrated either by the pre-stained protein ladder, or Ponceau S staining of all the protein samples on the membrane.
3.5.2.3 Immunodetection

3.5.2.3.1 Immunodetection with anti-GFP monoclonal antibody

The membrane was blocked with 5% skim fat milk in PBST (1 × PBS with 0.05% TWEEN-20) for 1 hour at room temperature, washed three times with PBST for 10 min. The primary antibody – anti-GFP monoclonal antibody (JL-8, Clontech 632380) was diluted in PBST at 1:1000 and incubated with the membrane for 2 hours at room temperature. The membrane was then washed three times with PBST for 10 min, and incubated in PBST with the 1:5000 diluted secondary antibody, anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma A4416), for 1 hour at room temperature. After washed four times with PBST for 10 min, the membrane was treated with peroxidase substrate (ECL, Amersham; or SuperSignal, PIERCE, Rockford, IL, USA) to detect the fluorescence signal (Fuji Medical X-ray film, Tokyo, Japan; or Hyperfilm ECL, Amersham).

3.5.2.3.2 Immunodetection with anti-DsRed monoclonal antibody

The membrane was blocked with 5% skimmed milk in PBST for 1 hour at room temperature, and washed three times with PBST for 10 min. The primary antibody, anti-DsRed monoclonal antibody (Clontech 632393), was diluted 1:100 in PBST and incubated with the membrane for 2 hours at room temperature. The membrane was then washed three times with PBST for 10 min, and incubated in PBST with a 1:5000-diluted secondary antibody, anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma A4416), for 1 hour at room temperature. After having been washed four times with PBST for 10 min each, the membrane was incubated in peroxidase substrate to detect the fluorescence signal.

3.5.2.3.3 Immunodetection with anti-FLAG M2 monoclonal antibody

The membrane was blocked with 5% skimmed milk in PBST for 1 hour at room temperature, and then washed twice with PBST for 5 min. The conjugated primary antibody, anti-FLAG M2 monoclonal antibody peroxidase conjugate (Sigma A8592), was diluted in PBST at 1:500 and incubated with the membrane for 1 hour at room temperature. The membrane was then washed six times with PBST for 5 min, and treated with peroxidase substrate to detect the fluorescence signal.
3.6 Immunoprecipitation

HEK 293 cells, 2 – 3 days after cotransfection with cDNAs encoding stargazin-FLAG and EGFP-tagged AMPA receptor subunit (GluR1 or GluR2) fusion proteins, were used for immunoprecipitation. It was carried out with FLAG Tagged Protein Immunoprecipitation Kit (Sigma FLAGIPT-1) according to the correlated technical bulletin (Sigma). The kit contains ANTI-FLAG-M2 affinity gel, which consists of a highly specific monoclonal antibody covalently attached to an agarose resin. Coprecipitated fusion proteins were eluted with elution buffer and then processed for Western blot analysis with anti-GFP monoclonal antibody.

3.7 Immunocytochemistry

For a control experiment designed to test the efficiency of fluorescent protein detection, HEK 293 cells transfected with cDNA of the ECFP-labeled kainate receptor subunit KA2, KA2-ECFP, were immunostained on day 5 after transfection. We used KA2 instead of GluR6 for this control as for this subunit a reliable antibody is commercially available. Briefly, cells were fixed with 4% paraformaldehyde for 20 minutes followed by membrane permeabilization with 0.1% Triton for 20 minutes. The cells were blocked with 5% goat serum at room temperature for 2 hours, incubated overnight with anti-KA2 antibody (1:500, Tocris, Bristol, United Kingdom) at 4°C, and then incubated with Alexa 488-conjugated goat anti-rabbit IgG (1:500, Invitrogen, Karlsruhe, Germany) at room temperature for 40 minutes.

3.8 Digital image acquisition, processing, and analysis

3.8.1 Live cell confocal imaging

All images were 8-bit-encoded digital images acquired on a Leica TCS SP2 AOBS confocal microscope (Leica). Live cell imaging was carried out with a micro Live Cell Incubation System (H.Saur, Reutlingen, Germany). To avoid cross talk or bleed through in dual and multiple color imaging, a sequential scanning method was employed so that all the emission spectra are sufficiently separated from each other at the initial step of image acquisition. The sequential
acquisition method was performed by exciting only one fluorophore at a time, and detecting within the range of the emission spectra of the fluorophore concomitantly.

### 3.8.2 Colocalization analysis by confocal microscopy

For the purpose of colocalization analysis, confocal images were acquired with an aqueous immersion objective (63×, NA 1.2) in live cell studies. A sequential acquisition method was usually applied in order to unambiguously distinguish the different emission spectra of different fluorophores in the sample. Selected dual or triple color composite images were analyzed with the Leica Confocal Software (LCS, Leica) for colocalization of different fluorophores. This digital analysis of colocalization of two or more fluorescent molecules provides information as to whether the fluorescence signals occupy the same pixel in the analyzed image. Compared to commonly used ‘visualized colocalization’ by simple overlapping, the digital colocalization analysis presents precise spatial localization and accurate fluorescence intensities of individual fluorescent molecules at each pixel in the entire field of recording. The colocalization measurement performed in this work is based on a statistical approach that performs intensity correlation coefficient-based (ICCB) analysis. In brief, the pixel grey values of each fluorophore in the image are plotted against each other and displayed in a pixel distribution diagram, called the fluorogram. For each given pixel in the multicolor image, the intensity value of fluorophore 1 is used as the x-coordinate and the intensity value of fluorophore 2 (and fluorophore 3, if required) as the y-coordinate (and the z-coordinate) of a two-dimensional (or three-dimensional) fluorogram. Thus the dimmer pixels in the image are close to the origin of the fluorogram, while the brighter pixels are farther out. The pixels with only one fluorescence signal are clustering toward the axes of the fluorogram, and the pixels with both (or all the three) fluorescence signals are clustering toward the centre of the fluorogram. Those clusters of pixels with fluorescence signals are called ‘clouds of signals’ below. Taking advantage of digital analysis, the colocalizing or non-colocalizing pixels were replotted for visualization of the original spatial information, as only those selected pixels are presented in the so-called masked image.
In addition to the commercial software (LCS, Leica), a set of MatLab programs were written for special colocalization analysis in this work. In brief, these programs offer the possibility to analyze multicolor xyzt four-dimensional image series, as well as determine the threshold values for each channel in each image according to the factor of photobleaching caused by long-term image acquisition. More details, including the theoretical background and formulas, can be found in Chapter 4.2.4.4.

### 3.8.3 Fluorescence Resonance Energy Transfer (FRET) by confocal microscopy

FRET, also known as Förster Resonance Energy Transfer, is a photophysical phenomenon involving the radiationless energy transfer from a donor fluorophore to an appropriately positioned acceptor fluorophore (Förster, 1948; Förster, 1965; Herman, 1989; Stryer, 1978; Van Der Meer et al., 1994; Wu and Brand, 1994). FRET occurs only if the spectral, dipole orientation, and distance criteria of both donor and acceptor fluorophores are satisfied. The efficiency of resonance energy transfer is defined by the formula

\[
E = \frac{R_0^6}{(R_0^6 + r^6)}
\]

where \( r \) is the distance between the two fluorophores, and \( R_0 \) is the distance at which 50% energy transfer takes place.

Excitation of the donor fluorophore results in quenching of donor emission and increased, sensitized acceptor emission. Intensity-based FRET measurement techniques are based on these effects, which have been applied in this work for detection of protein-protein interaction in living cells by confocal microscopy. In brief, nine 12-bit-encoded digital images are required for an analysis by the sensitized acceptor emission method, including donor only, acceptor only, and double-labeled samples for three channel-imaging: donor emission upon donor excitation (donor channel), acceptor emission upon acceptor excitation (acceptor channel), and acceptor emission upon donor excitation (FRET channel). The approach is based on the assumption of same spectral bleed-through (SBT) dynamics for single- and double-labeled cells under same acquisition
conditions. The formula used to calculate true FRET values and FRET efficiencies (FRETeff) are shown below:

\[
\text{FRET} = B - b \times A - (c - a \times b) \times C
\]

\[
\text{FRET}_{\text{eff}} = \frac{\text{FRET}}{C}
\]

where

\[A = \text{channel 1 = Donor emission (by excitation of the donor)}\]
\[B = \text{channel 2 = FRET emission (by excitation of the donor)}\]
\[C = \text{channel 3 = Acceptor emission (by excitation of the acceptor)}\]

\[A, B, \text{and } C \text{ are background corrected 12 bit values.}\]

\[a = \text{Correction factor of acceptor only measurement}\]
\[= \frac{\text{Donor emission (by excitation of the donor)}}{\text{acceptor emission (by excitation of the acceptor)}}\]

\[b = \text{Correction factor of donor only measurement}\]
\[= \frac{\text{Acceptor emission (by excitation of the donor)}}{\text{donor emission (by excitation of the donor)}}\]

\[c = \text{Correction factor of acceptor only measurement}\]
\[= \frac{\text{Acceptor emission (by excitation of the donor)}}{\text{acceptor emission (by excitation of the acceptor)}}\]

3.8.4 Dynamic FRET measurement by fluorescence microscopy

HEK 293 cells were mounted on an Axiovert 200 inverted microscope (Zeiss, Jena, Germany) with an oil immersion objective (100×), a dual emission photometric system (TILL Photonics, Planegg, Germany), and Polychrom V (TILL Photonics). Cells were illuminated at 435 nm (beam splitter DCLP 460, Chroma Technologies, Rockingham, USA) for ECFP excitation, or > 515 nm (beam splitter DCLP 505, Chroma Technologies) for EYFP excitation. To determine dynamic changes in FRET over time, cells were illuminated < 20 ms at a frequency of 1 to 10 Hz to minimize
Materials and methods

photobleaching. ECFP and EYFP emissions were recorded continuously, and FRET was determined as the ratio of EYFP / ECFP. Absolute FRET between ECFP and EYFP was determined by measuring donor quenching after acceptor photobleaching for 5 min by illumination at 500 nm. Fluorescence intensities were measured with a HEKA EPC-10 amplifier controlled by Patchmaster software (HEKA).

3.9 Instruments

Amplifier: EPC-9, HEKA, Lambrecht
EPC-10, HEKA, Lambrecht

Application system: Workshop, Faculty of Chemistry, Ruhr University Bochum

Autoclave: Varioklav, Dampfsterilisator Type 400, H+P Labortechnik, Oberschleißheim

Balance: 2004 MP6E, Sartorius, Göttingen
BP211D, Sartorius, Göttingen
Kilomat, Sartorius, Göttingen
P-120, Mettler, Greifensee, CH

Beam splitter: DCLP 460, Chroma Technologies, Rockingham, USA
DCLP 505, Chroma Technologies, Rockingham, USA

Centrifuge: Biofuge stratos, Heraeus, Hanau
Centrifuge 5415 C, Eppendorf, Hamburg
Labofuge 400R, Heraeus, Hanau
RC 5B, Sorvall

Computer: Apple Power Mac G4
Apple iMac
Asus W5G00A

Confocal microscope: TCS SP2 AOB, Leica, Mannheim

Dual emission photometric system: TILL Photonics, Planegg, Germany

Freezer –20 °C: GS 3183, Liebherr, Ochsenhausen
GS 3704, Liebherr, Ochsenhausen
GSS 3123, Liebherr, Ochsenhausen

Freezer –80 °C: HFU 686, Heraeus, Hanau

Gel electrophoresis chamber: MGU-202T (7 cm × 10 cm), C.B.S., Del Mar, CA, USA
SGE-014 (14 cm × 20 cm), C.B.S., Del Mar, CA, USA
SGE-020 (20 cm × 20 cm), C.B.S., Del Mar, CA, USA
Materials and methods

Glas pipette puller: PIP 5, HEKA, Lambrecht
Helios gene gun: Biorad, Munich
Ice machine: Scotsman AF-30
Incubator: BK 6120, Heraeus, Hanau
Heracell, Heraeus, Hanau
T 6120, Heraeus, Hanau
Live cell incubation system: H.Saur, Reutlingen
Magnetic stirrer: Ikamag, REC-G, Ika, Staufen
Microscope: Stemi SV6, Carl Zeiss, Jena
DM IRE 2, Leica, Mannheim
Microwave: R-204, Sharp, Osaka, Japan
Nucleofector: amaxa, Köln
Objective at confocal microscope: HC PL Fluorat 5× 0.15 Dry, Leica, Mannheim
HC PL APO CS 10× 0.4 IMM, Leica, Mannheim
HC PL APO Ibd.BL 20× 0.7 IMM/Corr, Leica, Mannheim
HCX PL APO CS 63× 1.4 Oil, Leica, Mannheim
HCX PL APO CS 63× 1.2 W Corr, Leica, Mannheim
Oscillation bench: TMC, Peabody, MA, USA
Pipette: Pipetman Gilson, Middleton, WI, USA
pH-Meter: Labor-pH-Meter 766, Knick, Berlin
Photometer: GeneQuant II, Pharmacia, Uppsala, Sweden
UV-160, Shimadzu, Kyoto, Japan
Piezo-manipulator: E-500.00, PI, Waldbronn
Pipette positioner: SM I, Luigs und Neumann, Ratingen
Power supply: PowerPac 3000, Bio-Rad, Hercules, CA, USA
Eletrophoresis Constant Power Supply ECPS 3000/150,
Pharmacia, Uppsala, USA
Refrigerator: FKS 3600, Liebherr, Ochsenhausen
Shaker: TM 130-6, HLC, Bovenden
Thermomixer 5436, Eppendorf, Hamburg
Software: Pulse 8.70, HEKA, Lambrecht
Patchmaster, HEKA, Lambrecht
Leica Confocal Software, Leica, Mannheim
Sterile bench: Herasafe HSP12, Heraeus, Hanau
BH NG, Gelman Sciences, Ann Arbor, MI, USA
### Materials and methods

**Thermocycler:**  PTC-100, MJ Research, Watertown, MA, USA  
PTC-200, MJ Research, Watertown, MA, USA

**Ultra pure water purification system:** Ultra Clear, SG, Barsbüttel

**UV lamp:** Polychrome IV, Photonics, Martinsried

**Vacuum concentrator:** Concentrator 5301, Eppendorf, Hamburg

**Vacuum pump:** MZ 2CE, Vacuubrand, Wertheim

**Valve (Electrophysiology):** Hamilton, Reno, NV, USA

**Vortex:** MS1 Minishaker, IKA, Staufen  
Vortex Genie 2, Bender & Hobein, Zürich, CH

**Preamplifier:** HEKA, Lambrecht  
Kilomat, Sartorius, Göttingen

**Water bath:** MP-5A, Julabo, Seelbach

### 3.10 Disposable materials

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<th>Item</th>
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<td>Microliter pipette tips</td>
<td>Starlab, Ahrensburg</td>
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<td>(10 – 5000 μl)</td>
<td>Biozym, Hess. Oldendorf</td>
</tr>
<tr>
<td>Microliter pipette filter tips</td>
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### Materials and methods

#### 3.11 Chemicals and enzymes

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<td>J.T. Baker</td>
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4. Results

4.1 Construction of recombinant proteins

For the following constructs, all the mutations and linker sites were verified by DNA sequencing.

> Table 4.1 List of constructs

<table>
<thead>
<tr>
<th>C-terminal labeled</th>
<th>Editing variant C-terminal labeled</th>
<th>N-terminal labeled</th>
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<td>Golgi-DsRed2</td>
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4.1.1 C-terminally fluorescently-labeled GluR1 constructs

GluR1 cDNA (X17184) was introduced into the four vectors pECFP-N1, pEGFP-N1, pEYFP-N1, and pDsRed2-N1 (BD Clontech, Heidelberg, Germany). In order to obtain in-frame fusion constructs, the stop codon of GluR1 was removed by PCR mutagenesis (primers P267 and M310), thereby introducing a Sal I restriction site (Figure 4.1.1). The constructs were named GluR1-ECFP, GluR1-EGFP, GluR1-EYFP, and GluR1-DsRed2.
4.1.2 C-terminally fluorescently-labeled GluR2(R) constructs

GluR2(R) cDNA (M85035) was inserted into the four vectors pECFP-N1, pEGFP-N1, pEYFP-N1, and pDsRed2-N1. In order to obtain in-frame fusion constructs, the stop codon of GluR2 was removed by PCR mutagenesis (primers P268 and M311), thereby introducing an Xho I restriction site (Figure 4.1.2). The constructs were named GluR2(R)-ECFP, GluR2(R)-EGFP, GluR2(R)-EYFP, and GluR2(R)-DsRed2.
4.1.3 C-terminally fluorescently-labeled GluR2(Q) constructs

A 966 bp DNA fragment including the pore region-encoding sequence was excised from GluR2(R586Q)/pSGEM with BstX I, and inserted into BstX I-digested GluR2(R)-ECFP and GluR2(R)-EYFP. The constructs were named GluR2(Q)-ECFP and GluR2(Q)-EYFP.

4.1.4 C-terminally fluorescently-labeled GluR6(Q) constructs

GluR6(Q) cDNA (Z11715) was introduced into the four vectors pECFP-N1, pEGFP-N1, pEYFP-N1, and pDsRed2-N1. In order to obtain in-frame fusion constructs, the stop codon of GluR6 was removed by PCR mutagenesis (primers P314 and M335a), thereby introducing an Age I restriction site (Figure 4.1.3). The constructs were named GluR6(Q)-ECFP, GluR6(Q)-EGFP, GluR6(Q)-EYFP, and GluR6(Q)-DsRed2.

4.1.5 C-terminally fluorescently-labeled GluR6(R) constructs

A 956 bp DNA fragment including the pore region encoding sequence was excised from GluR6(R)/pcDNA1 (Z11548) with BamH I, and inserted into BamH I-digested GluR6(Q)-ECFP, GluR6(Q)-EGFP, and GluR6(Q)-EYFP. The constructs were named GluR6(R)-ECFP, GluR6(R)-EGFP, and GluR6(R)-EYFP.

Figure 4.1.3. Cloning strategy of C-terminally fluorescently-labeled GluR6(Q) constructs. CMV IE: human cytomegalovirus immediate early promoter; FP: fluorescent protein; STOP: stop codon.
4.1.6 C-terminally fluorescently-labeled KA2 constructs

KA2 cDNA (U08258) was introduced into the four vectors pECFP-N1, pEGFP-N1, pEYFP-N1, and pDsRed2-N1. In order to obtain in-frame fusion constructs, the stop codon of KA2 was removed by PCR mutagenesis (primers P345 and M355), thereby introducing a Sal I restriction site (Figure 4.1.4). The constructs were named KA2-ECFP, KA2-EGFP, KA2-EYFP, and KA2-DsRed2.

Figure 4.1.4. Cloning strategy of C-terminally fluorescently-labeled KA2 constructs. CMV IE: human cytomegalovirus immediate early promoter; FP: fluorescent protein; STOP: stop codon.

4.1.7 C-terminally fluorescently-labeled stargazin constructs

Stargazin cDNA (AF077739, mus musculus) was inserted into the three vectors pECFP-N1, pEGFP-N1, and pDsRed2-N1. In order to obtain in-frame fusion constructs, the stop codon of stargazin was removed by PCR mutagenesis (primers TP90 and M339), thereby introducing a Sal I site. A myc tag inserted in the parent construct was deleted at the same time (Figure 4.1.5). The constructs were named stargazin-ECFP, stargazin-EGFP, and stargazin-DsRed2.

4.1.8 N-terminally fluorescently-labeled GluR1 constructs

ECFP or EYFP with stop codon deletion were inserted right after the signal peptide of GluR1, with an additional alanine introduced in front of the original start codon (methionine) of the fluorescent
4.1.5 Cloning strategy of C-terminally fluorescently-labeled mouse stargazin constructs. CMV IE: human cytomegalovirus immediate early promoter; FP: fluorescent protein; STOP: stop codon; myc tag.

In brief, a three-fragment overlap extension PCR was performed to obtain the fusion protein fragments shown in Figure 4.1.6. Each of the resulting fragments was inserted into GluR1/pcDNA3 between Hind III and Cla I restriction sites. The Kozak sequences of the fluorescent proteins were not maintained in the final constructs. The constructs were named ECFP-GluR1 and EYFP-GluR1.

4.1.9 Attempt to engineer N-terminally fluorescently-labeled GluR2(R) constructs

ECFP or EYFP with stop codon deletion were to be inserted right after the signal peptide of GluR2(R), with an additional valine introduced in front of the original start codon (methionine) of the fluorescent protein. A three-fragment overlap extension PCR was performed to obtain the fusion protein fragments as shown in Figure 4.1.7. Each of the resulting fragments was ligated into GluR2(R)/pcDNA3 between EcoR I and PpuM I restriction sites. However, restriction verification showed there was no correct clone. The construction was not continued because there was no fluorescence signal detectable at the plasma membrane in HEK 293 cells expressing N-terminally fluorescently-labeled GluR1 fusion proteins (data not shown).
Figure 4.1.6. Cloning strategy of N-terminally fluorescently-labeled GluR1 constructs. SP: signal peptide; CMV IE: human cytomegalovirus immediate early promoter; FP: fluorescent protein; STOP: stop codon.

Figure 4.1.7. Cloning strategy of N-terminally fluorescently-labeled GluR2(R) constructs. SP: signal peptide; CMV IE: human cytomegalovirus immediate early promoter; FP: fluorescent protein; STOP: stop codon.
4.1.10 N-terminally fluorescently-labeled stargazin constructs

ECFP or EYFP with stop codon deletion were inserted right before the original start codon (methionine) of stargazin (*Rattus norvegicus*). In brief, an overlap extension PCR was performed to obtain the fusion protein fragments as shown in Figure 4.1.8. Each of the resulting fragments was inserted into stargazin/pcDNA3 between Acc65 I and BspE I restriction sites. The constructs were named ECFP-stargazin and EYFP-stargazin.

**Figure 4.1.8.** Cloning strategy of N-terminally fluorescently-labeled stargazin constructs. CMV IE: human cytomegalovirus immediate early promoter; FP: fluorescent protein; STOP: stop codon.

4.1.11 C-terminal FLAG-tagged stargazin construct

Stargazin cDNA (AF077739, mus musculus) was inserted into the vector pcDNA4-TO-FLAG. In brief, a 469 bp fragment (BamH I – Pst I) from stargazin/pRK5 and a 572 bp fragment (Pst I – Sal I) from stargazin-EGFP were inserted into pcDNA4-TO-FLAG between BamH I / Pst I and Pst I / Xho I sites, respectively, to obtain an in-frame fusion construct. The construct was named stargazin-FLAG.
4.1.12 DsRed2-Golgi construct

A 260 bp fragment (Nhe I – Age I) from pECFP-Golgi (Clontech) was inserted into the vector pDsRed2-N1 between Nhe I and Age I restriction sites. The final construct encodes a fusion protein of DsRed2 and a sequence encoding the N-terminal 81 amino acids of human beta 1,4-galactosyltransferase (beta 1,4-GT), which contains the membrane-anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus. The construct was named DsRed2-Golgi.
4.2 Monitoring assembly and intracellular trafficking of AMPA receptors

4.2.1 GluR1 fusion proteins are localized at the ER as well as plasma membrane of live cells

To verify expression of the fusion constructs, GluR1-ECFP, GluR1-EGFP, GluR1-EYFP, and GluR1-DsRed2 were transfected into HEK 293 cells and the fluorescence signals were detected by live cell imaging. Figure 4.2.1 shows the expression patterns of two of the fusion proteins, GluR1-ECFP and GluR1-EYFP. Western blot analysis confirmed the expression of GluR1 fluorescent fusion proteins at the expected molecular sizes in HEK 293 cells (Figure 4.2.1 C).

In order to test whether the fused fluorescent proteins alter the functional properties of GluR1, patch clamp recordings were performed. Cells expressing either of the fusion proteins of GluR1 with variants of EGFP respond to glutamate application as shown in Figure 4.2.1, confirming that
the fusion proteins form fully functional ion channels. Mean currents of cells transfected with GluR1-ECFP, GluR1-EGFP, and GluR1-EYFP cDNAs were 230 pA (n = 1), 243 ± 193 pA (n = 2), and 82 ± 63 pA (n = 2), respectively. However, there was no current with the cells expressing GluR1-DsRed2 fusion proteins, therefore this recombinant cDNA was excluded in the later work.

To determine whether the intracellular fluorescence signals of GluR1 fusion proteins are localized at the ER of HEK 293 cells, cotransfection of GluR1-EYFP and ECFP-ER was performed. Analyzing cells expressing both fusion proteins, an overlap of cyan (serving as ER marker) and yellow fluorescence signals (GluR1-EYFP) was found (Figure 4.2.2 A – C). This can be seen in the two-dimensional cytofluorogram (Figure 4.2.2 J), where the dots at a 45º angle carry both fluorescence signals at approximately the same intensity. The masked images of those dots prove the ER localization of the intracellular yellow fluorescence signals (Figure 4.2.2 D – F). Furthermore, when the dots along the yellow axis (= with yellow signals only) in the two-dimensional cytofluorogram were selected, the masked images showed that these signals are located at the cell boundary (Figure 4.2.2 G – I). These data visually demonstrated ER as well as plasma membrane localization of GluR1 subunits in the living cells.

In addition, by confocal z-section scanning, GluR1 was shown to overlap with a lipid bilayer-specific stain (FM4-64, Figure 4.2.3). Figure 4.2.3 D – F show that the dots simultaneously presenting both green and red fluorescence signals (= colocalized fluorophores) are localized at the plasma membrane of the live cell. This further confirms the previously observed expression pattern of the GluR1 subunit. However, the portion of the plasma membrane-located GluR1 subunits is relatively small compared to the total pool of the subunits expressed in the entire cell.

*GluR1-ECFP, GluR1-EGFP, and GluR1-EYFP fusion proteins were expressed in HEK 293 cells and their electrophysiological properties were verified. GluR1 subunits were shown by ECFP-ER coexpression or FM4-64 staining to localize in the ER and at the cell surface.*
Figure 4.2.2. GluR1 fusion proteins localize at the ER as well as plasma membrane of HEK 293 cells. First row: a confocal cross section of HEK 293 cells cotransfected with ECFP-ER (A) and GluR1-EYFP (B); C, overlay of A and B. Second row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with yellow signals only: G, cyan channel; H, yellow channel; I, overlay of G and H. J, two-dimensional cytofluorogram; note one large cloud of fluorescence signals at 45° as well as another much smaller cloud at 0° relative to the Y axis (= yellow axis). Scale bar = 10 μm.
Figure 4.2.3. Plasma membrane expression of GluR1 fusion proteins. First row: a confocal cross section of HEK 293 cells transfected with GluR1-EGFP (A) and stained with FM4-64 (B); C, overlay of A and B. Second row: analysis of masked images of selected dots with both green and red signals at approximately same intensity values: D, green channel; E, red channel; F, overlay of D and E. G, two-dimensional cytofluorogram; note the three clouds of fluorescence signals at 45° as well as at 0° relative to the X axis (= green axis) and the Y axis (= red axis). Scale bar = 10 μm.
Results

4.2.2 Retention of GluR2(R) fusion proteins in the ER

When the fluorescently labeled recombinant constructs of GluR2, namely GluR2(R)-ECFP, GluR2(R)-EGFP, GluR2(R)-EYFP, GluR2(R)-DsRed2 were transfected into HEK 293 cells, the fusion proteins encoded were found to be quantitatively retained in the ER (Figure 4.2.4).

![Figure 4.2.4](image)

Figure 4.2.4. HEK 293 cells transiently transfected with C-terminally fluorescently-labeled GluR2(R) cDNAs: GluR2(R)-ECFP (A), GluR2(R)-EGFP (B), and GluR2(R)-EYFP (C). D, Western blot analysis of cell lysates from transfected HEK 293 cells, probed with the monoclonal anti-EGFP antibody JL-8. Scale bars = 10 μm.

Analyzing cells expressing both GluR2(R)-EYFP and ECFP-ER fusion proteins, a perfect overlap of cyan (serving as ER markers) and yellow fluorescence signals (GluR2(R)-EYFP) was found (Figure 4.2.5 A – C). This can be seen in the two-dimensional cytofluorogram (Figure 4.2.5 M), where the dots at a 45° angle carry both fluorescence signals at approximately the same intensity. The masked images of those dots prove the ER localization of almost all of the yellow fluorescence signals (Figure 4.2.5 D – F). As a control, in the same recording field there were cells expressing exclusively ECFP-ER. When the dots along the cyan axis (= with cyan signals only) in the two-dimensional cytofluorogram were selected, the masked images showed the image of these
Figure 4.2.5. Colocalization analysis demonstrates ER retention of GluR2(R) subunits. First row: a confocal cross section of HEK 293 cells cotransfected with ECFP-ER (A) and GluR2(R)-EYFP (B); C, overlay of A and B. Second row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with cyan signals only: G, cyan channel; H, yellow channel; I, overlay of G and H. Fourth row: a confocal cross section of HEK 293 cells cotransfected with DsRed2-ER (J) and GluR2(R)-EYFP (K); L, overlay of J and K. M, two-dimensional cytofluorogram of the images of the first row; note the cloud of fluorescence signals at 45° as well as another cloud at 0° relative to the X axis (= cyan axis). N, two-dimensional cytofluorogram of the images of the fourth row; note the cloud of fluorescence signals at 45° relative to the X axis (= cyan axis). Scale bars = 10 μm.
cells only in the cyan channel while no signal was found in the yellow channel (Figure 4.2.5 G and H). Similar analysis was also performed with cells expressing GluR2(R)-EYFP and another ER marker, DsRed2-ER, fusion proteins, where a perfect overlap was found for yellow and red fluorescence signals (Figure 4.2.5 J – L). In the two-dimensional cytofluorogram for colocalization analysis (Figure 4.2.5 N), a cloud of dots carrying both fluorescence signals is shown at a 45° angle.

When those coexpressing cells were stained with FM4-64 (serving as plasma membrane marker), there was no overlap between the red fluorescence signals with cyan or yellow signals as shown in Figure 4.2.6 A – D, whereas almost all of the yellow fluorescence signals were colocalized with cyan signals (Figure 4.2.6 E).

![Figure 4.2.6](image.png)

Figure 4.2.6. Colocalization analysis demonstrates ER retention of GluR2(R) subunits. A confocal cross section of HEK 293 cells cotransfected with ECFP-ER (A) plus GluR2(R)-EYFP (B), and stained with FM4-64 (C). D, overlay of A, B, and C. E, two-dimensional cytofluorogram of the images of A and B; note the cloud of fluorescence signals at 45° as well as another cloud at 0° relative to the X axis (cyan axis). Scale bar = 10 μm.
A further plasma membrane colocalization analysis was performed with HEK 293 cells transfected with GluR2(R)-EGFP-encoding cDNA and stained with the dye FM4-64 (Figure 4.2.7). Well-separated green and red pixels in the 2D cytofluorogram (Figure 4.2.7 D) indicate that GluR2(R)-EGFP fusion proteins are not present at the plasma membranes of the cells.

Figure 4.2.7. GluR2(R) fusion proteins are not localized at the cell surface. A confocal cross section of HEK 293 cells transfected with GluR2(R)-EGFP (A) and stained with the plasma membrane marker FM4-64 (B); C, overlay of A and B. D, two-dimensional cytofluorogram; note the two clouds of fluorescence signals at 0° relative to the X axis (= green axis) and the Y axis (= red axis). Scale bar = 10 μm.

*GluR2(R)-ECFP, GluR2(R)-EGFP, GluR2(R)-EYFP, and GluR2(R)-DsRed2 fusion proteins were expressed in HEK 293 cells. GluR2(R) subunits were shown by ECFP-ER or DsRed2-ER coexpression and/or FM4-64 staining to be retained in the ER without cell surface expression.*
4.2.3 GluR2(Q) subunits are predominantly retained in the ER, with a small amount present at the cell surface

In order to monitor and verify the expression and intracellular localization of un-edited version of GluR2, cDNAs encoding fluorescently-labeled GluR2(Q) subunits were transiently transfected into HEK 293 cells. In Figure 4.2.8 A – C, expression patterns of three of the fusion proteins, GluR2(Q)-ECFP, GluR2(Q)-EGFP, and GluR2(Q)-EYFP are shown. Western blot analysis confirmed the expression of the fusion proteins at the expected molecular sizes in HEK 293 cells (data not shown). In order to test whether the fused fluorescent proteins alter the functional properties of GluR2(Q), patch clamp recordings were performed. Cells expressing GluR2(Q)-EGFP fusion proteins respond to glutamate application as shown in Figure 4.2.8 D, confirming that the fusion proteins form fully functional ion channels. Mean currents were 44 ± 21 pA (n = 3). The other fusion proteins are expected to be functional as well because the few differences in the coding sequence are considered minor.

Figure 4.2.8. HEK 293 cells transiently transfected with C-terminally fluorescently-labeled GluR2(Q) cDNAs: GluR2(Q)-ECFP (A), GluR2(Q)-EGFP (B), and GluR2(Q)-EYFP (C). D, representative whole-cell recording of glutamate-evoked currents of HEK 293 cells transfected with GluR2(Q)-EGFP cDNA. Scale bars = 10 μm.
To determine whether the intracellular fluorescence signals of GluR2(Q) fusion proteins are localized at the ER of the cells, cotransfection of GluR2(Q)-EYFP and ECFP-ER was carried out. Analyzing cells expressing both fusion proteins, an overlap of cyan (serving as ER marker) and yellow fluorescence signals (GluR2(Q)-EYFP) was found (Figure 4.2.9 A – C). This can be seen in the 2D cytofluorogram (Figure 4.2.9 D), where the dots at a 45° angle carry both fluorescence signals at approximately the same intensity. Similar analysis was also performed with cells coexpressing GluR2(Q)-EYFP and another ER marker, DsRed2-ER, where an overlap was found for yellow and red fluorescence signals (Figure 4.2.9 E – H).

However, contrary to similar colocalization analyses with ER markers performed for GluR1 subunits (Chapter 4.2.1 Figure 4.2.2), there was no visually observable pixels carrying only yellow signals (= GluR2(Q) subunits) in these experiments. The data show that almost all of the GluR2(Q) subunits are retained in the ER. Thus, to further test whether there are any cell surface-
targeted GluR2(Q) subunits, colocalization analysis was performed with cells expressing GluR2(Q)-EGFP and stained with FM4-64 dyes (serving as plasma membrane marker). As shown in Figure 4.2.10, a representative recording field with several GluR2(Q)-EGFP-expressing cells was analyzed. At the upper right corner of the 2D cytofluorogram (Figure 4.2.10 G, indicated with a white arrow), there is a distinguishable cloud of dots carrying both green and red fluorescence signals at approximately same intensity values. When those dots were selected and analyzed for their intracellular distribution (Figure 4.2.10 D – F), they were predominantly found to be localized at the plasma membrane of the cells where FM4-64 dyes are expected to bind. Interestingly, the rate of fluorescence intensity values between the masked area and the complete recording field for green and red channels were 1.32% and 1.28%, respectively. The range of these rates varied from approximately 0.5% to 1.5% when more cells were analyzed (data not shown). This indicates that only a very small amount of GluR2(Q), maybe around 1% of the total pool, are transported to the cell surface while the majority is still retained intracellularly. However, this small amount of GluR2(Q) molecules is able to form functional ion channels and thus serves a physiological role in living cells, which was proven by patch clamp recordings.

*GluR2(Q)-ECFP, GluR2(Q)-EGFP, and GluR2(Q)-EYFP fusion proteins were expressed in HEK 293 cells, and the electrophysiological functional properties were verified for GluR2(Q)-EGFP and GluR2(Q)-EYFP. GluR2(Q) subunits were shown by ECFP-ER or DsRed2-ER coexpression to be retained predominantly in the ER. By FM4-64 staining there was a very small amount of GluR2(Q) expressed at the cell surface detected.*
4.2.4 Surface expression of AMPA receptors is regulated by auxiliary subunits

4.2.4.1 Cell surface expression of N- and C-terminally fluorescently-labeled stargazin fusion proteins

In order to verify the expression of the C-terminally fluorescently-labeled stargazin (*Mus musculus*) the cDNAs for the recombinant proteins (stargazin-ECFP, stargazin-EGFP, and stargazin-DsRed2) were transfected into HEK 293 cells and the fluorescence signals were detected by live cell imaging. A robust plasma membrane expression of the fusion proteins was observed (Figure 4.2.11 A), and there were no significant differences in the subcellular expression patterns of the three different fluorescent protein-tagged stargazin proteins. A Western blot performed with a monoclonal anti-EGFP antibody, JL-8, which recognizes EGFP and its variants such as ECFP, indicated the expression of stargazin fusion proteins with EGFP variants at the expected molecular size in transfected cells (Figure 4.2.11 G). By confocal z-section scanning, the expression of
stargazin at the cell surface was shown to overlap with a lipid bilayer-specific stain (FM4-64) (Figure 4.2.11 A – C). Similar surface expression was demonstrated for N-terminally fluorescently-labeled stargazin (*Rattus norvegicus*) in HEK 293 cells (Figure 4.2.11 H).

When stargazin-EGFP was transfected into cultured cortical neurons, a robust plasma membrane expression of the fusion proteins was observed in both soma and dendrites (Figure 4.2.11 D – F).

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Figure 4.2.11. Cell surface expression of C-terminally fluorescently-labeled stargazin fusion proteins. A – C, a confocal cross section of HEK 293 cells transfected with stargazin-EGFP (A) and stained with FM4-64 (B); C, overlay of A and B. D – F, confocal cross sections of a cultured cortical neuron transfected with stargazin-EGFP. G, Western blot analysis of cell lysates from transfected HEK 293 cells, probed with the monoclonal anti-EGFP antibody JL-8. H, a confocal cross section of HEK 293 cells transfected with N-terminally fluorescently-labeled stargazin-encoding cDNA (EYFP-stargazin). Scale bars = 10 μm in C and H, and 40 μm in F.
Results

*Plasma membrane expression of C-terminally fluorescently-labeled stargazin fusion proteins was demonstrated in both HEK 293 cells and cultured neurons. Similarly, N-terminally fluorescently-labeled stargazin fusion proteins were shown to express at the plasma membrane of HEK 293 cells.*

4.2.4.2 Stargazin enhances surface expression of AMPA receptor subunits in live HEK 293 cells

After verifying the surface expression of stargazin fusion proteins, they were used for coexpression with AMPA receptor subunits in mammalian cells. Confocal microscopy applied with digital colocalization analysis as well as patch clamp recordings of cotransfected HEK 293 cells were analyzed to detect whether the surface expression of selected representative AMPA receptor subunits are enhanced by stargazin, and if yes, to which extent.

At first, GluR1-EGFP- and stargazin-DsRed2-coding cDNAs were transiently cotransfected into HEK 293 cells (Figure 4.2.12). Strikingly, there was a boosted surface expression of GluR1 subunits (compared to Chapter 4.2.1 Figure 4.2.1) when coexpressed with stargazin (for example, the cells indicated with white arrows in Figure 4.2.12 C and I). For these cells, almost all of the GluR1 subunits were translocated to the plasma membrane by stargazin. Thus, there was a significant increase of surface GluR1 when coexpressed with stargazin compared to when it was expressed alone (see Chapter 4.2.1). The significant amount of ER-retained GluR1 subunits (when expressed alone) in Figure 4.2.1 – 4.2.3 was not observed in those cells marked by white arrows in Figure 4.2.12. There were also some other cells coexpressing both fusion proteins where they colocalized at the plasma membrane while retaining a significant amount of GluR1 intracellularly (cells indicated with yellow arrows, Figure 4.2.12 C). This might result from different expression levels of the two exogenous proteins in individual cells, and the duration allowed for protein association and trafficking before the recording. Nevertheless, the 2D cytofluorograms (Figure 4.2.12 J and K), indicating intensity values of both green and red channels of each pixel in both recordings (Figure 4.2.12 C and I, respectively), show a good separation of the two channels as well as a clear colocalization of both surface-localized fusion proteins.
Figure 4.2.12. Colocalization analysis demonstrates enhanced surface expression of GluR1 subunits when coexpressed with stargazin. First row: a confocal cross section of HEK 293 cells cotransfected with GluR1-EGFP (A) and stargazin-DsRed2 (B); C, overlay of A and B. Second row: analysis of masked images of selected dots with both green and red signals at approximately same intensity values: D, green channel; E, red channel; F, overlay of D and E. Third row: a confocal cross section of HEK 293 cells cotransfected with GluR1-EGFP (G) and stargazin-DsRed2 (H); I, overlay of G and H. J, two-dimensional cytofluorogram of the images of the first row; note the two dense clouds of fluorescence signals at 0° relative to the X axis (= green axis) and the Y axis (= red axis), and the pool of dots marked with a white arrow indicating colocalizing signals. K, two-dimensional cytofluorogram of the images of the third row; note the cloud of fluorescence signals at 0° relative to the X axis (= green axis) indicating dots with green signals only, and the cloud at 45° indicating colocalizing signals. Scale bars = 10 μm.
When GluR2(R), the edited version of GluR2 subunit that lacks surface expression if expressed alone in HEK 293 cells (Chapter 4.2.2), was coexpressed with stargazin, a robust surface expression of the subunit was observed. The experiments were carried out with HEK 293 cells coexpressing GluR2(R)-EGFP plus stargazin-DsRed2, or GluR2(R)-EYFP plus stargazin-ECFP fusion proteins.

Figure 4.2.13. Surface expression of GluR2(R) subunits when coexpressed with stargazin in HEK 293 cells. First row: HEK 293 cells cotransfected with GluR2(R)-EGFP (A) and stargazin-DsRed2 (B); C, overlay of A and B, shows the distinguishable populations of cells expressing different fusion proteins in the field; D, two-dimensional cytofluorogram, note the two clouds of fluorescence signals at 0° relative to either axis indicating well-separated signals from two channels. Second row: HEK 293 cells coexpressing both GluR2(R)-EGFP (E) and stargazin-DsRed2 (F); G, overlay of E and F; H, two-dimensional cytofluorogram, note the pool of fluorescence signals at upper right corner. Third row: analysis of masked images of selected dots with both green and red signals at approximately same intensity values: I, green channel; J, red channel; K, overlay of I and J. Scale bars = 50 μm in C, and 10 μm in G.
Results

For the former combination, a control analysis was first performed with a recording field where most of the cells were expressing only one of the fusion proteins (Figure 4.2.13 A – C). In the 2D cytofluorogram the two well-separated pools of fluorescence signal dots close to either the X or the Y axis, indicate well distinguished signals from green and red channels (Figure 4.2.13 D). Based on this perfect separation of distinct fluorescence signals, the colocalization analysis of coexpressing cells was performed. Surprisingly, contrary to the GluR2(R) mainly intracellular expression pattern when transfected alone, there is a highlighted plasma membrane structure clearly visible in both green (GluR2(R)-EGFP) and red (stargazin-DsRed2) channels (Figure 4.2.13 E – G). By digital colocalization analysis shown in Figure 4.2.13 H, the pixels with colocalized fluorophores indeed map to the cell surface (Figure 4.2.13 I – K).

Another representative image (Figure 4.2.14) shows two cells with one of them coexpressing both of the fusion proteins (indicated by the white arrow, C) while the other one expressing only GluR2(R)-EGFP (indicated by the yellow arrow, C). Compared to the yellow-arrowed cell, the white-arrowed cell presents a strong surface expression of GluR2(R) subunits that colocalize with stargazin at the plasma membrane (Figure 4.2.14 D – F). Interestingly, there is also an intracellular colocalization of both fusion proteins at an area adjacent to the nucleus, which may suggest early association of the two proteins at this compartment. Further analysis selecting pixels with only one of the fluorescence signals also provide important information of the expression pattern. In Figure 4.2.14 G – I, GluR2(R)-EGFP proteins that do not colocalize with stargazin-DsRed2 proteins are presented: both cells show intracellular GluR2(R) expression only. However, the amount of the intracellular GluR2(R) subunits in the cell marked by a white arrow is much smaller than in the cell marked by a yellow arrow where the subunits are predominantly retained in the ER. There is no surface GluR2(R) present in this analysis (green signals only), which suggests that association with stargazin is required for surface expression of GluR2(R) subunits, and moreover, surface GluR2(R) subunits remain associated with stargazin even after they reach their final destination.
Figure 4.2.14. Colocalization analysis of GluR2(R) and stargazin at the plasma membrane and intracellular region. First row: HEK 293 cells were cotransfected with GluR2(R)-EGFP (A) and stargazin-DsRed2 (B); C, overlay of A and B: note the cell indicated by a white arrow coexpresses both fusion proteins while the cell indicated by a yellow arrow expresses only GluR2(R)-EGFP. Second row: colocalization analysis: masked images of selected dots with both green and red signals at approximately same intensity values, both fusion proteins are expressed and colocalized at the plasma membrane of the cell marked by the white arrow: D, green channel; E, red channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with green signals only, cells marked by white and yellow arrow present intracellular GluR2(R) fusion proteins while the amount is smaller in the cell marked by the white yellow: G, green channel; H, red channel; I, overlay of G and H. Fourth row: analysis of masked images of selected dots with red signals only, there are cell surface stargazin fusion proteins not colocalized with GluR2(R) subunits in the cell marked by the white arrow: J, green channel; K, red channel; L, overlay of J and K. M, two-dimensional cytofluorogram. Scale bar = 20 μm.
Finally, as shown in Figure 4.2.14 J – L, there is a considerable amount of surface stargazin proteins that do not colocalize with GluR2(R) subunits, which suggests constitutive expression and independent surface targeting of stargazin proteins. Similar results were obtained with another combination of fluorescent labels, GluR2(R)-EYFP and stargazin-ECFP. Visible plasma membrane-localized membrane-localized GluR2(R)-EYFP was observed in coexpressing cells (Figure 4.2.15). Digital analysis also indicates that both of the fusion proteins are colocalized at the cell surface (Figure 4.2.15 D – G).

In addition, coexpression of stargazin and GluR2(Q), the un-edited version of GluR2 subunit, was performed. Two combinations of differently-tagged fusion proteins were chosen for the cotransfection experiments, namely GluR2(Q)-EGFP plus stargazin-DsRed2, and GluR2(Q)-EYFP plus stargazin-ECFP. The fluorophore combinations are thus comparable to the GluR2(R)/stargazin coexpression experiments described above. Colocalization analyses of both combinations, as shown in Figure 4.2.16, indicate that GluR2(Q) and stargazin do overlap and
Results

colocalize at the plasma membranes of the cells. Moreover, in some of the cells, such as the cell indicated by the white arrow in Figure 4.2.16 C, there is an intracellular perinuclear region abundant with both of the fusion proteins. This region might be suggested to be the Golgi apparatus. It will be discussed in detail in the next chapter (Chapter 4.2.5).

Figure 4.2.16. Plasma membrane colocalization of GluR2(Q) and stargazin fusion proteins. First row: a confocal cross section of HEK 293 cells cotransfected with GluR2(Q)-EGFP (A) and stargazin-DsRed2 (B); C, overlay of A and B, note the perinuclear region abundant of both proteins show in the cell marked by a white arrow. Second row: analysis of masked images of selected dots with both green and red signals at approximately same intensity values: D, green channel; E, red channel; F, overlay of D and E. G, two-dimensional cytofluorogram. Third row: a confocal cross section of HEK 293 cells cotransfected with stargazin-ECFP (H) and GluR2(Q)-EYFP (I); J, overlay of H and I. Fourth row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: K, cyan channel; L, yellow channel; M, overlay of K and L. N, two-dimensional cytofluorogram. Scale bars = 10 μm.
Furthermore, it has been shown that GluR2(Q) can exit the ER by itself and express at the cell surface, though at a fairly small amount that was difficult to be distinguished from the ER marker (Chapter 4.2.3 Figure 4.2.9). If stargazin coexpression would strongly enhance surface expression of GluR2(Q) subunits, it might be possible to detect surface GluR2(Q) subunits.

Triple transfections of three recombinant cDNAs, GluR2(Q)-EYFP, stargazin-ECFP, and DsRed2-ER, were therefore carried out. Figure 4.2.17 A – D shows a representative recording field of the coexpressing cells, where stargazin-ECFP was mainly expressed at the plasma membrane, GluR2(Q)-EYFP was expressed at the plasma membrane as well as intracellular compartments, and DsRed2-ER was retained in the ER. Similar to the analysis above, colocalization of GluR2(Q)-EYFP and stargazin-ECFP at the cell surface could be shown (Figure 4.2.17 E – H). Interesting results were obtained analyzing colocalization patterns of GluR2(Q)-EYFP and DsRed2-ER: when the pixels carrying only yellow fluorescence signals were selected (= expressing GluR2(Q)-EYFP only) and replotted to visualize their intracellular localization, they were found to be reside the plasma membrane. Thus, the surface GluR2(Q) subunits were significantly increased by coexpression of stargazin and became detectable at our imaging system.

Enhanced surface expression of GluR2(Q) was also confirmed by patch clamp recordings as shown in Figure 4.2.18. As a control experiment for multicolor imaging, HEK 293 cells with triple expression of GluR2(R)-EYFP, stargazin-ECFP, and DsRed2-ER were also analyzed. Figure 4.2.19 shows a selected cell where stargazin was present at the cell surface while the other two proteins were still retained in the ER. Colocalization analysis shows perfect overlap between GluR2(R)-EYFP and DsRed2-ER fusion proteins, in addition to distinguishable localization of stargazin-ECFP in both 2D and 3D cytofluorograms.
Figure 4.2.17. Detection of surface GluR2(Q) subunits when coexpressed with stargazin. First row: a confocal cross section of HEK 293 cells triply transfected with stargain-ECFP (A), GluR2(Q)-EYFP (B), and DsRed2-ER (C); D, overlay of A, B, and C. Second row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values, shows colocalization of GluR2(Q) and stargazin at the plasma membranes of the cells: E, cyan channel; F, yellow channel; G, overlay of E and F. H, two-dimensional cytofluorogram (cyan and yellow channels). Third row: analysis of masked images of selected dots carrying yellow but not red signals, shows exit of GluR2(Q) from the ER: I, yellow channel; J, red channel; K, overlay of I and J. L, two-dimensional cytofluorogram (yellow and red channels). Fourth row: two-dimensional cytofluorogram (M) and three-dimensional cytofluorograms (N and O). Scale bar = 10 μm.
Figure 4.2.18. Whole cell recordings of glutamate-evoked currents.  
A, HEK 293 cells transfected with GluR1-EYFP (left trace), or cotransfected with GluR1-EYFP and stargazin-EGFP (right trace).  
B, HEK 293 cells transfected with GluR2(Q)-EGFP (left trace), or cotransfected with GluR2(Q)-EGFP and stargazin-EGFP (right trace).  
C, HEK 293 cells transfected with GluR2(R)-EGFP (left trace), or cotransfected with GluR2(R)-EGFP and stargazin-EGFP (right trace).  
Note that stargazin-EGFP not only potentiated the glutamate-evoked currents of AMPARs, but also altered the desensitization patterns of those receptors.

Figure 4.2.19. Multicolor analysis. First row: a confocal cross section of HEK 293 cells triply transfected with stargain-ECFP (A), GluR2(R)-EYFP (B), and DsRed2-ER (C); D, overlay of A, B, and C. Second row: two-dimensional cytofluorogram (E) and three-dimensional cytoflorograms (F and G). Scale bar = 10 μm.
Finally, expression patterns of heteromeric AMPA receptor complexes consisting of GluR1 and GluR2 subunits expressed in the presence of stargazin was investigated. Three recombinant cDNAs, GluR1-EGFP, GluR2(R)-DsRed2, and stargazin-ECFP, were transiently transfected into HEK 293 cells. Overlap of the three fusion proteins was found at the cell surface as shown in Figure 4.2.20. Two- and three-dimensional cytofluorograms further confirmed the colocalization of these proteins which might suggest protein-protein interactions between the proteins at the plasma membrane. Thus, we were able to demonstrate glutamate receptor complexes consisting of GluR1 and GluR2(R) associated with stargazin to be expressed at the cell surface.

![Figure 4.2.20](image)

*Figure 4.2.20. Stargazin translocates heteromeric AMPA receptor complexes to the cell surface. A confocal cross section of HEK 293 cell triply transfected with stargain-ECFP (A), GluR1-EGFP (B), and GluR2(R)-DsRed2 (C). D, overlay of A, B, and C. E, two-dimensional cytofluorogram. F, three-dimensional cytoflorogram. Scale bar = 10 μm.*

*Different combinations of fluorescently-labeled stargazin and one of the AMPA receptor subunits, GluR1, GluR2(Q), and GluR2(R), were coexpressed in HEK 293 cells. With confocal live imaging recorded in dual or triple channels, and digital colocalization analysis, cell surface expression levels of AMPA receptor subunits were shown to be vastly enhanced.*

Receptor
complexes consisting of GluR1 and GluR2(R) associated with stargazin were also demonstrated to be expressed at the cell surface.

4.2.4.3 Stargazin interacts with AMPA receptors at the cell surface as demonstrated by FRET analysis

FRET is a photophysical phenomenon where energy is transferred from a donor fluorophore to an acceptor fluorophore with an efficiency governed by the formula

\[ E = \frac{R_0^6}{R_0^6 + r^6} \]

where \( r \) is the distance between the two fluorophores, and \( R_0 \) is the distance at which 50% energy transfer takes place, which is typically 2 to 6 nm.

Here, an intensity-based method, namely the sensitized emission method, was used for FRET analysis on live HEK 293 cells expressing C-terminally ECFP- (as donor) and EYFP- (as acceptor) tagged stargazin and AMPA receptor subunits GluR1, GluR2(R), or GluR2(Q). These experiments can provide evidence of in vivo assembly of stargazin-AMPAR complexes recorded at high resolution. In addition, experiments were carried out with cells coexpressing either C-terminally ECFP-tagged or N-terminally EYFP-tagged stargazin fusion proteins. This is a novel approach in demonstrating protein-protein interaction between TARPs and AMPARs in vivo. In brief, four combinations of fusion proteins were tested for this purpose (Table 4.2), and used to analyze protein interactions as well as where interactions occur in live cells. For each cell analyzed in this work, 3 – 6 repeat recordings were carried out, lasting around 40 to 100 seconds in total, in order to exclude any possible random false signal. The final FRET data for each cell is thus presented as mean ± SEM from the repetitive measurements. The FRET data for each combination of subunits is presented as mean ± SEM based on all the tested cells in the group, if not specified otherwise.

Essential control experiments were carried out with HEK 293 cells coexpressing wild type ECFP and EYFP fluorescence proteins, as well as HEK 293 cells coexpressing stargazin-ECFP and GluR6(Q)-EYFP fusion proteins. Details of the control experiments can be found in Chapter
4.3.5.1. The FRET and FRET efficiency values of the second combination as negative control were 0.67 ± 0.58 (n = 10) and 0.19 ± 0.16% (n = 10).

Table 4.2 Summary of FRET analysis performed with fluorescently-labeled stargazin and AMPA receptor subunits in live cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>n</th>
<th>Plasma membrane</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stargazin-ECFP</td>
<td>GluR6(Q)-EYFP</td>
<td>10</td>
<td>-</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>Stargazin-ECFP</td>
<td>GluR1-EYFP</td>
<td>6</td>
<td>+</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>Stargazin-ECFP</td>
<td>GluR2(Q)-EYFP</td>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stargazin-ECFP</td>
<td>GluR2(R)-EYFP</td>
<td>31</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stargazin-ECFP</td>
<td>EYFP-stargazin</td>
<td>10</td>
<td>+</td>
<td>Not analyzed</td>
</tr>
</tbody>
</table>

Three combinations of fluorescently-labeled AMPA receptor subunit- and stargazin-encoding cDNAs: stargazin-ECFP plus GluR1-EYFP (Figure 4.2.21), stargazin-ECFP plus GluR2(Q)-EYFP (Figure 4.2.21), and stargazin-ECFP plus GluR2(R)-EYFP (Figure 4.2.21), were transiently cotransfected into HEK 293 cells and tested for FRET. For all three combinations, significant FRET signals were observed at specific regions of the cells, such as the plasma membrane as well as intracellular membrane compartments. The FRET values at the plasma membrane of the cells were 71.04 ± 28.42 (n = 6), 133.40 ± 35.65 (n = 8), and 338.52 ± 64.28 (n = 31), respectively, significantly higher than the negative control ($p < 0.05$, see Figure 4.2.22 A and Table 4.4). Based on the fluorescence level of the EYFP-fusion protein, serving as the acceptor, there were significant FRET efficiencies as well ($p < 0.01$, see Figure 4.2.22 B and Table 4.4). These data for the first time demonstrated the protein-protein interaction between stargazin and different AMPA receptor subunits at the plasma membrane of living mammalian cells (Ma and Hollmann, 2004a; Ma and Hollmann, 2004b).
Figure 4.2.21. Images of FRET analysis in live HEK 293 cells coexpressing ECFP-labeled stargazin and EYFP-labeled AMPAR subunits or stargazin. The columns from left to right of each row present the confocal images of the cyan channel (donor), the yellow channel (acceptor), the FRET channel, and the visualized true FRET value. HEK 293 cells were cotransfected with stargazin-ECFP plus GluR1-EYFP (A – D), stargazin-ECFP plus GluR2(Q)-EYFP (E – H), stargazin-ECFP plus GluR2(R)-EYFP (I – L), or stargazin-ECFP plus EYFP-stargazin (M – P).
The three combinations of coexpressed fusion proteins were tested as well for their electrophysiological properties. Whole cell recordings were carried out with cells coexpressing both donor and acceptor proteins, and glutamate-evoked currents were obtained for all three combinations. Compared to when either of the AMPA receptor subunits was expressed alone (see Chapter 4.2.4.2 Figure 4.2.18), stargazin-ECFP not only potentiated the glutamate-evoked currents of AMPARs, but also altered the desensitization patterns of those receptors (Figure 4.2.23).

In addition, FRET analysis was performed with cells coexpressing differently-tagged stargazin fusion proteins in order to test whether there is possible dimer formation or protein-protein interaction between stargazin proteins. Interestingly, when C-terminally ECFP-tagged stargazin (*Mus musculus*) served as donor, and N-terminally EYFP-tagged stargazin (*Rattus norvegicus*) served as acceptor, FRET signals were detectable at the plasma membrane of live cells, as shown in Figure 4.2.21. The FRET and FRET efficiency values at the plasma membrane were found to be significantly higher than that of the negative control ($0.001 \leq p < 0.01$, see Figure 4.2.22 and Table 4.4) even though at pretty low counts, such as $12.87 \pm 3.37 \, (n = 10)$ and $0.65 \pm 0.19\% \, (n = 10)$, respectively. This may suggest possible stargazin-stargazin interaction present at the cell surface, though stargazin-AMPAR interactions seem to be prefered *in vivo*.

In summary, the true FRET and FRET efficiency values are presented in Tables 4.3 and 4.4. Table 4.3 presents the original data calculated from all the cells measured, while in Table 4.4 those cells with negative FRET values were excluded for the final calculations. Notably, there is no significant increase in either FRET or FRET efficiency values after the selection. The values in Table 4.4 are also shown in Figure 4.2.22 as a bar diagram.
Results

Figure 4.2.22. FRET and FRET efficiency values measured at the plasma membrane of live HEK 293 cells coexpressing stargazin-ECFP plus GluR1-EYFP, stargazin-ECFP plus GluR2(Q)-EYFP, stargazin-ECFP plus GluR2(R)-EYFP, stargazin-ECFP plus EYFP-stargazin, and stargazin-ECFP plus GluR6(Q)-EYFP. Asterisks indicate significant differences, *** indicates $p < 0.001$, ** indicates $0.001 \leq p < 0.01$, * indicates $0.01 \leq p < 0.05$. Stg: stargazin.

Fluorescence Resonance Energy Transfer (FRET) analysis was performed with live cells coexpressing stargazin-ECFP and AMPAR-EYFP fusion proteins. Selected AMPA receptor subunits were GluR1, GluR2(Q), and GluR2(R). Significant FRET signals were detected at the plasma membranes of the cells, indicating in vivo interactions between stargazin and AMPA receptor subunits at the cell surface. Additional experiments with stargazin-ECFP and EYFP-stargazin indicate possible dimer formation of stargazin proteins at the cell surface, though stargazin-AMPA interaction seems to be preferred.
Table 4.3 Summary of FRET analysis (derived from all cells measured)

<table>
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<tr>
<th></th>
<th>stargazin / GluR1</th>
<th>stargazin / GluR2(Q)</th>
<th>stargazin / GluR2(R)</th>
<th>stargazin / stargazin</th>
<th>stargazin / GluR6(Q)</th>
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<td><strong>A</strong></td>
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<td>47.96</td>
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</table>

| **B**   |                   |                      |                      |                      |                     |
| FRET efficiency |                   |                      |                      |                      |                     |
| PM       | 12.49             | 9.69                 | 51.88                | 0.43                 | 0.19                |
| n        | 6                 | 10                   | 31                   | 15                   | 10                  |
| SEM      | 5.03              | 3.24                 | 10.92                | 0.15                 | 0.16                |
| Intracellular (ER and/or Golgi) | 32.52            | 3.86                 |                      |                      |                     |
| n        | 7                 | 2                    |                      |                      |                     |
| SEM      | 8.19              | 1.95                 |                      |                      |                     |

Table 4.4 Summary of FRET analysis (those cells with negative FRET values excluded)

<table>
<thead>
<tr>
<th></th>
<th>stargazin / GluR1</th>
<th>stargazin / GluR2(Q)</th>
<th>stargazin / GluR2(R)</th>
<th>stargazin / stargazin</th>
<th>stargazin / GluR6(Q)</th>
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</table>

| **B**   |                   |                      |                      |                      |                     |
| FRET efficiency |                   |                      |                      |                      |                     |
| PM       | 12.49             | 12.40                | 51.88                | 0.65                 | 0.19                |
| n        | 6                 | 8                    | 31                   | 10                   | 10                  |
| SEM      | 5.03              | 3.40                 | 10.92                | 0.19                 | 0.16                |
| Intracellular (ER and/or Golgi) | 37.95          | 3.86                 |                      |                      |                     |
| n        | 6                 | 2                    |                      |                      |                     |
| SEM      | 7.26              | 1.95                 |                      |                      |                     |

PM: plasma membrane; ER: endoplasmic reticulum; Golgi: Golgi apparatus
n: number of cells measured, SEM: standard error of mean
4.2.4.4 Dynamics of in vivo stargazin-AMPAR association upon L-glutamate application

Protein-protein interactions between AMPA receptor subunits and stargazin at the plasma membrane of live HEK 293 cells were demonstrated by FRET as described above. A more challenging task came up as to whether the two proteins stay associated or separate upon application of agonists as has been postulated by Tomita et al. (Nakagawa et al., 2005; Tomita et al., 2004; Vandenberghe et al., 2005b). Utilizing colocalization analysis, accurate calculations of colocalized molecules were performed to monitor dynamic changes of the stargazin-AMPAR complexes at live cell surfaces with the digital information obtained by confocal imaging.

In brief, coexpression experiments were carried out with three different fusion protein combinations in HEK 293 cells: GluR1 plus stargazin, GluR2(R) plus stargazin, and GluR2(Q) plus stargazin, all fused with fluorescent proteins. For each coexpressing cell analyzed in this work, four-dimensional confocal image series were acquired, which supply information at different focal planes at a specific time after agonist application. 300 μM L-glutamate was applied at t₀ = 0.

To correct for possible photobleaching of each fluorophore (for an example of photobleaching, see the curves of the total intensity sum for the yellow channel in Figures 4.2.25 E and F) during this long-lasting image acquisition procedure (20 – 40 minutes), the threshold value of each channel was calculated independently for each focal plane at each time point, according to the following equation:

\[
\text{Threshold} (z_a, \text{channel}_b, t_{c+1}) = \frac{\text{Threshold} (z_a, \text{channel}_b, t_c) \times \text{Total intensity sum} (z_a, \text{channel}_b, t_{c+1})}{\text{Total intensity sum} (z_a, \text{channel}_b, t_c)}
\]

where the value of the total intensity sum is calculated from the entire recording field. For t₀ = 0, a threshold value is preset for each channel so that all the colocalized pixels with good signal to noise ratios are selected for analysis. For t > 0, the colocalized pixels are selected by comparing to the values of threshold (zₐ, channelₗ, tₖ₊₁). As described above, those selected pixels form a ‘mask’
image indicating colocalization, and the following values were further calculated for each image in the xyzt series:

Mask area rate \((z_a, \text{channel}_b, t_c) = \frac{\text{Number of masked pixels} (z_a, \text{channel}_b, t_c)}{\text{Number of total pixels} (z_a, \text{channel}_b, t_c)}

Mask intensity rate \((z_a, \text{channel}_b, t_c) = \frac{\text{Mask intensity value} (z_a, \text{channel}_b, t_c)}{\text{Total intensity value} (z_a, \text{channel}_b, t_c)}

Above calculations were coded as a set of MatLab programs and thus applied to the data analysis. In keeping with previous FRET analyses, coexpression of ECFP-labeled stargazin and EYFP-labeled GluR1 was the first combination tested. For each cell analyzed, only those focal planes where stargazin expressed mostly at the cell surface were selected for quadratic curve calculation. This is to ensure that most of the pixels presenting both fluorescence signals and selected as ‘colocalized pixels’ are localized at the cell surface.

In Figure 4.2.24, images of a typical cell at a single focal plane recorded before \((t_0 = 0 \text{ s})\) and after L-glutamate application are shown. It is noticeable that the intracellular cyan signal (= stargazin) is increased from A to H. Importantly, the intensity sum in the cyan channel remained stable (Figure 4.2.25 E), which implies no photobleaching of the ECFP fluorophores and suggests that the same amount of stargazin-ECFP protein was recorded at this focal plane in the entire experiment. The dynamic curves of the colocalization analysis of this representative cell (corresponding to Table 4.5 cell 1) are shown in Figure 4.2.25 (first column), where an additional focal plane of the same cell was also included (second column). Table 4.5 presents the formulas calculated for mask area rate, and mask intensity rates of both channels. The formulas generated are highly consistent not only within different focal planes of the same cell, but also for different cells analyzed, which indicates the high reliability of the data derived from this approach. The down-regulated quadratic curves of mask area rate indicate a dramatic reduction upon agonist application in the number of colocalized pixels carrying both cyan and yellow signals. Quadratic
curves were also generated for mask intensity rates of both channels. Similar data was obtained for a second combination of the same subunits with different fluorescent labels, namely stargazin-DsRed2 and GluR1-EGFP (data not shown).

Figure 4.2.24. Investigation of kinetics of cell surface stargazin-GluR1 fusion protein complexes upon L-glutamate application. Confocal cross sections at a single focal plane of a representative HEK 293 cell coexpressing stargazin-ECFP (A – H) and GluR1-EYFP (I – P) fusion proteins, and incubated with 300 μM L-glutamate from $t_0 = 0$ s to $t_{14} = 1512$ s. Both fusion proteins are highly expressed at the plasma membrane of the cell. This set of images corresponds to cell 1, Z₁ in Table 4.5, and Figures 4.2.25 A, C, and E.
Functional properties of those cell surface stargazin-GluR1 complexes were confirmed by patch clamp recordings, and more importantly, the amount of the surface complexes was investigated as well. In brief, a single HEK 293 cell coexpressing both stargazin-ECFP and GluR1wt proteins was held on the patch electrode with constant application of 600 μM L-glutamate. Whole cell recordings were performed every five minutes. The reduction of the peak of the current is apparent during glutamate incubation (Figure 4.2.26 A). The kinetics of the currents were fitted, and the best fit generated the quadratic function of t (Figure 4.2.26 B) was

\[ y = 0.3819t^2 - 24.909t + 663.93 \quad (R^2 = 0.9887) \]

where \( y \) corresponds to the whole cell recording currents. This indicates that the kinetics of dissociation of functional stargazin-GluR1 complexes follows a quadratic function, in keeping with similar formulas generated from colocalization analysis.

Taken together, the above data demonstrate that cell surface stargazin dissociates from GluR1 upon L-glutamate application under predictable kinetics that follow a quadratic function of time (refer to Table 4.5 first column, mask area rate).

In addition, C-terminally fluorescently-tagged GluR2 subunits, both edited and un-edited versions, were coexpressed with stargazin fusion proteins in HEK 293 cells. Similar approaches of recording dual color four-dimensional image series after agonist application were carried out with these cells. After correction of possible photobleaching as described above, similar quadratic functions were generated for stargazin-GluR2(R) and stargazin-GluR2(Q) complexes (Figure 4.2.27, Table 4.6, and Table 4.7). From these experiments, it may be concluded that functional stargazin-AMPAR complexes localized at the cell surface dissociate after L-glutamate application.

More interestingly, for a quadratic function of \( t \):

\[ y = at^2 + bt + c \quad (a > 0) \]

there is a minimum \( y \) value called as vertex. At the vertex, \( t \) can be calculated as:
\[ t = \frac{-b}{2a} \]

Therefore, for all the quadratic functions generated from colocalization analysis the \( t \) value at each vertex was calculated: \( t = 1025 \pm 77 \text{ s (n = 9)} \) for the GluR1 plus stargazin combination, \( t = 1150 \pm 150 \text{ s (n = 2)} \) for the GluR2(R) plus stargazin combination, and \( t = 2068 \pm 260 \text{ s (n = 3)} \) for the GluR2(Q) plus stargazin combination. When all the quadratic functions for all three combinations were averaged, the mean value of \( t \) was \( 1266 \pm 136 \text{ s (n = 14)} \). Furthermore, the \( t \) value at the vertex of the quadratic function generated by the whole cell recording currents was 1957 s. Maximal dissociation of \textit{in vivo} stargazin-AMPAR surface complexes was found at approximately 1000 – 2000 s of agonist incubation.

However, preliminary experiments with dynamic FRET measurement by donor dequenching after acceptor photobleaching gave different results. In Figure 4.2.28 a representative dynamic FRET measurement is shown, where the intensities of both ECFP and EYFP fluorescence signals in the entire recording field are shown according to the recording time. With the method of donor dequenching after acceptor photobleaching, if FRET increases or decreases during agonist application, these two intensity lines of ECFP and EYFP wouldn’t go parallel anymore but get separated or come closer together, respectively. Thus, the change of the ratio between the whole recording field fluorescence intensity sums of EYFP and ECFP was determined as the change in FRET. There was no significant change in FRET during L-glutamate incubation. Control experiments with this acceptor photobleaching method at the same microscope set-up showed an increase of stargazin-ECFP (donor) intensity after GluR2(R)-EYFP (acceptor) photobleaching, which confirmed the interaction between stargazin-ECFP and GluR2(R)-EYFP was detectable at this set-up, though the increase of cyan channel intensity was not large (Figure 4.2.28). The differing results obtained from this set of experiments compared to previous colocalization and patch clamp recording experiments may be caused by different microscope set ups: the dynamic FRET analysis was performed at a non-confocal microscope where out-of-focus signals can not be excluded from the analysis, so that all the fluorescence signals in the entire recording field were...
added into the count and thus couldn’t be restricted to cell surface signals only; whereas the colocalization analysis was carried out with each single focal plane, so that only in focus signals were analyzed, and intracellular or cell surface localization (where the major expression of stargazin-ECFP is expected) could thus be picked out for analysis by selecting the recording field.

Figure 4.2.25. Kinetics of dissociation of surface stargazin-ECFP / GluR1-EYFP fusion protein complexes in a single live HEK 293 cell upon application of 300 µM L-glutamate (corresponds to cell 1 in Table 4.5). A and B, quadratic curves of mask area rates at two different focal planes (Z₁ and Z₂, respectively). C and D, quadratic curves of mask intensity rates for both channels at two different focal planes (Z₁ and Z₂, respectively). E and F, total intensity sums of the entire recording field for both channels correlated to the recording time duration at two different focal planes (Z₁ and Z₂, respectively). Representative confocal images from where the curves in A, C, and E were derived are shown in Figure 4.2.24.
### Table 4.5 Formulas of the quadratic curves generated for colocalization dynamics upon 300 μM L-glutamate application, with live HEK 293 cells coexpressing stargazin-ECFP and GluR1-EYFP

<table>
<thead>
<tr>
<th>Focal plane</th>
<th>Mask area rate</th>
<th>Mask intensity rate (cyan channel)</th>
<th>Mask intensity rate (yellow channel)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1</td>
<td>y = 5E-07t² - 0.0012t + 1.8158</td>
<td>R² = 0.9802</td>
<td>y = 3E-06t² - 0.008t + 12.275</td>
</tr>
<tr>
<td></td>
<td>y = 5E-07t² - 0.0012t + 1.5338</td>
<td>R² = 0.9795</td>
<td>y = 4E-06t² - 0.0093t + 11.636</td>
</tr>
<tr>
<td>Z2</td>
<td>y = 2E-06t² - 0.0035t + 3.0012</td>
<td>R² = 0.968</td>
<td>y = 1E-05t² - 0.0237t + 17.46</td>
</tr>
<tr>
<td></td>
<td>y = 2E-06t² - 0.0033t + 2.315</td>
<td>R² = 0.9821</td>
<td>y = 2E-05t² - 0.0262t + 15.711</td>
</tr>
<tr>
<td></td>
<td>y = 2E-06t² - 0.0033t + 2.1638</td>
<td>R² = 0.9907</td>
<td>y = 1E-05t² - 0.0247t + 14.714</td>
</tr>
<tr>
<td><strong>Cell 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1</td>
<td>y = 1E-06t² - 0.0029t + 1.4854</td>
<td>R² = 0.9815</td>
<td>y = 1E-05t² - 0.027t + 13.644</td>
</tr>
<tr>
<td></td>
<td>y = 2E-06t² - 0.0035t + 2.2531</td>
<td>R² = 0.995</td>
<td>y = 2E-05t² - 0.033t + 19.673</td>
</tr>
<tr>
<td>Z2</td>
<td>y = 2E-06t² - 0.0046t + 2.7002</td>
<td>R² = 0.998</td>
<td>y = 1E-05t² - 0.0405t + 22.345</td>
</tr>
<tr>
<td>Z3</td>
<td>y = 2E-06t² - 0.0033t + 1.5748</td>
<td>R² = 0.9769</td>
<td>y = 1E-05t² - 0.0303t + 13.805</td>
</tr>
<tr>
<td><strong>Cell 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1</td>
<td>y = 1E-06t² - 0.0029t + 1.4854</td>
<td>R² = 0.9815</td>
<td>y = 1E-05t² - 0.027t + 13.644</td>
</tr>
<tr>
<td></td>
<td>y = 1E-06t² - 0.0019t + 9.9684</td>
<td>R² = 0.9924</td>
<td>y = 2E-05t² - 0.033t + 19.673</td>
</tr>
<tr>
<td>Z2</td>
<td>y = 9E-06t² - 0.0196t + 12.281</td>
<td>R² = 0.9995</td>
<td>y = 2E-05t² - 0.0405t + 22.345</td>
</tr>
<tr>
<td>Z3</td>
<td>y = 2E-06t² - 0.0033t + 1.8404</td>
<td>R² = 0.9777</td>
<td>y = 1E-05t² - 0.0303t + 13.805</td>
</tr>
</tbody>
</table>

y, rate of colocalization.

Representative confocal images of Cell 1, Z1 are shown in Figure 4.2.24.

The quadratic curves of Cell 1 (Z1 and Z2) are shown in Figure 4.2.25.

Note for all quadratic functions of t: y = at² + bt + c, the values of a, b, and c are very similar.

---

Figure 4.2.26. Whole cell recordings of glutamate-evoked currents with a single HEK 293 cell cotransfected with stargazin-ECFP and GluR1 cDNAs, during constant application of 600 μM L-glutamate. A, representative currents according to the duration of glutamate incubation. B, quadratic curve of the peak current related to the duration of glutamate incubation.
Figure 4.2.27. Dynamic dissociation of surface stargazin and GluR2 fusion proteins in live HEK 293 cells upon application of 300 μM L-glutamate. First row, quadratic curves of mask area rates. Second row, quadratic curves of mask intensity rates for both channels. The formulas of curves in A and C are shown in Table 4.6, Cell 1. The formulas of curves in B and D are shown in Table 4.7, Z1.

Figure 4.2.28. Approaches in investigation of dynamic dissociation between stargazin-ECFP / GluR2(R)-EYFP fusion protein complexes in live HEK 293 cells. A, dynamic FRET measurement upon glutamate application; note there is no significant change in FRET value (= YFP/CFP ratio), or intensity values in either ECFP or EYFP channels. B, control experiment: FRET analysis with acceptor photobleaching method; note a slight increase of ECFP intensity indicating the existence of resonance energy transfer between stargazin-ECFP and GluR2(R)-EYFP that implies protein-protein interactions.
Table 4.6 Formulas of the quadratic curves generated for colocalization dynamics upon 300 μM L-glutamate application, with live HEK 293 cells coexpressing stargazin and GluR2(R) fusion proteins

<table>
<thead>
<tr>
<th>Focal plane</th>
<th>Mask area rate</th>
<th>Mask intensity rate (channel 1: stargazin)</th>
<th>Mask intensity rate (channel 2: GluR2(R))</th>
</tr>
</thead>
</table>
| Cell 1 Z1   | $y = 5E-07t^2 - 0.0013t + 1.3871$  
$R^2 = 0.8644$ | $y = 3E-06t^2 - 0.0079t + 8.1815$  
$R^2 = 0.8656$ | $y = 2E-06t^2 - 0.0049t + 4.8455$  
$R^2 = 0.8526$ |
| Cell 2 Z1   | $y = 2E-07t^2 - 0.0004t + 0.5704$  
$R^2 = 0.948$ | $y = 1E-06t^2 - 0.003t + 5.7642$  
$R^2 = 0.9165$ | $y = 1E-06t^2 - 0.0027t + 4.5155$  
$R^2 = 0.923$ |

y, rate of colocalization.

Cell 1, coexpressing stargazin-ECFP and GluR2(R)-EYFP fusion proteins, the quadratic curves are shown in Figures 4.2.27 A and C.

Cell 2, coexpressing stargazin-DsRed2 and GluR2(R)-EGFP fusion proteins.

Note for all quadratic functions of t: $y = at^2 + bt + c$, the values of a, b, and c are very similar.

Table 4.7 Formulas of the quadratic curves generated for colocalization dynamics upon 300 μM L-glutamate application, with live HEK 293 cells coexpressing stargazin-ECFP and GluR2(Q)-EYFP fusion proteins

<table>
<thead>
<tr>
<th>Focal plane</th>
<th>Mask area rate</th>
<th>Mask intensity rate (cyan channel)</th>
<th>Mask intensity rate (yellow channel)</th>
</tr>
</thead>
</table>
| Cell 1 Z1   | $y = 5E-07t^2 - 0.0024t + 10.053$  
$R^2 = 0.9597$ | $y = 3E-06t^2 - 0.0149t + 46.368$  
$R^2 = 0.9682$ | $y = 2E-06t^2 - 0.0107t + 33.106$  
$R^2 = 0.9742$ |
| Z2          | $y = 4E-07t^2 - 0.0018t + 9.9663$  
$R^2 = 0.8594$ | $y = 4E-06t^2 - 0.0108t + 46.692$  
$R^2 = 0.9051$ | $y = 1E-06t^2 - 0.0055t + 33.865$  
$R^2 = 0.8252$ |
| Z3          | $y = 9E-07t^2 - 0.0028t + 9.617$  
$R^2 = 0.9364$ | $y = 4E-06t^2 - 0.0123t + 43.165$  
$R^2 = 0.9363$ | $y = 4E-06t^2 - 0.0109t + 33.652$  
$R^2 = 0.9137$ |

y, rate of colocalization.

The quadratic curves of $Z_i$ are shown in Figures 4.2.27 B and D.

Note for all quadratic functions of t: $y = at^2 + bt + c$, the values of a, b, and c are very similar.

**Utilizing confocal dual-channel four-dimensional (xyzt) image series recorded from living cells and employing custom-made MatLab programs made it possible to perform colocalization analysis with these image series. Dynamic changes of cell surface stargazin-AMPAR complexes upon L-glutamate application were demonstrated. Stargazin was found to dissociate from AMPA receptor subunits, namely GluR1, GluR2(Q), or GluR2(R), immediately after agonist application following a similar quadratic function of time. Moreover, duration of agonist incubation resulting in maximal dissociation of surface stargazin-AMPAR complexes was calculated to be in the range of approximately 1000 – 2000 s.**
4.2.5 The role of the endoplasmic reticulum and the Golgi apparatus in intracellular trafficking of AMPA receptors

4.2.5.1 FRET measurements show that subunits assemble in the ER

FRET analysis was carried out as a novel approach in order to test whether AMPA receptor subunit oligomerization occurs as early as in the ER.

As a model of homomeric AMPA receptor complexes, GluR2(R)-ECFP and GluR2(R)-EYFP fusion proteins were transiently coexpressed in HEK 293 cells. As shown in Chapter 4.2.2, GluR2(R) subunits are retained in the ER without cell surface expression. Analyzing the live cells coexpressing both proteins, there were detectable FRET signals occurring exclusively in the ER (Figure 4.2.29). This suggests the interaction between GluR2(R) subunits takes place in the ER where the assembly of the receptor complexes probably starts though they are not transported to the cell surface.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>FRET channel (mixed signals)</th>
<th>FRET value (true signals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
</tbody>
</table>

Figure 4.2.29. Images of FRET analysis in live HEK 293 cells coexpressing GluR2(R)-ECFP and GluR2(R)-EYFP fusion proteins. The images from left to right present the confocal images of the cyan channel (donor), the yellow channel (acceptor), the FRET channel, and the visualized true FRET value.

As for heteromeric AMPA receptor complexes, GluR2(R)-ECFP and GluR1-EYFP fusion proteins were transiently coexpressed in HEK 239 cells. Unfortunately, there was no significant FRET signal detectable (data not shown), neither at the cell surface nor intracellularly. Since resonance energy transfer between acceptor (ECFP) and donor (EYFP) only occurs if a number of
crucial conditions are satisfied, including not only the distance between the two fluorophores, but also dipole orientation, the undetectable FRET in this case might be caused by improper angles between the dipoles. Another possible reason could be the length difference between the intracellular C-terminal domains of GluR1 and GluR2 to which the fluorophores are fused, so that even the physical distance criteria for FRET to occur might not be fulfilled.

*FRET analysis performed with cells coexpressing GluR2(R)-ECFP and GluR2(R)-EYFP fusion proteins demonstrated oligomerization of AMPA receptor subunits in the ER of living cells.*

### 4.2.5.2 An endoplasmic reticulum – Golgi apparatus – plasma membrane pathway for AMPA receptor complexes

As a model of the heteromeric AMPA receptor complexes, GluR1 and GluR2(R) subunits fused with fluorescent tags were chosen to be coexpressed in HEK 293 cells. Two pairs of subunits labeled with various fluorescent proteins were tested in order to exclude any possible differences caused by different fluorescence tags fused to the subunits. When GluR1-EYFP- and GluR2(R)-ECFP-encoding cDNAs were cotransfected into HEK 293 cells, a perfect overlap of both cyan and yellow fluorescence signals were observed in the ER of the cells (Figure 4.2.30 A – C). A similar expression pattern was observed after coexpression of both GluR1-EGFP and GluR2(R)-DsRed2 fusion proteins. A perfect overlap of both green and red fluorescence signals was seen in the ER of the cells (Figure 4.2.30 D – F), with a further confirmation of colocalization of both proteins demonstrated by the two-dimensional cytofluorogram (Figure 4.2.30 G).
An interesting observation was made when another 24 hours were allowed to pass after the transfection. As shown in Figure 4.2.31, indicated by white arrow heads, there is an intracellular solid structure abundant with both green (GluR1-EGFP) and red (GluR2(R)-DsRed2) fluorescence signals localized at a perinuclear region. This structure may represent the Golgi apparatus.

Figure 4.2.30. GluR1 subunits overlap with GluR2 subunits at intracellular membrane compartments (probably ER) of live mammalian cells. First row: HEK 293 cells cotransfected with GluR2(R)-ECFP (A) and GluR1-EYFP (B); C, overlay of A and B. Second row: HEK 293 cells cotransfected with GluR1-EGFP (D) and GluR2(R)-DsRed2 (E); F, overlay of D and E; G, two-dimensional cytofluorogram, note the cloud of fluorescence signals at 45º relative to both axes. Scale bars = 10 μm.

Figure 4.2.31. GluR1 subunits colocalize with GluR2 subunits at intracellular membrane compartments (probably ER, and the Golgi apparatus) of live mammalian cells. HEK 293 cells cotransfected with GluR1-EGFP (A) and GluR2(R)-DsRed2 (B); C, overlay of A and B, the white arrow points to the perinuclear region abundant of both proteins; D, two-dimensional cytofluorogram, note the cloud of fluorescence signals at 45º relative to both axes. Scale bar = 10 μm.
In order to determine whether this intracellular aggregation of GluR1/GluR2 subunits indeed occurs at the Golgi apparatus, additional triple coexpression experiments were carried out with ECFP-Golgi, GluR1-EYFP, and GluR2(R)-DsRed2 cDNAs. In a typical recording field, there were two cells coexpressing all three fluorescence signals (Figure 4.2.32). For the cell presented in Figure 4.2.32 A – F, there was overlap between GluR1-EYFP and ECFP-Golgi (A – C), as well as an apparent abundant expression of GluR2(R)-DsRed2 at the Golgi apparatus (D – F). This indicates that GluR1 and GluR2(R) subunits are both transported and localized at the Golgi complex after exiting from the ER. The overlap between GluR1 and the Golgi marker was not significant in the second cell (Figure 4.2.32 G – L), while GluR2(R)-DsRed2 still overlaps with the Golgi marker intensively. Since this high expression of GluR2(R) subunits at the Golgi complex was not observed if the subunit was expressed alone, this may further suggest that GluR2(R) subunits are transported to the Golgi apparatus *en route* to the plasma membrane when coexpressed with GluR1 subunits.

*Colocalization of GluR1 and GluR2 fusion proteins in the ER as well as the Golgi apparatus was demonstrated.*
Figure 4.2.32. GluR1 subunits overlap with GluR2 subunits at the Golgi apparatus of live mammalian cells. A – F, and G – L: two confocal cross sections of the same recording field: HEK 293 cells triply transfected with ECFP-Golgi (first column), GluR1-EYFP (B and H), and GluR2(R)-DsRed2 (E and K). Third column, overlays. Scale bar = 10 μm.
### 4.2.5.3 The role of the Golgi apparatus in stargazin-regulated AMPA receptor surface delivery

In the previous chapters, stargazin-associated AMPA receptor surface insertion was demonstrated with confocal live imaging and colocalization analysis. Furthermore, with FRET analysis and patch clamp recording, it has also been shown that stargazin interacts with AMPA receptor subunits at the plasma membrane of HEK 293 cells. However, it remains unclear at which stage and which intracellular compartments that stargazin-AMPA interaction first occurs. Following the coexpression pattern of fluorescently-tagged stargazin and AMPA receptor subunit in living cell may give evidences of the intracellular protein-protein interaction between the two proteins.

As shown in Chapter 4.2.4.2, it was suggested that stargazin may express at the cell surface constitutively and thus independently from AMPAR coexpression. A perinuclear region abundant with both stargazin and AMPA receptor subunits, namely GluR2(Q) or GluR2(R), was also observed. Therefore, colocalization analysis which may suggest association between stargazin and AMPA receptor subunits was performed. Fluorescently-tagged stargazin was therefore coexpressed with each of the fluorescently-tagged AMPA receptor subunits, GluR1, GluR2(Q), and GluR2(R). Representative images are shown in Figure 4.2.33, where coexpression experiments of stargazin-DsRed2 plus GluR1-EGFP, stargazin-DsRed2 plus GluR2(R)-EGFP, and stargazin-ECFP plus GluR2(Q)-EYFP were carried out. The fourth column in the figure shows 2D cytofluorograms of the colocalization analyses performed with each coexpression. The last row in the figure shows only those colocalized pixels replotted when analyzing images I – J. Stargazin is thus shown to indeed colocalize with AMPA receptor subunits not only at the plasma membrane but also the suggested perinuclear region intracellularly.

Furthermore, FRET analysis was carried out with live cells coexpressing stargazin-ECFP plus GluR2(Q)-EYFP, or stargazin-ECFP plus GluR2(R)-EYFP fusion proteins. As shown in Chapter 4.2.4.3, significant intracellular FRET and FRET efficiency values were obtained with both combinations (Table 4.4). These data indicate protein-protein interaction between stargazin and AMPA receptor subunits occurs before they are transported to the cell surface. In Figure 4.2.21 H, also as a representative example for intracellular FRET measurement, there were detectable FRET
signals at the perinuclear regions (indicated by white arrows), which may imply stargazin-AMPAR interaction at the Golgi apparatus.

Again, in order to determine whether this is indeed the Golgi apparatus, triple coexpression of stargazin, an AMPA receptor subunit, and a Golgi marker was carried out. In Figure 4.2.34, a typical recording field is presented with cells coexpressing ECFP-Golgi, stargazin-DsRed2, and GluR2(R)-EGFP fusion proteins. At this focal plane, apparent aggregation of GluR2(R)-EGFP at the Golgi apparatus was observed in three cells, with stargazin-DsRed2 aggregation at the Golgi apparatus in one of the cells. 2D and 3D cytofluorograms are shown as well (Figure 4.2.34 E and F) for the digital analysis. The masked images present colocalized pixels which carry cyan and red, cyan and green, or green and red signals in G – I, J – L, and M – O, respectively. Another combination of triple expression with ECFP-Golgi, stargazin-DsRed2, and GluR2(R)-EYFP was also tested and similar results were obtained (Figure 4.2.35). Analyzing this single cell coexpressing all three fusion proteins, the masked images presenting colocalized pixels which carry cyan and red, cyan and green, or green and red signals are shown in G – I, J – L, and M – O, respectively. These masked images in G – O show the same intracellular structure, the Golgi apparatus, where stargazin colocalizes with GluR2(R) subunits, suggesting the association of stargazin and GluR2(R) at this organelle.

*Triple expression of ECFP-Golgi, stargazin-DsRed2, and GluR2(R)-EGFP; or ECFP-Golgi, stargazin-DsRed2, and GluR2(R)-EYFP fusion proteins demonstrated the association of stargazin and AMPA receptor subunits at the Golgi apparatus before AMPA receptor subunits are translocated to the cell surface by stargazin. FRET analysis further confirmed protein-protein interaction between stargazin and AMPA receptor subunits at a perinuclear region.*
Figure 4.2.33. AMPA receptor subunits colocalize with stargazin at intracellular membrane compartments (probably the Golgi apparatus) of live mammalian cells. First row: a confocal cross section of HEK 293 cells cotransfected with GluR1-EGFP (A) and stargazin-DsRed2 (B); C, overlay of A and B; D, two-dimensional cytofluorogram: note the cloud of fluorescence signals at 45º relative to both axes. Second row: a confocal cross section of HEK 293 cells cotransfected with GluR2(R)-EGFP (E) and stargazin-DsRed2 (F); G, overlay of E and F; H, two-dimensional cytofluorogram. Third row: a confocal cross section of HEK 293 cells cotransfected with stargazin-ECFP (I) and GluR2(Q)-EYFP (J); K, overlay of I and J; L, two-dimensional cytofluorogram. Fourth row: analysis of masked images of selected dots with both cyan and yellow signals from images I – K, at approximately same intensity values: M, cyan channel; N, yellow channel; O, overlay of M and N. Scale bars = 10 μm in C, G, and K.
Figure 4.2.34. Stargazin colocalizes with the AMPA receptor subunit GluR2(R) at the Golgi apparatus of live mammalian cells: analysis with EGFP-labeled GluR2(R). First row: a confocal cross section of HEK 293 cells triply transfected with Golgi-ECFP (A), GluR2(R)-EGFP (B), and stargazin-DsRed2 (C). Second row: D, overlay of A, B, and C; E, two-dimensional cytofluorogram; F, three-dimensional cytofluorogram. Third row: analysis of masked images of selected dots with both cyan and red signals at approximately same intensity values: G, cyan channel; H, red channel; I, overlay of G and H. Fourth row: analysis of masked images of selected dots with both cyan and green signals at approximately same intensity values: J, cyan channel; K, green channel; L, overlay of J and K. Fifth row: analysis of masked images of selected dots with both green and red signals at approximately same intensity values: M, green channel; N, red channel; O, overlay of M and N. Scale bar = 10 μm.
Figure 4.2.35. Stargazin colocalizes with the AMPA receptor subunit GluR2(R) at the Golgi apparatus of live mammalian cells: analysis with EYFP-labeled GluR2(R). First row: a confocal cross section of HEK 293 cells triply transfected with Golgi-ECFP (A), GluR2(R)-EYFP (B), and stargazin-DsRed2 (C). Second row: D, overlay of A, B, and C; E, two-dimensional cytofluorogram; F, three-dimensional cytofluorogram. Third row: analysis of masked images of selected dots with both cyan and red signals at approximately same intensity values: G, cyan channel; H, red channel; I, overlay of G and H. Fourth row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: J, cyan channel; K, yellow channel; L, overlay of J and K. Fifth row: analysis of masked images of selected dots with both yellow and red signals at approximately same intensity values: M, yellow channel; N, red channel; O, overlay of M and N. Scale bar = 5 μm.
4.3 Monitoring assembly and intracellular trafficking of kainate receptors

4.3.1 Monitoring intracellular trafficking of GluR6(Q) in live mammalian cells

4.3.1.1 Functional GluR6(Q) fusion proteins express mainly at the plasma membrane

To verify expression of the fusion constructs, GluR6(Q)-ECFP, GluR6(Q)-EGFP, GluR6(Q)-EYFP, and GluR6(Q)-DsRed2 encoding cDNAs were transfected into HEK 293 cells and observed the plasma membrane localization of the fusion proteins by live imaging. As shown in Figure 4.3.1 there were no significant differences in the subcellular expression patterns of the four different fluorescent protein-tagged GluR6(Q) subunits. When GluR6(Q)-EGFP was transfected into cultured hippocampal neurons, a strong expression of the subunit was observed at the plasma membrane of the soma as well as in dendrites (Figure 4.3.1 E – F).

![Image of HEK 293 cells and cultured hippocampal neurons expressing GluR6(Q) fusion proteins](image)

By confocal z-section scanning, a robust expression of GluR6(Q) at the plasma membrane was demonstrated (Figure 4.3.2 B and E) and shown to overlap with a lipid bilayer-specific stain (FM4-64, Figure 4.3.2 C and F). To my knowledge, this was the first time that fluorescently-labeled...
GluR6 expression was shown at the plasma membrane in live mammalian cells. This distribution matches previous observations by immunocytochemistry (Ren et al., 2003a; Yan et al., 2004). According to further pixel-based colocalization analysis, most of the pixels carrying a green signal show a red signal at the same time (Figure 4.3.2 H).

Figure 4.3.2. A robust plasma membrane expression of GluR6(Q) fusion proteins. A, HEK cells stained with 2 μM FM4-64 which labels the plasma membrane of live cells. B – D, a confocal cross section of HEK 293 cells transfected with GluR6-EYFP (B) and stained with FM4-64 (C); D, Overlay, note the plasma membrane localization of both yellow and red fluorescence signals for those cells pointed out with white arrows. E – H, colocalization analysis with a confocal cross section of HEK 293 cells transfected with GluR6-EGFP (E) and stained with FM4-64 (F); G, Overlay; H, two-dimensional cytofluorogram, note one cloud of fluorescence signals at 45° as well as another cloud at 0° angle to the Y axis (= red axis). Scale bars = 50 μm in A and D, 15 μm in G.
A western blot performed with a monoclonal anti-EGFP antibody, JL-8, which recognizes EGFP and its variants such as ECFP and EYFP, demonstrated the expression of GluR6(Q)-EGFP-variant fusion proteins at the expected molecular size in transfected cells (Figure 4.3.3 C). In order to test whether the fused fluorescent proteins alter the functional properties of GluR6, patch clamp recordings were performed. Cells expressing either of the GluR6 fusion proteins respond to glutamate application as shown in Figure 4.3.3 A and B, confirming that all fusion proteins form fully functional ion channels in the plasma membrane.

Figure 4.3.3. Confirmation of expression of functional GluR6(Q) fusion proteins. A – B, patch clamp recordings confirmed electrophysiological function of GluR6(Q) fusion proteins. A, representative whole-cell recordings of glutamate-evoked currents: HEK 293 cells transfected with GluR6 wild type or various fluorescently-labeled GluR6 cDNAs. B, mean glutamate-evoked currents of HEK 293 cells transfected with GluR6 wild type, GluR6-ECFP, GluR6-EGFP, GluR6-EYFP, and GluR6-DsRed2 cDNAs were 1960 ± 643 pA (n = 3), 1185 ± 251 pA (n = 3), 789 ± 305 pA (n = 4), 1159 ± 647 pA (n = 3), and 1021 ± 347 pA (n = 4), respectively. C, western blot analysis of cell lysates from transfected HEK 293 cells blotted with the monoclonal anti-EGFP antibody JL-8.

After verification of membrane localization and functional integrity of GluR6 fusion proteins, these constructs were used to analyze the mechanisms of assembly and intracellular trafficking of homomeric kainate receptor complexes, and those of heteromeric receptor complexes (together
with GluR6(R) and KA2 fusion proteins) in live HEK 293 cells. In addition, these constructs were also used to estimate individual and coexpression efficiencies of glutamate receptor subunits in HEK 293 cells.

_GluR6(Q)-ECFP, GluR6(Q)-EGFP, GluR6(Q)-EYFP, and GluR6(Q)-DsRed2 fusion proteins were expressed in HEK 293 cells and their electrophysiological properties were verified. Utilizing ECFP-ER coexpression or FM4-64 staining, GluR6(Q) subunits were shown to localize mainly at the cell surface._

4.3.1.2 GluR6(Q) fusion proteins localize at a cytosolic network-like compartment and a perinuclear region before their transport to the plasma membrane

Utilizing the fluorescently-labeled GluR6(Q) subunits and the live imaging technique, the intracellular trafficking pathway of the subunits in HEK 293 cells was monitored. As shown in Figures 4.3.4, 4.3.5 and 4.3.6, the fluorescently-labeled GluR6(Q) fusion proteins showed a similar pattern of intracellular trafficking before plasma membrane insertion of the subunits. Cytosolic localization of the fusion proteins was observed, with fluorescence signals revealing a network-like structure that suggests the endoplasmic reticulum (Figure 4.3.5, Figure 4.3.6 D); as well as a perinuclear region with an abundance of the labeled subunits that may suggest the Golgi apparatus (Figure 4.3.6, B and E).

_Abundance of GluR6(Q) fusion proteins at the cytosolic and perinuclear membrane compartments was observed before their cell surface expression._
Figure 4.3.4. GluR6(Q) fusion proteins localize at a cytosolic network-like compartment and a perinuclear region before their insertion into the plasma membrane. Confocal cross sections of HEK 293 cells transfected with GluR6(Q)-DsRed2. Scale bar = 10 μm.
Figure 4.3.5. GluR6(Q) fusion proteins localize at a cytosolic network-like compartment before their insertion into the plasma membrane. A, confocal cross sections of HEK 293 cells transfected with GluR6(Q)-ECFP. B, confocal cross sections of HEK 293 cells transfected with GluR6(Q)-EGFP. C, a confocal cross section of HEK 293 cells transfected with GluR6(Q)-EYFP. Scale bars = 20 μm in A – C.
Figure 4.3.6. GluR6(Q) fusion proteins localize at a cytosolic network-like compartment and a perinuclear region before their insertion into the plasma membrane. A, a confocal cross section of HEK 293 cells transfected with GluR6(Q)-EYFP. B, zoom in confocal cross sections of selected HEK 293 cells in A. C, a confocal cross section of HEK 293 cells transfected with GluR6(Q)-EGFP. D – E, zoom in confocal cross sections of selected HEK 293 cells in C. Scale bars = 40 μm in A and C.
4.3.1.3 GluR6(Q) fusion proteins colocalize with intracellular membrane compartments

One possible approach to identify the intracellular membrane compartments in live mammalian cells is to stain the cells with BODIPY TR-C5-Ceramide. Ceramide belongs to the class of sphingolipids, a lipid family retained at the Golgi apparatus, and thus can serve as a fluorescent structure marker for the Golgi complex as well as to outline cellular boundaries. The BODIPY TR fluorophore exhibiting red fluorescence (617 nm) is well separated from EGFP green fluorescence by confocal microscopy (Figure 4.3.7).

Figure 4.3.7. GluR6(Q)-EGFP overlaps with intracellular membrane compartments in live cells. HEK 293 cells transiently transfected with GluR6(Q)-EGFP (A), and incubated with 5 μM BODIPY TR-C5-Ceramide for 30 minutes at 4 ºC (B). C, Overlay. Scale bar = 20 μm.

Furthermore, the colocalization of green and red fluorescence signals for selected cells was analyzed as shown in Figure 4.3.8. In the CF2D cytofluorogram (Figure 4.3.8 G), which is a dot diagram that visualizes the joint distribution of the intensity values of two detection channels, there is a cloud of fluorescence dots at a 45º angle which represents the pixels with both fluorescence signals at approximately same intensity values. Those dots are singled out and presented in Figure 4.3.8 D – F, which shows their intracellular localization. This perinuclear structure has a highly concentrated BODIPY TR-C5-Ceramide staining: therefore it probably represents the Golgi complex of the cell.

The labelling of intracellular membrane compartments with BODIPY TR-C5-Ceramide, however, does not distinguish between ER and Golgi. Seeking another method in order to label
either ER or Golgi apparatus separately may give further advantages when exploring the trafficking of the receptors in live cells.

Figure 4.3.8. GluR6(Q)-EGFP colocalizes with intracellular membrane compartments at specific structures in live cells. HEK 293 cells transiently transfected with GluR6(Q)-EGFP (A), and incubated with 5 μM BODIPY TR-C5-Ceramide for 30 minutes at 4 °C (B); C, Overlay of A and B. Colocalization analysis shows selected dots with both fluorescence signals at approximately same intensity values: D, green channel; E, red channel; F, overlay of D and E. F, two-dimensional cytofluorogram, note that there are three clouds of fluorescence signals: one at 0°, one at 45°, and another one at 90° with respect to the X axis (= green axis). Scale bar = 15 μm.

Utilizing live staining of intracellular membrane compartments with BODIPY TR-C5-Ceramide, GluR6(Q) fusion proteins were demonstrated to colocalize at these compartments. The next step shall be distinguishing between ER and Golgi apparatus membranes (Figures 4.3.9 and 4.3.10).
Colocalization analysis demonstrates an ER – Golgi apparatus – plasma membrane trafficking pathway for GluR6(Q) subunit

In order to label distinct intracellular membrane compartments, such as ER or Golgi apparatus, ECFP-ER or ECFP-Golgi encoding cDNA was transfected into HEK 293 cells and monitored the expression of those fusion proteins in live cells. Given enough time for protein expression and re-localization (usually after 24 hours for ECFP-ER, and 48 hours for ECFP-Golgi), the fluorescent fusion proteins were readily detected at specific intracellular structures as expected.

ECFP-ER is incorporated into the endoplasmic reticulum (ER) as shown by the bright cyan fluorescence in Figure 4.3.9.

Figure 4.3.9. ECFP-ER fusion protein is targeted to the endoplasmic reticulum (ER) in HEK 293 cells. Cells were transiently transfected with pECFP-ER. ECFP fluorescence is localized exclusively to the ER.
The ECFP-Golgi fusion protein is targeted to the trans-medial region of the Golgi apparatus in HEK 293 cells. Cells were transiently transfected with pECFP-Golgi. ECFP fluorescence is localized exclusively to the Golgi apparatus.

A, confocal cross sections of a single cells expressing ECFP-Golgi; B, 2D image generated by merging all the image sections in A; C, panel B shown in a depth-color coded mode, shows a 3D reconstruction model of the cell. D, confocal cross sections of a single cells expressing ECFP-Golgi; E, 2D image generated by merging all the image sections in D; F, panel E shown in a depth-color coded mode, shows a 3D reconstruction model of the cell. G – H, four cells expressing ECFP-Golgi: G, 2D image generated by merging confocal image sections (not shown); H, panel G shown in a depth-color coded mode, shows 3D reconstruction model of the cells. Note different morphology of individual Golgi apparatuses as shown in C, F, and H. Scale bars = 10 μm in C, F and H.
ECFP-Golgi is incorporated into the Golgi apparatus as shown by bright cyan fluorescence in Figure 4.3.10. By three-dimensional reconstruction of several live cells expressing ECFP-Golgi fusion protein, the Golgi apparatus of each cell was demonstrated by cyan fluorescence (Figure 4.3.10 A, B, D, E, and G) as well as a color-coded 3D model according to the depth of the z-sections (Figure 4.3.10 C, F, and H).

With the capability to distinguish ER and Golgi apparatus verified, the intracellular trafficking of the GluR6(Q) subunit was then monitored and analyzed by live imaging. The first trafficking step is the localization of GluR6(Q) subunits at the ER demonstrated by the overlapping cyan and yellow signals (Figure 4.3.11 A – C). By further digital analysis, it is shown that GluR6(Q)-EYFP colocalized with ECFP-ER intracellularly (Figure 4.3.11 D – F), and expressed at the plasma membrane as well (Figure 4.3.11 J – L). After being exported from ER, GluR6(Q) subunits were found to be abundant at the Golgi apparatus as shown in Figure 4.3.12 where GluR6(Q)-EYFP colocalizes with Golgi-ECFP in two cells in the middle of the images. Interestingly, there were also additional intracellular yellow signals observed to be adjacent to cyan signals (Figure 4.3.12 and Figure 4.3.13). Since Golgi-ECFP actually is targeted to the trans-medial region of the Golgi apparatus, this neighbouring aggregation of GluR6(Q)-EYFP beside Golgi-ECFP may occur at the cis-Golgi or the trans Golgi network.

*Utilizing coexpression with ECFP-ER and ECFP-Golgi fusion proteins and digital colocalization analysis, GluR6(Q) subunits were shown to be abundant first in the ER and then in the Golgi apparatus before being expressed at the cell surface.*
Figure 4.3.11. Colocalization analysis indicates the first step of GluR6(Q) intracellular trafficking is its localization at the ER. First row: HEK 293 cells were cotransfected with ECFP-ER (A) and GluR6(Q)-EYFP (B); C, overlay of A and B. Second row: colocalization analysis: masked images of selected dots with both cyan and yellow signals at approximately same intensity values; D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with cyan signals only: G, cyan channel; H, yellow channel; I, overlay of G and H. Fourth row: analysis of masked images of selected dots with yellow signals only: J, cyan channel; K, yellow channel; L, overlay of J and K. M, two-dimensional cytofluorogram, note there are three clouds of fluorescence signals: one at 0º, one at 45º, and another one at 90º relative to the X axis (= cyan axis). Scale bar = 10 μm.
Figure 4.3.12. GluR6(Q) is abundant at the Golgi apparatus before its insertion into plasma membrane. First row; HEK 293 cells were cotransfected with ECFP-Golgi (A) and GluR6(Q)-EYFP (B); C, overlay of A and B. Second row: colocalization analysis: masked images of selected dots with both cyan and yellow signals at approximately same intensity values; D, cyan channel; E, yellow channel; F, overlay of D and E. G, two-dimensional cytofluorogram, note there are three clouds of fluorescence signals: one at 0°, one at 45°, and another one at 90° relative to the X axis (= cyan axis). Scale bar = 15 μm.
Figure 4.3.13. GluR6(Q) is abundant at, as well as adjacent to, the trans-medial region of the Golgi apparatus before its insertion into plasma membrane. First row: 3D reconstruction of HEK 293 cells cotransfected with ECFP-Golgi (A) and GluR6(Q)-EYFP (B); C, overlay of A and B. Second row: a confocal cross section of same cells: D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values; G, cyan channel; H, yellow channel; I, overlay of G and H. J, two-dimensional cytofluorogram, note there are two dominant clouds of fluorescence signals: one at 0º and another at 90º relative to the X axis (= cyan axis), some dots also appear at a 45º angle. Scale bar = 15 μm.
4.3.2 Monitoring intracellular trafficking of GluR6(R) in live mammalian cells

4.3.2.1 GluR6(R) fusion proteins form functional homomeric receptors at the plasma membrane

To verify expression of the fusion constructs, GluR6(R)-ECFP, GluR6(R)-EGFP, or GluR6(R)-EYFP was transfected into HEK 293 cells and the fusion proteins were observed to be localized at the plasma membrane of the cells by live imaging. As shown in Figure 4.3.14 A – C there were no significant differences in the subcellular expression patterns of the three different fluorescent protein-tagged GluR6(R) subunits. In order to test whether the fused fluorescent proteins alter the functional properties of GluR6(R), patch clamp recordings were performed. Cells expressing GluR6(R)-ECFP respond to glutamate application (Figure 4.3.14 D), mean glutamate-evoked current was $105 \pm 42$ pA ($n = 3$). This confirms that the fusion protein forms fully functional ion channels in the plasma membrane. The other two fusion proteins are expected to be functional as well because of the minimal differences in the coding sequences of GFP variants.

![Image](image-url)

Figure 4.3.14. Plasma membrane expression of functional GluR6(R) fusion proteins. HEK 293 cells transiently transfected with either of the three C-terminal fluorescently-labeled GluR6(R) cDNAs GluR6(R)-EGFP (A), GluR6(R)-EYFP (B), and GluR6(R)-ECFP (C). D, representative whole cell recording of glutamate-evoked currents: HEK 293 cells transfected with GluR6(R)-ECFP. E, western blot analysis of cell lysates from transfected HEK 293 cells blotted with the monoclonal anti-EGFP antibody JL-8. Scale bars = 20 μm in A – C.
GluR6(R)-ECFP, GluR6(R)-EGFP, and GluR6(R)-EYFP fusion proteins were expressed in HEK 293 cells and functional properties were verified electrophysiologically for GluR6(R)-ECFP. GluR6(R) subunits were shown to mainly express at the cell surface.

4.3.2.2 An ER – Golgi apparatus – plasma membrane trafficking pathway for GluR6(R) subunit

To test whether the intracellular trafficking of GluR6(R) also follows the ‘ER – Golgi apparatus – plasma membrane’ pathway in mammalian cells, live imaging of GluR6(R) fusion proteins was performed at different time points before the insertion of the receptors at the plasma membrane occurred.

At an early time point, GluR6(R)-ECFP or GluR6(R)-EYFP was observed to localize exclusively at ER-like intracellular structures as shown in Figure 4.3.15 A and B. In order to verify that this was indeed ER localization of GluR6(R) subunit, cotransfection of GluR6(R)-EYFP and the ER marker ECFP-ER was carried out in HEK 293 cells. GluR6(R)-EYFP was found to overlap with ECFP-ER (Figure 4.3.15 C – E). The colocalization analysis (Figure 4.3.15 F) showed a single cloud of fluorescence signals at a 45º angle in the two-dimensional cytofluorogram, which demonstrates a 100% colocalization of cyan signals (serving as ER marker) and yellow signals (GluR6(R) subunits) in these two cells.

After exiting from ER, GluR6(R) subunits were then localized at a perinuclear region demonstrated by the highly concentrated fluorescence signals of the fusion proteins: GluR6(R)-ECFP or GluR6(R)-EGFP (Figure 4.3.16 A and B, respectively). Further analysis was carried out with cells cotransfected with GluR6(R)-EYFP and ECFP-Golgi (Figure 4.3.16 C – P). Here, two typical observations are shown as panels C – I, and J – P. In Figure 4.3.16 C – E, the yellow fluorescence signals (GluR6(R)-EYFP) are shown to overlap with cyan signals (serving as marker of the trans-medial region of the Golgi apparatus), which is also shown as the cloud of fluorescent dots at a 45º angle in the two-dimensional cytofluorogram (Figure 4.3.16 F). These selected dots with approximately same intensities of both fluorescence signals were localized exactly at the Golgi apparatus of the cotransfected cells (Figure 4.3.16 G – I). Similar analysis is shown in
Figure 4.3.16 J – P, where in addition to localization of yellow fluorescence signals (GluR6(R)-EYFP) at the trans-medial region of the Golgi apparatus, there were highly concentrated yellow signals expressed adjacent to it. This observation is in fact consistent with that of GluR6(Q) subunit as described before (Chapter 4.3.1.4).

The final destination of the subunits is the plasma membrane. Coexpression of GluR6(R)-EYFP and ECFP-ER in HEK 293 cells showed no overlap of cyan and yellow fluorescence signals (Figure 4.3.17).

![Image of Figure 4.3.15: Colocalization analysis indicates the first step of GluR6(R) intracellular trafficking is its localization at the ER. First row: HEK 293 cells transiently transfected with either one of the C-terminally fluorescently-labeled GluR6(R) cDNAs, GluR6(R)-ECFP (A) or GluR6(R)-EYFP (B). Second row: HEK 293 cells cotransfected with ECFP-ER (C) and GluR6(R)-EYFP (D). Third row: colocalization analysis: E, overlay of C and D, showing 100% overlap of cyan and yellow fluorescence signals; F, two-dimensional cytofluorogram analyzing fluorescence signals of the cyan channel (C) and the yellow channel (D). Note the only cloud of fluorescence signals at a 45º angle. Scale bars = 10 μm in A, B and E.]
Figure 4.3.16. Colocalization analysis indicates GluR6(R) exits from the ER to the Golgi apparatus. A–B, confocal cross sections of HEK 293 cells transiently transfected with either one of the C-terminally fluorescently-labeled GluR6(R) cDNAs, GluR6(R)-ECFP (A) or GluR6(R)-EGFP (B); note the structure with highly concentrated cyan (or green) fluorescence signals at a perinuclear region. C–F, HEK 293 cells cotransfected with ECFP-Golgi (C) and GluR6(R)-EYFP (D); E, overlay of C and J; F, CF2D cytofluorogram. G–I, colocalization analysis of panels C and D: masked images of selected dots with both cyan and yellow signals at approximately same intensity values; G, cyan channel; H, yellow channel; I, overlay of G and H. J–M, HEK 293 cells cotransfected with ECFP-Golgi (J) and GluR6(R)-EYFP (K); L, overlay of J and K; M, CF2D cytofluorogram. N–P, colocalization analysis of panels J and K: masked images of selected dots with both cyan and yellow signals at approximately same intensity values; N, cyan channel; O, yellow channel; P, overlay of N and O. Note there are three clouds of fluorescence signals: one at 0°, one at 45°, and another one at 90° relative to the X axis (= cyan axis) in panels F and M. Scale bars = 10 μm in A, B, E, and L.
Results

Figure 4.3.17. Plasma membrane expression of the GluR6(R) subunit. HEK 293 cells cotransfected with ECFP-ER (A) and GluR6(R)-EYFP (B). C, overlay. Scale bar = 10 μm in C.

Utilizing coexpression with ECFP-ER and ECFP-Golgi fusion proteins and digital colocalization analysis, GluR6(R) subunits were shown to be abundant first in the ER and then in the Golgi apparatus before being expressed at the cell surface.

4.3.3 Retention of KA2 fusion proteins in the ER

Homomerically expressed KA2 subunits are known as non-functional receptors so far (Hollmann, 1999). When the fluorescently labeled recombinant constructs, namely KA2-ECFP, KA2-EGFP, KA2-EYFP, and KA2-DsRed2, were transfected into HEK 293 cells, the fusion proteins encoded were found to be quantitatively retained in the ER (Figure 4.3.18 A – C).

Western blot analysis confirmed the expression of KA2 fluorescent fusion proteins at the expected molecular sizes in HEK 293 cells (Figure 4.3.18 D). The functional integrity of these fusion proteins was verified by patch clamp recordings of GluR6 / KA2 heteromeric complexes generated by coexpression of GluR6(Q) or GluR6(R) and KA2 fusion proteins in HEK 293 cells (data shown in Chapter 4.3.4.3).
Figure 4.3.18. Expression of KA2 fusion proteins. HEK 293 cells transiently transfected with either one of the C-terminal fluorescently-labeled KA2 cDNAs KA2-EGFP (A), KA2-EYFP (B), and KA2-ECFP (C). D, western blot analysis of cell lysates from transfected HEK 293 cells blotted with the monoclonal anti-EGFP antibody JL-8. Scale bars = 10 μm in A, B, and C.

To visualize the differences of the intracellular localization of KA2 and GluR6 subunits under high magnification, the plasma membrane or the ER was labeled in live cells simultaneously expressing KA2 fusion protein. As shown in Figure 4.3.19, the plasma membrane of cells transfected with KA2-EGFP was stained by the dye FM4-64. However, there was no overlap of green (KA2-EGFP) and red fluorescence signals (serving as marker for plasma membrane), as also indicated by the two clearly separated clouds of green and red dots along the two the axes in the two-dimensional cytofluorogram (Figure 4.3.19 D). Thus, a significantly different intracellular localization of KA2 and GluR6 (Figure 4.3.19 and Figure 4.3.2, respectively) was confirmed.
Results

Figure 4.3.19. KA2 fusion protein does not localize at the plasma membrane of mammalian cells. Colocalization analysis with a confocal cross section of HEK 293 cells transfected with KA2-EGFP (A) and stained with FM4-64 (B). C, Overlay; D, two-dimensional cytofluorogram; note the two separated clouds of fluorescence signals at 0° and 90° relative to the X axis (= green axis). Scale bar = 10 μm in C.

Analyzing cells expressing both KA2-EYFP and ECFP-ER fusion proteins, a perfect overlap of cyan (serving as ER marker) and yellow fluorescence signals (KA2-EYFP) was found (Figure 4.3.20 A – C). This can be seen in the two-dimensional cytofluorogram (Figure 4.3.20 J), where the dots at a 45° angle carry both fluorescence signals at approximately the same intensity. The masked images of those dots prove the ER localization of almost all of the yellow fluorescence signals (Figure 4.3.20 D – F). As a control, in the same recording field there was a cell expressing exclusively ECFP-ER. When the dots along the cyan axis (= with cyan signals only) in the two-dimensional cytofluorogram were selected, the masked images showed the image of this cell only in the cyan channel (Figure 4.3.20 G) while no signal was found in the yellow channel (Figure 4.3.20 H).
An additional coexpression experiment was carried out with KA2-EYFP and ECFP-Golgi cDNAs. In a typical recording field, there were several cells coexpressing both fluorescence signals (Figure 4.3.21 C and G). Analyzed by two-dimensional cytofluorograms (Figure 4.3.21 D and H) according to individual confocal z-sections, the great majority of the yellow fluorescence signal (KA2-EYFP) is not colocalized with the cyan signal (serving as marker of the trans-medial region of the Golgi apparatus) (Figure 4.3.21 A – D). However, there is a small amount of yellow fluorescence signal that does colocalize with the cyan signals in one of the cells (Figure 4.3.21 E – H, cell indicated by an arrow). Taken together with the finding that KA2 subunits are not detectable in the plasma membrane, as shown before, this observation indeed suggests a possible dynamic transport of KA2 subunits between the ER and the Golgi apparatus -- exiting of KA2 subunits from ER to the Golgi apparatus and a retrieve route in the opposite direction.

KA2-ECFP, KA2-EGFP, KA2-EYFP, and KA2-DsRed2 fusion proteins were expressed in HEK 293 cells and their electrophysiological properties were verified in coexpression with GluR6(Q) or GluR6(R) fusion proteins. Utilizing ECFP-ER coexpression or FM4-64 staining, KA2 subunits were shown to be retained in the ER quantitatively, with no cell surface expression at all. A small amount of KA2 subunits are shown to localize at the Golgi apparatus suggesting a possible dynamic transport between the ER and the Golgi apparatus.
Figure 4.3.20. Colocalization analysis confirms ER retention of KA2 subunits. First row: a confocal cross section of HEK 293 cells cotransfected with ECFP-ER (A) and KA2-EYFP (B); C, overlay of A and B; note two of the three cells coexpressing both fusion proteins while the other one expresses ECFP-ER only. Second row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with cyan signals only: G, cyan channel; H, yellow channel; I, overlay of G and H. J, two-dimensional cytofluorogram; note one large cloud of fluorescence signals at 45° as well as another much smaller cloud at 0° relative to the X axis (= cyan axis). Scale bar = 10 μm.
Figure 4.3.21. Analysis to determine whether KA2 subunits colocalize with the Golgi apparatus. First row: a confocal cross section of HEK 293 cells cotransfected with ECFP-Golgi (A) and KA2-EYFP (B); C, overlay of A and B. Second row: another confocal cross section of the same HEK 293 cells cotransfected with ECFP-Golgi (D) and KA2-EYFP (E); F, overlay of D and E. Third row: analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: G, cyan channel; H, yellow channel; I, overlay of G and H. J, two-dimensional cytofluorogram of the images of the first row; note the two separated clouds of fluorescence signals at 0° and 90° relative to the X axis (= cyan axis). K, two-dimensional cytofluorogram of the images of the second row; note the two separated clouds of fluorescence signals at 0° and 90° angle, and a much less intense third cloud of the signals at 45° relative to the X axis (= cyan axis). Scale bar = 10 μm.
4.3.4 Monitoring intracellular trafficking of GluR6 / KA2 heteromeric complexes in live mammalian cells

4.3.4.1 Colocalization of GluR6 and KA2 subunits in the ER

Heteromeric receptor complexes containing both GluR6 and KA2 subunits have been reported to respond to the agonist glutamate as well as AMPA, while homomeric GluR6 complexes have no AMPA-induced currents and homomeric KA2 complexes give no current at all. However, the rules of assembly and intracellular trafficking of kainate receptors remained largely unknown. In the previous chapters, homomeric kainate receptors of GluR6(Q), GluR6(R), and KA2 were expressed and monitored in live mammalian cells. Whether the suggestion (Chapters 4.3.1.4 and 4.3.2.2) that the receptor subunits start to assemble into oligomers in the ER is also true for the heteromeric kainate receptors remained to be demonstrated. Therefore, transient cotransfection of GluR6 and KA2 cDNAs fused with fluorescent tags were carried out in HEK 293 cells.

Figure 4.3.22. KA2 and GluR6(Q) colocalize in the ER at a time point before their insertion into the plasma membrane. A confocal cross section of a HEK 293 cell cotransfected with KA2-ECFP (A) and GluR6(Q)-EYFP (B); C, overlay of A and B, showing 100% overlap of cyan and yellow fluorescence signals; D, two-dimensional cytofluorogram; note a single cloud of fluorescence signals at a 45° angle. Scale bar = 10 μm.
The first heteromeric subunit combination tested was KA2 plus GluR6(Q). In order to exclude any possible differences caused by different fluorescent tags fused to the subunits, three pairs of subunits labeled with different fluorescent proteins were tested. In Figure 4.3.22, the coexpression pattern of KA2-ECFP and GluR6(Q)-EYFP before either subunit reaches the plasma membrane is demonstrated. Both cyan (KA2-ECFP) and yellow (GluR6(Q)-EYFP) fluorescence signals are localized in the ER of the cell, and a perfect overlap is proven by colocalization analysis (Figure 4.3.22 D). Similar colocalization of both subunits within the ER was also observed with cells coexpressing KA2-EGFP plus GluR6(Q)-DsRed2 (data not shown) and KA2-DsRed2 plus GluR6(Q)-EGFP (Figure 4.3.24).

Another heteromeric combination chosen was KA2 coexpressed with GluR6(R), the edited version of GluR6. When GluR6(R)-EYFP was coexpressed with KA2-ECFP in HEK 293 cells, the overlap and colocalization of both fluorescence signals in the ER was found at a time point before either of the fusion proteins was expressed at the cell surface, as well (Figure 4.3.25 D – F).

**Digital analysis demonstrated that KA2 subunits colocalize with GluR6(Q) or GluR6(R) subunits in the ER, which may suggest that oligomerization of heteromeric kainate receptor complexes occurs in the ER.**

### 4.3.4.2 A significant delay of plasma membrane-targeted GluR6 trafficking by coexpression with KA2

Apart from the colocalization of both KA2-ECFP and GluR6(Q)-EYFP fusion proteins in the ER of cotransfected cells, a very interesting observation is that at the same time point in cells transfected in parallel but only with GluR6(Q)-EYFP, GluR6(Q) subunits were detected mainly at the plasma membrane (data not shown). In those cells coexpressing both fusion proteins, after some time plasma membrane-inserted GluR6(Q) subunits were also observed (Figure 4.3.23 G – I). However, there remained a considerable amount of intracellular GluR6(Q) colocalized with KA2 subunits which were still retained in the ER (Figure 4.3.23 D – F). This may point to a possible delay of plasma membrane-targeted GluR6(Q) trafficking by KA2 subunit coexpression. A similar delay
Figure 4.3.23. Colocalization analysis of KA2 and GluR6(Q) subunits. First row: a confocal cross section of HEK 293 cells cotransfected with KA2-ECFP (A) and GluR6(Q)-EYFP (B); C, overlay of A and B; note that there is intracellular overlap of cyan and yellow fluorescence signals in three cells coexpressing both fusion proteins, while the other cells expressing only GluR6(Q)-EYFP show yellow signals exclusively at plasma membrane. Second row: colocalization analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with yellow signals only: G, cyan channel; H, yellow channel; I, overlay of G and H. J, two-dimensional cytofluorogram; note one cloud of fluorescence signals at 45º as well as another at 90º relative to the X axis (= cyan channel). Scale bar = 10 μm.
was also observed when two other recombinant subunit pairs, KA2-EGFP plus GluR6(Q)-DsRed2 and KA2-DsRed2 plus GluR6(Q)-EGFP, were cotransfected into HEK 293 cells (Figure 4.3.24).

Figure 4.3.24. A confocal cross section of HEK 293 cells cotransfected with GluR6-EGFP (A) and KA2-DsRed2 (B); C, overlay of A and B. Note that there is intracellular overlap of green and red fluorescence signals in the cells coexpressing both fusion proteins, while the other two cells expressing only GluR6(Q)-EGFP show green signal exclusively at the plasma membrane (indicated by white arrow heads). Scale bar = 12 μm.

For the combination of KA2-ECFP plus GluR6(R)-EYFP, although colocalization of both subunits is shown for the coexpressing cells, there are, however, several other cells in the same recording field not carrying a cyan signal (= expressing only GluR6(R)-EYFP, but not KA2-ECFP), and where the GluR6(R) subunits are mostly expressed at the plasma membrane (Figure 4.3.25 G – I). This is in agreement with the previously shown data of the heteromeric combination of KA2 and GluR6(Q).

The cell surface expression of both edited and non-edited versions of GluR6 subunits, GluR6(R) and GluR6(Q), is significantly delayed when coexpressed with KA2 subunits in HEK 293 cells.
Figure 4.3.25. KA2 and GluR6(R) colocalize in the ER at a certain time point before their insertion into the plasma membrane. First row: a confocal cross section of HEK 293 cells cotransfected with KA2-ECFP (A) and GluR6(R)-EYFP (B); C, overlay of A and B, showing overlap of cyan and yellow fluorescence signals in the cell coexpressing both fusion proteins, while the other cells express only GluR6(R)-EYFP show yellow signals exclusively at plasma membrane. Second row: colocalization analysis of masked images of selected dots with both cyan and yellow signals at approximately same intensity values: D, cyan channel; E, yellow channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with yellow signals only: G, cyan channel; H, yellow channel; I, overlay of G and H. J, two-dimensional cytofluorogram; note one cloud of fluorescence signals at 45° as well as another at 90° relative to the X axis (= cyan channel). Scale bar = 10 μm.
4.3.4.3 KA2 subunits are transported to the plasma membrane by coexpression with GluR6 while a significant amount of the subunits are retained in the ER

Though KA2 subunits could be shown to be retained in the ER of live mammalian cells when expressed alone (Chapter 4.3.3), they must finally reach the plasma membrane to serve as a constitutive part of functional heteromeric kainate receptors. Apparently, this requires one of the low affinity kainate receptor subunits, namely GluR5, GluR6, or GluR7. Continual observation of HEK 293 cells cotransfected with KA2-EGFP and GluR6(Q)-DsRed2 was thus performed in an attempt to visualize the pattern of translocation of KA2 by GluR6 subunits. In addition, whole-cell recordings of cells coexpressing both KA2 and GluR6(Q/R) fusion proteins were carried out to electrophysiologically confirm the formation of functional heteromeric receptors.

![Images of KA2 and GluR6(Q) colocalization](image)

Figure 4.3.26. KA2 and GluR6(Q) colocalize in the ER at a certain time point before their insertion into the plasma membrane. First row: a confocal cross section of HEK 293 cells cotransfected with KA2-EGFP (A) and GluR6(Q)-DsRed2 (B); C, overlay of A and B. Second row: colocalization analysis of masked images of selected dots with both green and red signals at approximately same intensity values: D, green channel; E, red channel; F, overlay of D and E, showing overlap of green and red fluorescence signals in the two cells coexpressing both fusion proteins. G, two-dimensional cytofluorogram. Scale bar = 10 μm.
Consistent with the observations for the other two pairs of KA2 / GluR6(Q) fusion proteins (Chapter 4.3.4.1, Figures 4.3.22 – 24), KA2-EGFP and GluR6(Q)-DsRed2 were found to colocalize in the ER of HEK 293 cells as the first step of the formation of heteromeric complexes (Figure 4.3.26). Similarly, the next step was the plasma membrane-targeted GluR6(Q) transport (Figure 4.3.27 J – L), as well as the intracellular colocalization of KA2 and GluR6(Q) subunits (Figure 4.3.27 D – F). This is shown in Figure 4.3.27 A – C, where the cell express intracellularly localized KA2 as well as intracellularly and plasma membrane-localized GluR6(Q) fusion proteins. There was a considerable amount of intracellular KA2 subunits not colocalized with GluR6(Q) subunits (Figure 4.3.27 G – I), which suggests not all of the subunits were involved in the heteromeric interaction.

If another 24 hours were allowed to pass after transient transfection, KA2 subunits became detectable at the plasma membrane, overlapping with GluR6(Q) subunits, as the two cells indicated by white and yellow arrows show in Figure 4.3.28. The cell indicated with the white arrow shows overlap of almost all KA2 subunits (localized intracellularly or at the plasma membrane) with GluR6(Q) subunits (Figure 4.3.28 D – F). In addition, some GluR6(Q) is expressed at the plasma membrane without interaction with KA2 subunits (Figure 4.3.28 J – L). The cell indicated with the yellow arrow shows a similar pattern (Figure 4.3.28 D – F and J – L), but, with additional intracellular KA2 subunits not overlapping with GluR6(Q) subunits (Figure 4.3.28 G – I).

Despite having demonstrated the transport and insertion into the plasma membrane of both fusion proteins in live cells, the question whether those fusion proteins do form electrophysiologically functional ion channels had to be tested by patch clamp recordings.
Figure 4.3.27. Colocalization analysis of KA2 and GluR6(Q) subunits. First row: HEK 293 cells were cotransfected with KA2-EGFP (A) and GluR6(Q)-DsRed2 (B); C, overlay of A and B. Second row: colocalization analysis: masked images of selected dots with both green and red signals at approximately same intensity values; D, green channel; E, red channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with green signals only: G, green channel; H, red channel; I, overlay of G and H. Fourth row: analysis of masked images of selected dots with red signals only: J, green channel; K, red channel; L, overlay of J and K. M, two-dimensional cytofluorogram. Scale bar = 10 μm.
Figure 4.3.28. Colocalization analysis of KA2 and GluR6(Q) subunits. First row: HEK 293 cells were cotransfected with KA2-EGFP (A) and GluR6(Q)-DsRed2 (B); C, overlay of A and B. Second row: colocalization analysis: masked images of selected dots with both green and red signals at approximately same intensity values; D, green channel; E, red channel; F, overlay of D and E. Third row: analysis of masked images of selected dots with green signals only: G, green channel; H, red channel; I, overlay of G and H. Fourth row: analysis of masked images of selected dots with red signals only: J, green channel; K, red channel; L, overlay of J and K. M, two-dimensional cytofluorogram. Scale bar = 10 μm.
Results

Cells coexpressing one of the KA2 fusion proteins (tagged with ECFP, EGFP, or EYFP) and GluR6(Q)-EGFP respond to 200 μM AMPA application (Figure 4.3.29 A – C). The mean AMPA-evoked currents were $56 \pm 44$ pA (n = 2), $290 \pm 112$ pA (n = 3), and $447 \pm 429$ pA (n = 2), respectively. When the edited version of the GluR6 subunit, GluR6(R)-ECFP, was cotransfected with KA2-EYFP, cells responded to 200 μM AMPA application (Figure 4.3.29 D) as well, with mean currents of $130 \pm 63$ pA (n = 4). These data confirm not only electrophysiologically the functional integrity of the KA2 fusion proteins, but also the assembly of GluR6 and KA2 fusion proteins into heteromeric receptors in our heterologous expression system.

![Figure 4.3.29](image_url)

**Figure 4.3.29.** GluR6(Q/R) fusion proteins and KA2 fusion proteins form electrophysiologically functional ion channels in HEK 293 cells. Representative whole-cell recordings of AMPA-evoked currents: HEK 293 cells cotransfected with GluR6(Q)-EGFP and KA2-ECFP (A), GluR6(Q)-EGFP and KA2-EGFP (B), GluR6(Q)-EGFP and KA2-EYFP (C), and GluR6(R)-ECFP and KA2-EYFP (D).

**Investigation of coexpression patterns of KA2 and GluR6(Q) fusion proteins in HEK 293 cells demonstrated that KA2 is translocated to the cell surface by GluR6(Q). In the plasma membrane they form functional heteromeric receptors while a significant amount of KA2 is still retained in the ER. In addition, homomeric GluR6(Q) is expressed at the plasma membrane.**
4.3.5 Assembly and intracellular trafficking of kainate receptors analyzed by (FRET) in live cells

In Chapter 3.8.3 and 4.2.4.3, a brief introduction of FRET analysis has been supplied. An intensity-based method, namely the sensitized emission method, was used for FRET analysis on live HEK 293 cells expressing ECFP- (as donor) and EYFP- (as acceptor) tagged kainate receptor subunits: GluR6(Q), GluR6(R), and KA2. These experiments can provide evidence of assembly of homomeric and heteromeric receptors (at least dimer formation) recorded at high resolution. This is a novel approach in demonstrating the rule of intracellular trafficking of kainate receptors. In brief, six combinations of fusion proteins were tested for this purpose (Table 4.8), and used to analyze subunit interactions as well as where interactions occur in live cells. For each cell analyzed in this work, 3 – 6 repeat recordings were carried out, lasting around 40 to 100 seconds in total, in order to exclude any possible random false signal. The final FRET data for each cell is thus presented as mean ± SEM from the repetitive measurements. The FRET data for each combination of subunits is presented as mean ± SEM based on all the tested cells in the group if not specified otherwise.

Table 4.8 Summary of FRET analysis performed with fluorescently-labeled kainate receptor subunits in live cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>n</th>
<th>Plasma membrane</th>
<th>Intracellular</th>
<th>ER</th>
<th>Golgi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stargazin-ECFP</td>
<td>GluR6(Q)-EYFP</td>
<td>10</td>
<td>-</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>GluR6(Q)-ECFP</td>
<td>GluR6(Q)-EYFP</td>
<td>19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GluR6(R)-ECFP</td>
<td>GluR6(R)-EYFP</td>
<td>20</td>
<td>N.A.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KA2-ECFP</td>
<td>KA2-EYFP</td>
<td>11</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>N.A.</td>
</tr>
<tr>
<td>GluR6(Q)-ECFP</td>
<td>GluR6(R)-EYFP</td>
<td>20</td>
<td>+</td>
<td>+</td>
<td>N.A.</td>
<td>+</td>
</tr>
<tr>
<td>GluR6(R)-ECFP</td>
<td>KA2-EYFP</td>
<td>39</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GluR6(Q)-ECFP</td>
<td>KA2-EYFP</td>
<td>14</td>
<td>N.A.</td>
<td>+</td>
<td>+</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A.: Not analyzed.
4.3.5.1 FRET analysis indicates subunit interactions at the plasma membrane of live cells

HEK 293 cells coexpressing both wild type ECFP and EYFP fluorescence proteins were used as one of the negative controls for the FRET analysis. As shown in Figure 4.3.30 A – D, ECFP and EYFP were distributed in the entire cell, while there was no FRET signal detectable in those cells.

Electrophysiologically functional ion channels are localized in the plasma membrane of mammalian cells, where there is probably a relatively dense population of the receptor complexes with many receptors concentrated in a limited spatial region. To exclude the possibility that those closely spaced fluorophores of the fluorescently-labeled receptor subunits may cause false FRET signals, an ideal control experiment should be with two non-interacting fusion proteins that both insert into and highly concentrate in the plasma membrane of the cells. The pair of stargazin-ECFP and GluR6(Q)-EYFP was thus used as a negative control for the FRET analysis, based on convincing reports that there is no protein-protein interaction between stargazin and GluR6(Q) (Chen et al., 2003). In Figure 4.3.30 stargazin-ECFP is expressed at the plasma membrane of HEK 293 cells (Figure 4.3.30 E), and GluR6(Q)-EYFP is also expressed at the plasma membrane, with additional intracellular retention (Figure 4.3.30 F). The image of the FRET channel (Figure 4.3.30 G) theoretically contains mixed signals from three sources: true FRET signals, bleed through signals from the cyan channel to the FRET channel, and bleed through signals caused by cross excitation (the cyan fluorophores activated by the emission of the yellow fluorophores). The true FRET signals are thus visualized in Figure 4.3.30 H. The plasma membrane region of each cell was selected for FRET measurement, and the mean FRET value was calculated to be 0.67 ± 0.58 (n = 10) with the FRET efficiency at 0.19 ± 0.16% (n = 10).

Three combinations of fluorescently-labeled kainate receptor subunit-encoding cDNAs, which represent three possibilities of homomeric and heteromeric receptor complexes: GluR6(Q)-ECFP plus GluR6(Q)-EYFP (Figure 4.3.30 I – L), GluR6(Q)-ECFP plus GluR6(R)-EYFP (Figure 4.3.30 M – P), and GluR6(R)-ECFP plus KA2-EYFP (Figure 4.3.30 Q – T), were transiently cotransfected into HEK 293 cells and tested for FRET at the plasma membrane of the cells.
Figure 4.3.30. Images of FRET analysis with live HEK 293 cells coexpressing ECFP- and EYFP-labeled proteins. The columns from left to right of each row present the confocal images of the cyan channel (donor), the yellow channel (acceptor), the FRET channel, and the visualized true FRET value. HEK 293 cells were cotransfected with wild type ECFP plus wild type EYFP (A – D), stargazin-ECFP plus GluR6(Q)-EYFP (E – H), GluR6(Q)-ECFP plus GluR6(Q)-EYFP (I – L), GluR6(Q)-ECFP plus GluR6(R)-EYFP (M – P), or GluR6(R)-ECFP plus KA2-EYFP (Q – T).
As for the formation of homomeric GluR6(Q) complexes, FRET analysis was performed with live cells cotransfected with GluR6(Q)-ECFP- and GluR6(Q)-EYFP-encoding cDNAs. The FRET value at the plasma membrane was $82.34 \pm 24.12$ ($n = 17$), significantly higher than the negative control ($p < 0.05$). Despite the identical intensity values of the yellow fluorescence channel, based on the expression level of the same fusion protein, GluR6(Q)-EYFP, serving as the acceptor, there was a significant difference in FRET efficiency between the two groups ($p < 0.05$), with FRET efficiency at $2.75 \pm 0.69\%$ ($n = 17$) for the GluR6(Q) / GluR6(Q) interaction (Figure 4.3.31).

Similar analysis was carried out for GluR6(Q) / GluR6(R) heteromeric complexes by coexpressing GluR6(Q)-ECFP and GluR6(R)-EYFP in HEK 293 cells. The FRET and FRET efficiency value at the plasma membrane was significantly higher than that of the negative control ($p < 0.05$ for both values) at $42.15 \pm 11.81$ ($n = 20$) and $2.97 \pm 0.46\%$ ($n = 20$), respectively (Figure 4.3.31).

When GluR6(R)-ECFP and KA2-EYFP were coexpressed in HEK 293 cells, significantly higher values of FRET and FRET efficiency compared to the negative control ($p < 0.05$ for both values) were found with $100.49 \pm 52.92$ ($n = 5$) and $2.76 \pm 1.37\%$ ($n = 5$), respectively, and was localized at the plasma membrane of the cells (Figure 4.3.31).

The above data confirm the protein-protein interaction between kainate receptor subunits at the plasma membrane of HEK293 cells. In addition, the FRET analysis is thus shown to be a valuable method for identifying subunit interactions in live mammalian cells where not only the physiological condition is retained but the exact spatial information where interaction occurs can also be obtained.
Three combinations representing homomeric and heteromeric kainate receptor complexes, GluR6(Q)-ECFP plus GluR6(Q)-EYFP, GluR6(Q)-ECFP plus GluR6(R)-EYFP, and GluR6(R)-ECFP plus KA2-EYFP, were analyzed by FRET for subunit interactions at the plasma membrane of live cells. Significant FRET signals were obtained for the above combinations, compared to the negative control, stargazin-ECFP plus GluR6(Q)-EYFP.

4.3.5.2 FRET measurements show that subunits assemble in the ER

Although it has been shown in the previous chapters that kainate receptor subunits are transported from ER to the Golgi apparatus and finally to the plasma membrane if that is the final destination of the receptor complexes, it remains unclear whether the subunits are already assembled into dimers or tetramers on their way to the plasma membrane, and if yes, where. Utilizing FRET analysis as a novel approach to address this question for receptor complexes, at least for dimers, suggests the subunit assembly in the ER, followed by trafficking of the assembled complexes to the plasma membrane via the Golgi apparatus.

For homomeric GluR6(Q) or GluR6(R) receptor complexes, ECFP- and EYFP-tagged GluR6(Q/R) subunits were coexpressed in HEK 293 cells for intracellular FRET measurements. Before the subunits were transported to the plasma membrane, the live cells were used to analyze...
any potential subunit interaction as shown in Figure 4.3.32. The white arrows indicate the intracellular network-like structure, which is most probably the ER, where FRET signals could be detected in those cells coexpressing GluR6(Q)-ECFP plus GluR6(Q)-EYFP (Figure 4.3.32 A – D), or GluR6(R)-ECFP plus GluR6(R)-EYFP (Figure 4.3.32 E – H).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>FRET channel (mixed signals)</th>
<th>FRET value (true signals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
</tr>
</tbody>
</table>

Figure 4.3.32. Images of FRET analysis in live HEK 293 cells coexpressing ECFP- and EYFP-labeled GluR6(Q/R) subunits. The columns from left to right of each row present the confocal images of the cyan channel (donor), the yellow channel (acceptor), the FRET channel, and the visualized true FRET value. HEK 293 cells were cotransfected with GluR6(Q)-ECFP plus GluR6(Q)-EYFP (A – D), or GluR6(R)-ECFP plus GluR6(R)-EYFP (E – H). The white arrows indicate the intracellular network-like structures, mostly probably the ER of the cells. The yellow arrows indicate perinuclear, specifically structured regions, which probably represent the Golgi apparatus of the cells.

For heteromeric kainate receptor complexes assembled from both high and low affinity receptor subunits, ECFP-tagged GluR6(Q) or GluR6(R) fusion protein was coexpressed with KA2-EYFP in HEK 293 cells. The FRET signals for both combinations were detectable in the intracellular network-like structure (Figure 4.3.33 D and H). The high FRET and FRET efficiency demonstrating the interaction between GluR6(Q)-ECFP and KA2-EYFP at the possible ER region were 124.24 ± 30.6 (n = 14) and 3.24 ± 0.72% (n = 14), respectively. For GluR6(R)-ECFP and
Results

KA2-EYFP, the values were 115.5 ± 22.27 (n = 28) and 3.75 ± 0.60% (n = 28), respectively. There was no significant difference in FRET efficiencies ($p > 0.05$) of the two combinations. The data give evidences of assembly of heteromeric complexes in the ER of mammalian cells.

![Table Image]

**Figure 4.3.33.** Images of FRET analysis with live HEK 293 cells coexpressing ECFP-labeled GluR6(Q/R) and EYFP-labeled KA2 subunits. The columns from left to right of each row present the confocal images of the cyan channel (donor), the yellow channel (acceptor), the FRET channel, and the visualized true FRET value. HEK 293 cells were cotransfected with GluR6(Q)-ECFP plus KA2-EYFP (A – D), or GluR6(R)-ECFP plus KA2-EYFP (E – H).

Another revealing experiment performed was the coexpression of KA2-ECFP and KA2-EYFP fusion proteins. As we know from the colocalization analysis (Chapter 4.3.3), KA2 subunits are retained in the ER of HEK 293 cells. Analyzing the live cells coexpressing both proteins, there were detectable FRET signals as shown in Figure 4.3.34. The mean FRET value of the cells measured was 217.15 ± 51.05 (n = 11). The FRET efficiency was 6.65 ± 1.39% (n = 11). This suggests the interaction between KA2 subunits occurs in the ER, though without low affinity kainate receptor subunits they are not transported to the plasma membrane.
Results

Figure 4.3.34. Images of FRET analysis in live HEK 293 cells coexpressing KA2-ECFP and KA2-EYFP fusion proteins.
A, KA2-ECFP (donor); B, KA2-EYFP (acceptor); C, FRET channel; D, visualized real FRET value.

Five combinations representing homomeric and heteromeric kainate receptor complexes, GluR6(Q)-ECFP plus GluR6(Q)-EYFP, GluR6(Q)-ECFP plus GluR6(Q)-EYFP, KA2-ECFP plus KA2-EYFP, GluR6(Q)-ECFP plus GluR6(R)-EYFP, and GluR6(R)-ECFP plus KA2-EYFP, were analyzed by FRET for intracellular subunit interactions. Significant FRET signals were obtained for the above combinations, suggesting that the assembly of the receptor complexes, at least the dimer formation, occurs in the synthetic pathway as early as in the ER.

4.3.5.3 The role of the Golgi apparatus in receptor assembly and trafficking demonstrated by FRET analysis

The kainate receptor subunits assemble in the ER of mammalian cells, as shown in the previous chapter (Chapter 4.3.5.2), and then get transported to the Golgi apparatus on their way to the plasma membrane (Chapter 4.3.1 – 4.3.4). The pattern of the subunits at the Golgi apparatus, whether they are kept associated, however, hasn’t been explored before. By FRET analysis in live cells, it became possible to detect and even visualize subunit-subunit interaction at specific intracellular region.

When GluR6(Q/R)-ECFP and GluR6(Q/R)-EYFP fusion proteins were coexpressed in HEK 293 cells for homomeric GluR6(Q/R) receptors, there were intracellular FRET signals detectable. Beyond the white-arrowed ER regions, the perinuclear regions indicated by the yellow arrows in...
the cells where FRET also occurs are shown in Figure 4.3.32 D and H. These regions appear to have the specific structure of the Golgi apparatus as well as its intracellular localization (shown in Figure 4.3.10), and are thus suggested to represent the Golgi apparatus.

Similar analyses were performed with HEK 293 cells coexpressing GluR6(Q)-ECFP and GluR6(R)-EYFP fusion proteins. In Figure 4.3.35, the two fusion proteins overlap at the plasma membrane as well as specific intracellular regions (Figure 4.3.35 A and B), which probably represent the Golgi apparatus. Same regions are indicated by yellow arrows in Figure 4.3.35 D, where strong FRET signals are clearly visible. The mean FRET value was 1267.98 ± 254.42 (n = 7) and FRET efficiency was as high as 33.03 ± 6.53% (n = 7). This suggests an interaction between GluR6(Q) and GluR6(R) subunits at the specific perinuclear region, which is probably the Golgi apparatus.

When GluR6(R)-ECFP and KA2-EYFP fusion proteins were coexpressed in HEK 293 cells for analysis of a different possibility of heteromeric receptor complexe formation that involves both low and high affinity subunits, considerable FRET signals were also detectable in those specific regions indicated by the yellow arrows in Figure 4.3.35 H and L. The mean FRET value at those regions was 224.66 ± 72.22 (n = 10), and FRET efficiency was as high as 13.50 ± 5.81% (n = 10).

Five combinations representing homomeric and heteromeric kainate receptor complexes, GluR6(Q)-ECFP plus GluR6(Q)-EYFP, GluR6(R)-ECFP plus GluR6(R)-EYFP, KA2-ECFP plus KA2-EYFP, GluR6(Q)-ECFP plus GluR6(R)-EYFP, and GluR6(R)-ECFP plus KA2-EYFP, were analyzed by FRET for intracellular subunit interactions. Significant FRET signals at a perinuclear region were obtained for above combinations. Taken together with the colocalization analysis described in the previous chapters, those data suggest that the oligomers stay associated in the Golgi apparatus after exiting the ER en route to the plasma membrane.
Figure 4.3.35. Images of FRET analysis in live HEK 293 cells expressing heteromeric kainate receptor complexes. The columns from left to right of each row present the confocal images of the cyan channel (donor), the yellow channel (acceptor), the FRET channel, and the visualized true FRET value. HEK 293 cells were cotransfected with GluR6(Q)-ECFP plus GluR6(R)-EYFP (A – D), or GluR6(R)-ECFP plus KA2-EYFP (E – L). The second and third rows show two different confocal z-sections of the same cells. The yellow arrows indicate the specifically structured perinuclear regions, probably the Golgi apparatus of the cells.
4.3.5.4 Summary of FRET analysis with kainate receptor subunits

In summary, FRET analysis has been performed with live HEK 293 cells coexpressing ECFP- and EYFP-labeled kainate receptor subunits, GluR6(Q), GluR6(R) and KA2. Various combinations, including homomeric and heteromeric receptor complexes, were thus analyzed for subunit interactions at distinguishable intracellular membrane compartments with a high lateral resolution.

The true FRET and FRET efficiency values are presented in Table 4.9 and 4.10. Table 4.9 presents the original data calculated from all the cells measured, while in Table 4.10 those cells with negative FRET values were excluded for the final calculations. Notably, there is no significant increase in either FRET or FRET efficiency values after the selection.

A summary of the intracellular FRET and FRET efficiency values of Table 4.10 is also shown in Figure 4.3.36 as a bar diagram.

Figure 4.3.36. FRET and FRET efficiency values measured at intracellular membrane compartments of live HEK 293 cells coexpressing GluR6(Q)-ECFP plus GluR6(Q)-EYFP, GluR6(R)-ECFP plus GluR6(R)-EYFP, KA2-ECFP plus KA2-EYFP, GluR6(R)-ECFP plus KA2-EYFP, GluR6(Q)-ECFP plus KA2-EYFP, and GluR6(Q)-ECFP plus GluR6(R)-EYFP fusion proteins. The regions of interest are indicated below the columns.

A summary of total raw (Table 4.9) and selected (Table 4.10) data of FRET analysis with kainate receptor subunits is presented.
### Table 4.9 Summary of FRET analysis (derived from all cells measured)

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<th>A</th>
<th>FRET value</th>
<th>GluR6(Q) / GluR6(R)</th>
<th>GluR6(R) / GluR6(Q)</th>
<th>KA2 / KA2</th>
<th>GluR6(Q) / GluR6(R)</th>
<th>GluR6(R) / KA2</th>
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PM: plasma membrane  
ER: endoplasmic reticulum  
Golgi: Golgi apparatus  
n: number of cells measured  
SEM: standard error of mean
### Table 4.10 Summary of FRET analysis (those cells with negative FRET values excluded)

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PM: plasma membrane  
ER: endoplasmic reticulum  
Golgi: Golgi apparatus  
n: number of cells measured  
SEM: standard error of mean
4.4 Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes

After verification of membrane localization and functional integrity of GluR6 fusion proteins (Chapter 4.3.1.1), the four constructs GluR6(Q)-ECFP, GluR6(Q)-EGFP, GluR6(Q)-EYFP, and GluR6(Q)-DsRed2 were used to analyze individual (Figure 4.4.1C) and coexpression efficiencies of glutamate receptor subunits in HEK 293 cells.

Calcium phosphate method was applied for transient transfection into HEK 293 cells. When transfected with 4 µg of EGFP-encoding cDNA, around 85 – 95% of cells expressed the green fluorescent protein (Figure 4.4.1, A and B). In this work, five different combinations of constructs with various DNA ratios were tested in cotransfection experiments (Table 4.11).

Figure 4.4.1. Expression characteristics of fluorescently labeled kainate receptors. A – B, when transfected with pEGFP-N1, ≥ 85% HEK 293 cells expressed EGFP. A, bright field image; B, merge of confocal z-scan-series. C, expression efficiencies of GluR6-ECFP, GluR6-EGFP, GluR6-EYFP, and GluR6-DsRed2 at identical cDNA amount (6 µg) are 27.3 ± 3.9% (n = 4), 30.0 ± 3.4% (n = 4), 24.3 ± 4.1% (n = 4), and 17.9 ± 1.8% (n = 4), respectively. D – E, merge of confocal z-scan-series of HEK 293 cells transfected with KA2-ECFP. D, cyan fluorescence; E, green fluorescence of an anti-KA2 antibody labeled by an Alexa 488-conjugated secondary antibody; note perfect overlap of green fluorescence signals with cyan fluorescence signals. Scale bar = 600 µm in A, and 50 µm in D.
Table 4.11 Summary of cotransfection experiments performed

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<th>Number of independent transfections</th>
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a, EGFP: Enhanced Green Fluorescent Protein  
b, DsRed2: Discosoma sp. Red 2  
c, ECFP: Enhanced Cyan Fluorescent Protein  
d, EYFP: Enhanced Yellow Fluorescent Protein

4.4.1 Cumulative transfection efficiency is not dependent on the total amount of DNA, and varies with the proteins to be expressed

The combination of EGFP and GluR6-DsRed2 was selected to mimic a method commonly used in HEK cell electrophysiology, where EGFP is often cotransfected as an easily detectable marker for transfection and is believed to simultaneously indicate expression of the target protein. In the experiment, combinations of EGFP and GluR6-DsRed2 cDNAs at various ratios (1 + 5, 5 + 1, 1 + 1, and 3 + 3 µg) were used for cotransfection experiments 1 – 4, respectively (see Table 4.11). The cells expressing red or green fluorescence were counted separately to determine expression efficiencies of each individual protein. Cells expressing any fluorescence were counted as well, to obtain the cumulative expression efficiency (= number of cells expressing any fluorescent protein /
all cells). Experiment 1 (1 + 5 μg of EGFP and GluR6-DsRed2, respectively) showed the highest
eexpression efficiency of both individual proteins: 62.6 ± 4.6% for GluR6-DsRed2 expression (n =
12), and 59.7 ± 4.0% for EGFP expression (n = 12) (Figure 4.4.2 A). The cumulative expression
efficiency in this experiment was 75.1 ± 4.6% (n = 12) (Figure 4.4.2 B, first column). When the
same total amount but a different ratio of the two cDNAs was used for cotransfection (experiment
2, 5 + 1 μg of EGFP and GluR6-DsRed2, respectively), a significantly lower value was obtained
for the expression efficiency of GluR6-DsRed2 (p < 0.01, Figure 4.4.2 A), while there was no
significant difference for the expression efficiency of EGFP (p > 0.05, Figure 4.4.2 A) nor the
cumulative expression efficiency calculated as above (p > 0.05, Figure 4.4.2 B). However,
compared to experiment 1, significantly lower values were obtained for the individual expression
efficiency of GluR6-DsRed2 (p < 0.01, Figure 4.4.2 A) as well as cumulative expression efficiency
(p < 0.05, Figure 4.4.2 B), but not for the individual expression efficiency of EGFP (p > 0.05,
Figure 4.4.2 A) in experiment 4 (3 + 3 μg of EGFP and GluR6-DsRed2, respectively).
Furthermore, when the relative ratio between the two constructs was kept but the absolute amount
of DNA was reduced, such as in experiment 3 (1 + 1 μg of EGFP and GluR6-DsRed2,
respectively), there was no significant difference in individual (EGFP: p > 0.05; GluR6-DsRed2: p
> 0.05, Figure 4.4.2 A) or cumulative expression efficiency (p > 0.05) compared to experiment 4 (3
+ 3 μg of EGFP and GluR6-DsRed2, respectively). Those results suggest that within the tested
commonly used concentration range, the total amount of DNA used in cotransfection experiments
is not the decisive factor determining the expression efficiency.

To test whether expression efficiencies depend on individual proteins, the combinations of
GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-EYFP, and GluR6-ECFP / GluR6-DsRed2
were compared (Table 4.11, experiments 5 – 8, 9 – 13, and 14 – 16, respectively).

When GluR6-EGFP and GluR6-DsRed2 cDNAs were cotransfected, the cumulative expression
efficiencies were significantly lower than for the EGFP and GluR6-DsRed2 combination at
identical DNA amounts (Figure 4.4.2 B). Interestingly, the highest cumulative expression
efficiency of the former combination (41.8 ± 5.5%, n = 9) was obtained with a lower total amount
of cDNAs: 4 μg in experiment 7 (2 μg GluR6-EGFP + 2 μg GluR6-DsRed2), compared to 6 μg in experiments 5, 6 and 8 (GluR6-EGFP and GluR6-DsRed2 at cDNA amounts of 1 + 5, 5 + 1, and 3 + 3 μg).

There was no significant difference in cumulative expression efficiencies between experiments 9 (71.0 ± 3.4%, n = 11, for 1 + 5 μg of GluR6-ECFP and GluR6-EYFP, respectively) and 10 (68.3 ± 3.2%, n = 16, for 5 + 1 μg of GluR6-ECFP and GluR6-EYFP, respectively) (p > 0.05), nor between experiments 11 (46.5 ± 8.6%, n = 9, for 1 + 1 μg GluR6-ECFP and GluR6-EYFP, respectively) and 12 (50.9 ± 3.3%, n = 9, for 2 + 2 μg GluR6-ECFP and GluR6-EYFP, respectively) (p > 0.05) (Table 4.11, Figure 4.4.2 C). When GluR6-ECFP and GluR6-EYFP were cotransfected at cDNA amounts of 3 + 3 μg (experiment 13, Table 4.11), there was a significant increase in cumulative expression efficiencies with this further increase in the amount of cDNA (Figure 4.4.2 C) compared to experiments 11 and 12. However, the highest expression efficiency of each individual protein was reached in different experiments with different relative ratios of cDNAs.

When GluR6-ECFP and GluR6-DsRed2 cDNAs were cotransfected at the same total amount of cDNA but different relative ratios, the cumulative expression efficiencies ranged between 42.4 ± 7.2%, n = 9, (5 + 1 μg of GluR6-ECFP and GluR6-DsRed2, respectively) and 61.9 ± 9.1%, n = 8 (1 + 5 μg of GluR6-ECFP and GluR6-DsRed2, respectively) (Figure 4.4.2 D).

*Various coexpression experiments with wild type or GluR6-fused fluorescent proteins were carried out in HEK 293 cells, and the individual as well as the cumulative expression efficiencies were investigated. Within the commonly used concentration range tested here, the total amount of cDNA used in cotransfection is not the decisive factor determining the cumulative efficiency, while the individual proteins can significantly affect cumulative expression efficiency.*
**4.4.2 Coexpression efficiency is influenced by several factors**

While individual and cumulative transfection efficiencies are interesting parameters, the most important parameter for any coexpression study is the coexpression efficiency of the cotransfected constructs. Therefore, for all 16 experiments described above, counted those cells which expressed both proteins simultaneously were counted in order to determine cotransfection efficiencies. Notably, the maximal coexpression efficiency obtained under any of the 16 different conditions tested was a surprisingly low $47.8 \pm 2.7\%$ ($n = 16$), the minimal efficiency was a mere $16.7 \pm 4.2\%$ ($n = 12$).
Coexpression efficiencies for EGFP and GluR6-DsRed2 generally varied around 25%; however, in one experiment a significantly higher efficiency of 47.3% was found (Figure 4.4.3 A). The coexpression efficiency of EGFP and GluR6-DsRed2 was thus far better when 1 μg of EGFP and 5 μg of GluR6-DsRed2 were cotransfected, rather than the reverse combination of cDNA amounts, 5 μg of EGFP and 1 μg of GluR6-DsRed2.

![Figure 4.4.3](image_url)

Figure 4.4.3. Coexpression efficiencies (calculated as the percentage of coexpressing cells out of all cells) under various conditions of cotransfection in HEK 293 cells. A – D refer to EGFP / GluR6-DsRed2, GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-EYFP, and GluR6-ECFP / GluR6-DsRed2 combinations at the indicated cDNA ratios, respectively. Asterisks indicate significant differences, *** indicates $p < 0.001$, ** indicates $0.001 \leq p < 0.01$.

When two glutamate receptor subunits, GluR6-EGFP and GluR6-DsRed2, were cotransfected at cDNA amounts of $1 + 5$ and $5 + 1$ μg, the coexpression efficiencies were similarly low at around 17% for both conditions. A slightly but not significantly higher coexpression efficiency ($23.2 \pm 5.7\%$, $n = 6$) was obtained when $3 + 3$ μg of GluR6-EGFP and GluR6-DsRed2 cDNAs, respectively, were used (Figure 4.4.3 B).
In cells cotransfected with GluR6-ECFP and GluR6-EYFP as shown in Figure 4.4.3 C, no significant difference in coexpression efficiency was obtained at $1 + 1 \mu g$ of DNA ($20.1 \pm 4.4\%$, $n = 9$) compared to $2 + 2 \mu g$ of DNA ($24.5 \pm 2.2\%$, $n = 9$) ($p > 0.05$). The highest coexpression efficiency observed was $47.8 \pm 2.7\%$ ($n = 16$) when $5 \mu g$ of GluR6-ECFP and $1 \mu g$ of GluR6-EYFP were cotransfected.

For another combination of subunits, GluR6-ECFP and GluR6-DsRed2 (Figure 4.4.3 D), coexpression efficiencies were similarly low and varied in the range of merely $18.3 \pm 3.4\%$ ($n = 9$) to $29.8 \pm 3.4\%$ ($n = 9$) for coexpression of $1 + 5$, $5 + 1$, and $3 + 3 \mu g$.

For the 16 different subunit combinations tested in this work, coexpression efficiencies obtained were pretty low. The data suggest that coexpression efficiency is influenced by several factors, including the cDNA ratio, the nature of the expressed protein, and the specific combination of cotransfected cDNAs.

### 4.4.3 Evaluation of the marker protein cotransfection method commonly used in electrophysiological studies

It is standard procedure in electrophysiological experiments to cotransfect one target protein together with a fluorescent marker protein to spot cells expressing the target protein. This procedure is based on the assumption that any cell expressing the fluorescent marker protein will also express the target protein. Nevertheless, this assumption is rarely tested by independent means. Therefore EGFP was used as the marker protein and GluR6-DsRed2 as the target protein and the percentage of cells that express both proteins (= cells with both fluorescence signals) out of all EGFP-expressing cells (= green fluorescing cells) was determined.

The percentages of cells expressing both proteins out of the cells expressing the marker protein (= cells with both fluorescence signals versus cells with green signal, for the combination of EGFP and GluR6-DsRed2), were $79.9 \pm 3.4\%$ ($n = 12$), $54.5 \pm 5.4\%$ ($n = 12$), $61.4 \pm 8.1\%$ ($n = 10$), and $73.7 \pm 3.8\%$ ($n = 12$) in experiments 1 – 4, respectively (Figure 4.4.4 A, second columns).
Figures 4.4.5 A and D, the cells which expressed EGFP but not GluR6-DsRed2 are pointed out with white arrows. Notably, some of those arrow-marked cells showed a high intensity of the green fluorescence signal, indicating a high expression level of EGFP that is not paralleled by a high expression level of GluR6-DsRed2; in fact, for those cells GluR6-DsRed2 was not expressed at all. However, the percentages of green cells that express both fluorescence signals under certain conditions could be as high as 73 – 80% (for example, in experiments 1 and 4). Those data suggest that only in cases when the transfection efficiencies for the fluorescently-labeled marker protein approaches 100% is it possible to apply cotransfection of a target protein with a marker fluorescent protein and pick cells with the marker protein fluorescence signal that have a sufficiently high probability to also express the target protein.

The percentages of cells expressing both proteins versus cells expressing the target receptor subunit (= cells with both fluorescence signals versus cells with red signal) varied from 67.5 ± 5.6% (n = 10) to 81.6 ± 3.9% (n = 12) (Figure 4.4.4 A, third columns). In Figure 4.4.5 B and E, for example, the cells which expressed GluR6-DsRed2 but not EGFP are pointed out with a white arrow, which indicates the population of EGFP-expressing cells is not the same as the population of all transfected cells.

The percentage of coexpressing cells among transfected cells is shown in Figure 4.4.4 A. With the cumulative expression efficiency at 75.1 ± 4.6% (n = 12) in experiment 1 (1 + 5 μg of EGFP and GluR6-DsRed2, respectively, Figure 4.4.2 B), the percentage of all fluorescent cells (= cells expressing any fluorescence signal) that express both fluorescence signals simultaneously was only 62.9 ± 2.2% (n = 12, Figure 4.4.4 A, first column). Moreover, in additional experiments (experiments 2 – 4: EGFP and GluR6-DsRed2 at cDNA amounts of 5 + 1, 1 + 1, and 3 + 3 μg) even lower coexpression efficiencies were found with 47.7 ± 4.6% (n = 12), 49.3 ± 8.0% (n = 10), and 56.8 ± 3.1% (n = 12) (Figure 4.4.4 A, first columns of respective ratios).
Figure 4.4.4. Coexpression efficiencies (calculated as the percentage of coexpressing cells out of cells with any fluorescence signals) under various conditions of cotransfection in HEK 293 cells. A – D refer to EGFP / GluR6-DsRed2, GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-EYFP, and GluR6-ECFP / GluR6-DsRed2 combinations at the indicated cDNA ratios, respectively. E, coexpression efficiencies of HEK 293 cells after ternary transfection with ECFP, GluR6-EYFP, and GluR6-DsRed2 at cDNA amounts of 2 μg each. Asterisks indicate significant differences, *** indicates $p < 0.001$. 

156
**Results**

*Coexpression experiments with EGFP and GluR6-DsRed2 were performed to mimic standard procedures in electrophysiological experiments where the expression of cotransfected EGFP is utilized to identify cells expressing the target protein. Three distinct populations of cells carrying fluorescence signals were found to be present: EGFP-expressing only, GluR6-DsRed2-expressing only, and coexpressing cells.*

4.4.4 Coexpression efficiencies of two glutamate receptor subunits are well below 100%

Cotransfection of two or more receptor subunit cDNAs is routinely used for electrophysiological analysis of heteromeric receptor complexes. In such experiments it is generally assumed that any given cell expresses all subunits present during transfection. To scrutinize this, cotransfection of HEK 293 cells with two fluorescently-tagged GluR6 subunits, which allow simple visualization of the expression efficiencies of the receptor subunits, was analyzed.

When cotransfected with two differently tagged fusion proteins of approximately the same molecular mass, such as in experiments 5 – 8 (GluR6-EGFP plus GluR6-DsRed2 at cDNA amounts of 1 + 5, 5 + 1, 2 + 2, and 3 + 3 μg, respectively), the percentage of cells coexpressing both fluorescence signals out of cells expressing any of the two fluorescence signals was only 41.3 ± 3.8% (n = 14), 55.4 ± 4.2% (n = 12), 47.8 ± 6.6% (n = 9), and 60.8 ± 3.3% (n = 6), respectively (Figure 4.4.4 B).

Coexpression efficiencies for two other combinations of labeled glutamate receptor subunits were also calculated, GluR6-ECFP plus GluR6-EYFP, and GluR6-ECFP plus GluR6-DsRed2. The percentages of cells expressing both fluorescence signals versus cells expressing any fluorescence signal in the first case were 46.5 ± 5.5% (n = 11) and 70.2 ± 3.0% (n = 16) at cDNA amounts of 1 + 5 and 5 + 1 μg (Figure 4.4.4 C), respectively. When cotransfected with a different absolute amount but the same ratio of the two cDNAs (1 + 1, 2 + 2, and 3 + 3 μg), the percentages of cells expressing both fluorescence signals out of cells expressing any fluorescence signal were 43.1 ± 4.3% (n = 9), 48.8 ± 4.2% (n = 9), and 43.4 ± 4.1% (n = 16), respectively (Figure 4.4.4 C).
Figure 4.4.5. Coexpression patterns under various conditions of double transfection in HEK 293 cells. A – F, HEK 293 cells 3 days after cotransfection with EGFP and GluR6-DsRed2 at cDNA amounts of 1 + 5 μg (A – C), and 3 + 3 μg (D – F). Green fluorescence (first column, A and D), red fluorescence (second column, B and E), and both channels merged (third column, C and F). White arrows indicate some cells expressing only one of the two fluorescence signals. G – L, HEK 293 cells 3 days after cotransfection with GluR6-ECFP and GluR6-EYFP at cDNA amounts of 5 + 1 μg (G – I), and 3 + 3 μg (J – L). Cyan fluorescence (first column, G and J), yellow fluorescence (second column, H and K), and both channels merged (third column, I and L). White arrows indicate some cells expressing only yellow fluorescence signals. Scale bars = 50 μm.
example, as shown in Figure 4.4.5, third and fourth rows, a considerable population of cells expressed only one of the fluorescence signals. For GluR6-ECFP and GluR6-DsRed2, the percentages of cells expressing both fluorescence signals out of cells expressing any fluorescence signal were 34.2 ± 5.5% (n = 8), 41.5 ± 2.8% (n = 9), and 56.0 ± 4.4% (n = 9) at cDNA amounts of 1 + 5, 5 + 1, and 3 + 3 μg, respectively (Figure 4.4.4 D).

These data indicate that even very similar cDNAs, when cotransfected at various cDNA ratios or absolute cDNA amounts, can vary widely in their coexpression efficiencies within transfected cells.

We then asked whether it might be possible to optimize transfection conditions such that one of the fusion proteins could be used as a reliable marker for coexpression; for example, by adjusting the cDNA ratios used for cotransfection.

When identical amounts of each of two cDNAs were cotransfected, the percentage of cells with both fluorescence signals out of cells with either a green or a red signal were similar. It was found 62 – 70% (p > 0.05) for GluR6-EGFP and GluR6-DsRed2 (2 μg each) and 72 – 80% (p > 0.05) for GluR6-EGFP and GluR6-DsRed2 (3 μg each) (Figure 4.4.4 B, second and third columns). However, for the combination of GluR6-ECFP and GluR6-EYFP (Figure 4.4.4 C, second and third columns), the percentages of cells with both fluorescence signals out of cells with a cyan signal (85.7 ± 3.8%, n = 9; 91.4 ± 2.6%, n = 9; and 87.6 ± 2.8%, n = 16 at 1 + 1, 2 + 2, and 3 + 3 μg of cDNAs, respectively) were consistently higher than the percentages of cells with both fluorescence signals out of cells with a yellow signal (46.5 ± 4.6%, n = 9; 50.8 ± 4.0%, n = 9; and 46.2 ± 4.4%, n = 16, at 1 + 1, 2 + 2, and 3 + 3 μg of cDNAs, respectively). This is also shown in Figure 4.4.5 K, where the cells expressing GluR6-EYFP but not GluR6-ECFP are indicated by white arrows. A similar observation was made for the combination of GluR6-ECFP and GluR6-DsRed2 (3 + 3 μg of cDNAs), when a higher percentage of cyan cells express both fluorescence signals (86.5 ± 2.9%, n = 9) compared to red cells (60.9 ± 4.3%, n = 9, Figure 4.4.4 D).

Interestingly, when different amounts of cDNAs were used (1 μg GluR6-EGFP and 5 μg GluR6-DsRed2 were cotransfected), the percentage of green cells that carry both fluorescence...
signals was as high as 95.7 ± 1.5% (n = 14), compared to a relatively low percentage of red cells that carry both signals (42.3 ± 4.0%, n = 14). In the reverse experiment, when 5 μg GluR6-EGFP and 1 μg GluR6-DsRed2 were cotransfected, the reverse observation was made, with the percentage of red cells that carry both fluorescence signals now being as high as 94.8 ± 2.1% (n = 12), while the percentage of green cells that carry both signals was only 57.2 ± 4.4% (n = 12) (Figure 4.4.4 B).

In comparison, when another combination, 1 μg GluR6-ECFP and 5 μg GluR6-EYFP, was cotransfected, the percentage of cyan cells that express both fluorescence signals was as high as 94.6 ± 1.4% (n = 11), while the percentage of yellow cells that express both fluorescence signals was only 48.2 ± 6.1% (n = 11). In the reverse experiment, when 5 μg GluR6-ECFP and 1 μg GluR6-EYFP were cotransfected, the percentage of yellow cells that express both fluorescence signals was 76.4 ± 2.6% (n = 16), while the percentage of cyan cells that express both fluorescence signals was 89.2 ± 2.0% (n = 16) (Figure 4.4.4 C). As shown in Figure 4.4.5 H, there was a population of GluR6-EYFP-expressing cells not bearing the cyan signal, even though some of those cells showed a high intensity of yellow fluorescence signal. Thus, in the latter experiment there were almost 24% GluR6-EYFP-expressing cells not bearing cyan fluorescence, which demonstrates the difficulties of optimizing conditions (for example, by using a smaller amount of one of the cDNAs during cotransfection experiment) such that at least one of the fusion proteins becomes a reliable coexpression marker.

Consistent with the data from the GluR6-ECFP and GluR6-EYFP combination, when 1 μg GluR6-ECFP and 5 μg GluR6-DsRed2 was cotransfected, the percentage of cyan cells that carried both fluorescence signals was only 78.1 ± 7.7% (n = 8) (Figure 4.4.4 D).

Although the above results with only two coexpressed subunits already cast doubt on the reliability of ‘all-or-none’ dual expression of functional glutamate receptor subunits in mammalian cell culture, a triple protein expression experiment was carried out utilizing the possibility of multicolor imaging in living cells. The transfection was carried out with the three cDNAs ECFP, GluR6-EYFP, and GluR6-DsRed2 (Table 4.11, experiment 17). With a cumulative transfection
efficiency of 60.3 ± 7.0% (n = 10), the expression efficiency of triple-expressing cells among all those cells bearing any fluorescence signal was only 37.9 ± 3.5% (n = 10) (Figure 4.4.4 E, first column). Among those cells expressing a cyan signal (= expressing ECFP), only 49.5 ± 3.7% (n = 10) also expressed both GluR6 fusion proteins (Figure 4.4.4 E, fifth column). Thus, only around half of the cells expressing the ‘expression marker’ also expressed the targeted receptor complex. While the percentage of triple-expressing cells out of cells expressing both cyan and red signals was almost 100% (Figure 4.4.4 E, third column), the percentage of triple-expressing cells out of cells expressing both cyan and yellow signals was only 69.9 ± 4.2% (n = 10) (Figure 4.4.4 E, second column). In summary, among those cells coexpressing ECFP and one of the GluR6 fusion proteins, around 30% did not express the second GluR6 fusion protein (Figure 4.4.4 E, forth column). The distinct populations of cells expressing various combinations of fluorescent proteins are pictured in Figure 4.4.6, first column.

In addition, another combination of ternary transfection, using ECFP-Golgi, stargazin-EGFP, and DsRed2 cDNAs, was tested. The images in the second column of Figure 4.4.6 show cells clearly expressing different combinations of these fluorescent proteins. This suggests that current data with fluorescently-labeled GluR6 is valid for other receptor subunits, and also for auxiliary receptor subunits.

Finally, a quadruple transfection with cDNAs of ECFP-Golgi, GluR6-EGFP, GluR2-EYFP, and DsRed2 was carried out, where GluR6 belongs to the kainate receptor subfamily and GluR2 to the AMPA receptor subfamily. By successful separation and detection of four intracellular fluorescent signals in live cells (Figure 4.4.6, third column), the presence of several distinguishable populations of differentially transfected cells is demonstrated, which is in agreement with our conclusions for double transfections presented above and suggests an extended general validity for multiple subunit cotransfections.

Various double, ternary, and quadruple transfections were carried out in order to determine the coexpression efficiencies of two or more receptor subunits. Coexpression efficiencies were found to be well below 100%. Even for similar cDNAs, coexpression efficiencies can vary widely
at various cDNA ratios or absolute cDNA amounts. In addition, the data indicate the difficulties of optimizing transfection conditions such that at least one of the fusion proteins becomes a reliable coexpression marker.

4.4.5 Various alternative transfection methods also result in a variable range of coexpression efficiencies

As the calcium phosphate method still is the most popular transfection method, it was evaluated in great detail, as described above. However, there are numerous alternative transient transfection methods which are gaining increasing popularity. Therefore, two lipid-based methods and a biolistic method were tested for a single receptor subunit combination, GluR6-ECFP and GluR6-EYFP.

With both lipid-based methods tested, transfections with 2 μg of GluR6-ECFP or GluR6-EYFP cDNA under optimal conditions resulted in high expression efficiencies (> 76%) (Figure 4.4.7 A, B). When GluR6-ECFP and GluR6-EYFP were cotransfected at cDNA amounts of 2 μg each, the percentages of cells coexpressing both fluorescence signals out of cells expressing any fluorescence signal were 83.7 ± 2.5% (PolyFect) and 93.4 ± 0.7% (Metafectene Pro). For comparison, a biolistic delivery method was tested, using gold particles simultaneously coated with equal amounts of GluR6-ECFP and GluR6-EYFP cDNAs. Around 75% of those cells expressing any fluorescence signal were found coexpressing both cDNAs (data not shown).

Figure 4.4.6. Coexpression patterns of ternary and quadruple transfections in HEK 293 cells. First column (A, D, G, J), HEK 293 cells 3 days after ternary transfection with ECFP (A), GluR6-EYFP (D), and GluR6-DsRed2 (G) at cDNA amounts of 2 μg each. J, merge of A, D, and G. White arrows indicate some cells expressing only one of the three fluorescence signals, red arrows indicate some cells expressing both cyan and yellow but no red signal; the green arrow indicates a cell expressing both cyan and red but no yellow signal. Second column (B, E, H, K), HEK 293 cells 3 days after ternary transfection with ECFP-Golgi (B), stargazin-EGFP (E) and KA2-EYFP (O) at cDNA amounts of 2 μg each. K, merge of B, E, and K. Third column (C, F, I, L, P), HEK 293 cells 3 days after quadruple transfection with ECFP-Golgi (C), GluR6-EGFP (F), GluR2-EYFP (I), and DsRed2 (L) at cDNA amounts of 2 μg each. P, merge of C, F, I, and L. M – O, separation of intracellular ECFP, EGFP and EYFP by live imaging. HEK 293 cells 3 days after ternary transfection with ECFP-Golgi (M), stargazin-EGFP (N), and KA2-EYFP (O) at cDNA amounts of 2 μg each. Scale bar = 50 μm in J, 10 μm in K, 20 μm in P, and 4 μm in O.
Figure 4.4.7. Expression and coexpression efficiencies of single and double transfections in HEK 293 cells by lipid-based methods. A, HEK 293 cells transfected in the presence of PolyFect with 2 μg of GluR6-ECFP or GluR6-EYFP. B, HEK 293 cells transfected in the presence of Metafectene Pro with 2 μg of GluR6-ECFP or GluR6-EYFP. C, HEK 293 cells cotransfected in the presence of PolyFect with GluR6-ECFP and GluR6-EYFP at cDNA amounts of 2 μg each. D, HEK 293 cells cotransfected in the presence of Metafectene Pro with GluR6-ECFP and GluR6-EYFP at cDNA amounts of 2 μg each.

In addition to the calcium phosphate method, two lipid-based transfection methods as well as a biolistic transfection method were tested in this work. High individual expression efficiencies of both fusion proteins may result in high coexpression efficiencies.
5. Discussion

5.1 Assembly and intracellular trafficking of AMPA receptors

AMPA receptors mediate the fast excitatory synaptic transmission in the mammalian central nervous system. Native AMPA receptors at mature hippocampal excitatory synapses assemble mainly as GluR1/GluR2 heteromers, in some cases also forming GluR2/GluR3 heteromers (Wenthold et al., 1996). The cytoplasmic tails of GluR1 and GluR2 differ in length, GluR1 possessing a long tail and the predominant splice form of GluR2 a short carboxyl terminus, and are the determinants regulating interactions with distinct cytoplasmic proteins, such as scaffold and cytoskeletal proteins. Hence, the cytoplasmic tails play a critical role in receptor trafficking, synaptic targeting and stabilization, as well as internalization. The edited GluR2(Q586R) subunit alters the Ca$^{2+}$ permeability, the receptor affinity for glutamate, and the current/voltage relation of heteromeric AMPA receptors, so that the receptor’s electrophysiological properties are dominated by this particular subunit (Higuchi et al., 1993; Seeburg et al., 1998; Sommer et al., 1991). For these reasons, GluR1 and GluR2 subunits (both edited and non-edited forms) were selected to construct recombinant cDNAs fused with various fluorescent proteins in order to monitor the assembly and intracellular trafficking of AMPA receptor complexes in live cells by high lateral resolution confocal imaging and digital analysis. Both spatial and temporal information in receptor assembly and trafficking was collected in these experiments.

5.1.1 Distinct intracellular distribution but similar intracellular tetramerization of GluR1, GluR2(R), and GluR2(Q) subunits

Confocal live imaging and digital colocalization analysis were performed to determine the intracellular distribution and potential tetramerization of selected AMPA receptor subunits, GluR1, GluR2(R), and GluR2(Q), representing AMPA receptor subunits with differed intracellular C-termini, or different residues at the Q/R editing site in the pore region. A heterologous expression system, HEK 293 cell culture, was used for the current study. This choice of expression system was based on the absence of endogenous expression of ionotropic glutamate receptors or auxiliary
proteins, low expression of PDZ proteins, and the possibility to investigate cell biological pathways of receptor exocytosis in those cells. In addition, preliminary experiments were performed in dissociated primary neuronal cultures in order to establish that culture system as an alternative expression system, optimize transient transfection methods, and confirm the expression of the recombinant proteins for further studies.

When functional homomeric recombinant receptors composed of GluR1 were transiently expressed in HEK 293 cells, the majority of fluorescently-labeled GluR1 subunits were found to be colocalized with an endoplasmic reticulum marker, ECFP-ER or DsRed2-ER. However, an apparently plasma membrane-residing population of GluR1 homomers was demonstrated by excluding the fluorescence pixels colocalized with the ER marker. FM4-64 dyes, as a marker for plasma membrane, were shown colocalized with that latter population. This finding is perhaps not surprising since GluR1 as an ionotropic glutamate receptor subunit originally isolated by expression cloning, certainly can form functional ion channels in *Xenopus* oocytes, which implies the capability of GluR1 of cell surface expression of homomeric receptors (Hollmann et al., 1989). Nevertheless, the current work demonstrates a powerful method comprising the use of commercial fluorescent proteins in the construction of fusion proteins combined with advanced imaging techniques in investigating surface protein expression in living cells. Such analysis previously had only been possible with extracellularly HA-tagged receptor subunits analyzed by immunocytochemistry in fixed cells or, more recently, with receptor subunits fused to pHluorins, a promising pH-sensitive GFP mutant which, however, is not commercially available. Another recently developed method utilizing photostable semiconductor nanoparticles (or quantum dots, QD), coated with anti-GluR antibodies, further expands the possibility of live imaging of surface proteins. However, the QD complex consisting of primary and secondary antibodies is in the 50 nm size range, far bigger than GFP (~3nm), and thus can affect protein trafficking and reduce accessibility to target proteins.

As for GluR2 subunits, expression patterns of both edited and non-edited variants were analyzed. Both variants were found to be predominantly residing in the ER of HEK 293 cells. A very small amount of GluR2(Q), approximately around 1% of the total pool, could be detected at
the plasma membrane, whereas no surface GluR2(R) homomers were detectable in our system. The surface GluR2(Q) homomers, however, were so few that it was impossible to apply a similar analysis as performed with GluR1 homomers: excluding the colocalized pixels when coexpressed with an ER marker. However, this relatively small amount of surface GluR2(Q) subunits is capable of forming functional homomeric channels as demonstrated by patch clamp recording. In contrast, there was no measurable glutamate-evoked current in GluR2(R)-expressing cells, consistent with the exclusively intracellular distribution of this subunit.

Those observations appear in constant with previous report that Q/R editing determines AMPA receptor exit from the ER (Greger et al., 2002). Greger et al. also suggested in a later study that the ER exit regulation by the amino acid present at the Q/R editing site is mediated by receptor tetramerization (dimerization of dimers) as the second step of assembly. The first step of assembly dimer formation, does not seem to be affected (Greger et al., 2003). Comparing the different expression systems used in earlier reports (neurons) and the present study (non-neuronal cells), there is an apparent difference in the expression levels of the endogenous auxiliary subunits, TARPs, which may dramatically affect the surface expression level of AMPA receptors. To test this, coexpression experiments of fluorescently-labeled stargazin with AMPAR variants were carried out in HEK 293 cells. Surprisingly, coexpression patterns of GluR1 plus stargazin, GluR2(Q) plus stargazin, and GluR2(R) plus stargazin showed no significant differences. Stargazin is obviously capable of targeting all three subunits into the plasma membrane to a similar extent. It is generally believed that only correctly assembled and functional receptor complexes are transported to the cell surface. Therefore, all three subunits tested show their abilities to form homotetramers at least in the presence of stargazin in HEK 293 cells. In order to test the first step of tetramer assembly, dimer formation, FRET analysis was carried out with cells coexpressing GluR2(R)-ECFP and GluR2(R)-EYFP fusion proteins. Significant FRET signals were found intracellularly, most likely at the ER according to the diffuse network structure observed, which indicates dimer formation of GluR2(R) subunits. These data suggest that the first step of tetramer assembly, the formation of dimers of AMPA receptor subunits occurs early in the assembly pathway in the ER, regardless of the intracellular C-terminus of the subunit, Q/R editing in the pore
region, or the presence of TARPs. Furthermore, the Q/R editing site may determine AMPA receptor exit from the ER \textit{en route} to the plasma membrane, however, not via determining receptor tetramerization. The present study suggests that in the presence of stargazin both Q and R variants are capable of assembling into homotetramers and trafficking to the cell surface forming functional ion channels. It is therefore possible that stargazin mediates AMPA receptor tetramerization, especially at the second step of dimerization of dimers, and may thus play a critical role in the AMPA receptor assembly pathway. Stargazin-mediated AMPA receptor tetramerization may involve the Q/R editing site which regulates interaction of AMPA receptors and stargazin via a conformational change, as proposed most recently (Körber et al., 2007).

Preliminary experiments of transient expression of homomeric AMPA receptors were also carried out in dissociated primary hippocampal or cortical neuron culture (data not shown). The dendritic spines were not yet apparent in our cultures at the time of fluorescence recording, which is probably due to the transfection methods used in this work (nucleofection and calcium phosphate methods) which required immediate transfection after tissue dissociation and resulted in early cell death in the culture. Other transient transfection techniques, such as lipid-based methods or an optimized calcium phosphate method, shall therefore be carried out in the near future in order to continue the investigation of receptor trafficking in primary neurons. However, for all recombinant AMPAR subunits tested, in addition to their intracellular localization, expression of receptors was observed to be at the plasma membrane of soma as well as dendrites, perhaps due to regulated receptor trafficking mediated by endogenous AMPAR-associated proteins, such as TARPs.

5.1.2 Stargazin regulates AMPAR surface expression and interacts with AMPAR at the plasma membrane

In live HEK 293 cells, fluorescently-labeled stargazin fusion proteins dramatically enhanced the surface expression level of recombinant GluR1, GluR2(R), and GluR2(Q) subunits, as shown in Chapter 4.2.4.2. This is in agreement with previous studies that stargazin (γ2), a member of a family of tetraspanning transmembrane AMPA receptor regulatory proteins (TARPs) comprising
four related subunits (γ2, γ3, γ4, and γ8), is critical for bringing AMPA receptors to the neuronal surface and targeting them to synapses (Chen et al., 2000; Chetkovich et al., 2002; Schnell et al., 2002; Tomita et al., 2003). However, when the current study was performed, there was little evidence yet for the role of stargazin besides regulating surface and synaptic trafficking of AMPA receptors. One such study showed clustering of TARPs with AMPA receptors at the postsynaptic density in primary neuronal cultures, and that AMPA receptors were coimmunoprecipitated with TARPs from brain extracts, which suggested a surface interaction between TARPs and AMPARs (Tomita et al., 2003).

By utilizing digital colocalization analysis, and, more importantly, FRET analysis in live HEK 293 cells that allowed to obtain information with nanometer resolution, we were able to monitor the stargazin-AMPAR complexes and identify the subcellular location of the interactions. These experiments demonstrated direct protein-protein interactions which, strikingly, appeared to persist within the plasma membrane (Ma and Hollmann, 2004a; Ma and Hollmann, 2004b). FRET was carried out by coexpression of ECFP-stargazin and EYFP-AMPAR fusion proteins. The Förster distance for energy transfer between ECFP and EYFP is 4.9 nm (Patterson et al., 2000), suggesting that significant FRET signals observed indicate actual physical interaction between the two fusion proteins. Compared to two negative controls, wildtype ECFP plus EYFP and stargazin-ECFP plus GluR6-EYFP, significant FRET was obtained at the cell surface for stargazin plus GluR1, stargazin plus GluR2(Q), and stargazin plus GluR2(R) combinations. This novel approach indicated that stargazin stays associated with AMPA receptors after translocating them to the HEK 293 cell surface, apparently mediated by an activity-independent mechanism as it occurs in a non-neuronal cell line. Interpreting the present data on receptor synaptic trafficking, and taking into consideration the reported lateral movement of endogenous AMPA receptor subunits in the neuronal plasma membrane where they show high mobility at extrasynaptic sites but low mobility at postsynaptic sites (Borgdorff and Choquet, 2002), a two step synaptic trafficking model of AMPA receptors may be conceived: first, stargazin translocates AMPA receptors, likely GluR1/GluR2 heteromers, to extrasynaptic sites in the plasma membrane. This surface region is highly enriched with stargazin-AMPAR complexes which may serve as a ‘reserve pool’. Second,
under specific stimulation, such as synaptic activity that induces NMDA receptor activation, the extrasynaptic stargazin-AMPAR complexes rapidly diffuse to the postsynaptic density where they are anchored, thus regulating synaptic strength and synaptic plasticity. Moreover, a persistent cell surface interaction between stargazin and AMPA receptor subunits may imply concealed physiological functions of stargazin beside receptor trafficking regulation. This has later been seen independently in studies demonstrating that stargazin indeed regulates AMPA receptor desensitization, deactivation, and agonist efficacies, hence, electrophysiological properties (Kott et al., 2007; Priel et al., 2005; Tomita et al., 2005a).

As a control, FRET analysis was performed with a pair of differently tagged stargazin proteins so that stargazin serves both as donor and acceptor for the energy transfer. Although at a low absolute level, a significant FRET signal was obtained in the experiment, which may indicate a possible association between different stargazin protein subunits at the cell surface. This might suggest the stoichiometry of stargazin-AMPAR complexes to be AMPAR tetramers associated with more than one stargazin subunit.

Moreover, as suggested in the former chapter (Chapter 5.1.1), stargazin may play a role in AMPA receptor assembly. Tracing exocytosis of potential stargazin/AMPAR complexes before their plasma membrane insertion was therefore carried out in living HEK 293 cells. It was difficult to catch the moment when both stargazin and AMPA receptor subunits were colocalized in the ER, likely due to a rapid exit of AMPA receptors from the ER via a stargazin-mediated mechanism. An interesting observation is that AMPA receptor subunits colocalize with stargazin at a peri-nuclear region with a compact solid structure, most likely the Golgi apparatus, implying the presence of stargazin/AMPAR complexes. Coexpression of a Golgi-resident fluorescent protein indeed confirmed a persistent colocalization of stargazin and AMPA receptor subunits at the trans-medial region of the Golgi apparatus and adjacent intracellular compartments. It has previously been reported that glycosylation of AMPA receptors is abnormal in neurons lacking TARPs possibly due to reduced Golgi residing AMPA receptors (Nicoll et al., 2006; Tomita et al., 2007), suggesting that association with stargazin may be essential for AMPAR exit from the ER to the Golgi apparatus. Therefore stargazin may play a role in early biosynthetic pathway of AMPA receptors.
Taken together, the Golgi apparatus seems to play a critical role in stargazin-mediated AMPA receptor exocytosis, possibly as a determining intracellular compartment for sorting stargazin/AMPAR complexes en route to the plasma membrane.

As discussed in the former and the present chapters, a model of stargazin-mediated AMPA receptor assembly and exocytosis may be conceived as follows: AMPA receptor subunits form dimers in the ER, followed by association with stargazin involving/causing a conformational change of the receptor subunits. The interaction of stargazin and AMPAR dimers may mediate/facilitate the dimerization of dimers into tetramers. The tetramers exit the ER rapidly, likely facilitated by stargazin, to the Golgi apparatus where stargazin and AMPARs stay associated. Stargazin/AMPAR complexes then accumulate and persist at the Golgi apparatus where they are routed to their final destination. If this model is correct, the expression level of stargazin would be the determinant of stargazin/AMPAR dimer formation and, as a consequence of AMPA receptor tetramerization and surface trafficking (Vandenbergh et al., 2005b). This model may explain the contradictory suggestions for the role of the Q/R editing site made in the present study and a previous report (Greger et al., 2003). The amino acid at the Q/R editing site has been suggested to determine tetramerization of AMPA receptors such that the Q variants are ready to traffic to the plasma membrane while the R variants reside in the ER (Greger et al., 2003). However, the present study shows that both Q and R variants form functional ion channels robustly expressed at the plasma membrane of HEK 293 cells when coexpressed with stargazin. In the previous report, the retention of GluR2 in the ER of cultured neurons might not be due to the proposed incapability of GluR2 tetramerization; rather, the limited expression level of stargazin might play a critical role. Whereas in the present study, when both GluR2 and stargazin were expressed in HEK 293 cells, the expression level of stargazin may have been sufficient to complex GluR2 and thus influence GluR2 assembly and plasma membrane delivery.
5.1.3 Dynamics of dissociation of surface AMPAR/stargazin complexes

Excitatory synaptic plasticity is known to be correlated with the numbers of postsynaptic AMPA receptors. Trafficking of AMPA receptors to and from synapses therefore plays a critical role in regulating synaptic strength during LTP and LTD. Exploring the underlying dynamics and mechanisms of AMPA receptor endocytosis (= removal from the cell surface) would provide further understanding of the mechanisms of downregulation of excitatory transmission. TARPs are required for synaptic trafficking and stabilization of functional AMPA receptors in the plasma membrane, and are sometimes called ‘auxiliary subunits of AMPA receptors’ (Chen et al., 2000; Schnell et al., 2002; Tomita et al., 2003). Previous studies provided conflicting evidence as to whether TARPs dissociate from AMPARs upon binding of glutamate (Nakagawa et al., 2005; Tomita et al., 2004; Vandenberghe et al., 2005b). Investigating the dynamics of stargazin/AMPAR complexes, the current study presents evidence of ligand-induced dissociation of TARP/AMPAR complexes under physiological conditions in living mammalian cells.

For three combinations of AMPARs and stargazin tested in live HEK 293 cells, namely ECFP-labeled stargazin coexpressed with GluR1-EYFP, GluR2(R)-EYFP, or GluR2(Q)-EYFP fusion proteins, significant FRET signals were detected at the plasma membrane (discussed in Chapter 5.1.2), clearly demonstrating protein-protein interactions between stargazin and AMPA receptor subunits. Utilizing confocal imaging of those ‘live fluorescent proteins’ at high lateral resolution, digital colocalization analyses were carried out in order to investigate glutamate-induced changes in the physical distance between interacting protein subunits. In contrast to the higher resolution of FRET, digital colocalization analysis confirms the physical distance of two fluorophores only within 120 – 150 nm for the fluorophore combination ECFP/EYFP. While this lack of resolution in the 150 nm range is a disadvantage, the method offers the advantage that when differently-tagged fluorescent fusion protein molecules are identified as ‘non-colocalized’, they are most likely not involved in protein-protein interactions or associated in macrocomplexes. Upon application of L-glutamate, the native ligand of AMPA receptors, the colocalization patterns of stargazin-ECFP and AMPAR-EYFP were analyzed by the number of colocalized pixels carrying both cyan and yellow signals. Importantly, in order to correct for possible photobleaching of the fluorophores that
may take place during long-term fluorescence recording, a correction factor for each channel at
each time point and each focal plane was calculated and applied to the final calculation. Indeed, a
slight photobleaching was observed for the EYFP channel in some experiments, which
demonstrates the importance of monitoring the bleaching factor. Down-regulated colocalization
rates were obtained for all three combinations, as well as an additional pair of stargazin plus
GluR2(R) fused with DsRed2 and EGFP, respectively. These data indicate that the number of
colocalized protein molecules indeed was reduced dramatically after onset of glutamate
application. With this novel approach the present study directly shows that AMPA receptor
subunits dissociate from stargazin upon ligand binding. A recent study showed that a point
mutation in the glutamate-binding region of GluR1 corresponding to the lurcher mutation of the
glutamate receptor subunit δ2 eliminated stargazin-mediated receptor trafficking and channel
gating, suggesting a functional interaction of TARPs with the extracellular glutamate-binding
domain of AMPA receptors (Tomita et al., 2007). Ligand binding may therefore induce a
conformational change in AMPA receptor or stargazin/AMPAR complexes, followed by the
dissociation of stargazin/AMPAR complexes. It has also been shown that AMPA receptors
internalize in cultured hippocampal neurons upon AMPA or glutamate application, partly by
activation of voltage-dependent calcium channels that results in a rise in postsynaptic Ca\(^{2+}\), and
partly by ligand binding independent of receptor activation (Lin et al., 2000; Zhou et al., 2001).
Presumably, AMPA receptor endocytosis is following the initial dissociation of AMPA receptors
from stargazin at the neuronal surface, and, especially at postsynaptic regions, released AMPA
receptors may rapidly diffuse in the plasma membrane to extrasynaptic sites, followed by selective
internalization. Moreover, the finding that competitive antagonists of AMPA receptors (such as
CNQX/DNQX), but not non-competitive antagonist (such as CYKI52466), induced receptor
internalization and partially inhibited AMPA-induced receptor internalization in cultured neurons
(Lin et al., 2000), provides indirect evidence that an intricate AMPA receptor endocytosis
mechanism is set off with the ligand-induced conformational change of surface stargazin/AMPAR
complexes.
It is, however, interesting to note that deletion of the membrane-proximal region at the intracellular C-terminal tail of GluR2, which is highly conserved for GluR1 – 4 and thus conceivably is the intracellular interaction domain for stargazin (Tomita et al., 2007), reduced GluR2 internalization in response to AMPA or DNQX in cultured neurons (Lin et al., 2000). According to our hypothesis, deletion of this intracellular segment that mediates GluR2 association with stargazin should lead to an increased release of surface GluR2 from associated stargazin. Consequently, the competitive antagonist-induced internalization of GluR2 would be expected to increase. A possible explanation of the discrepancy could be the intricate mechanism of AMPA receptor endocytosis in which the internalization or the accumulation of internalized receptors is regulated by several independent protein-protein interactions via the intracellular tail of AMPA receptor subunit. For example, this membrane-proximal region at C-terminal tail of GluR2 involves interactions with two other cytoplasmic proteins, NSF and AP2, which play a role in synaptic maintenance and endocytosis of AMPA receptors, respectively (Lee et al., 2002; Luthi et al., 1999; Nishimune et al., 1998; Noel et al., 1999; Osten et al., 1998; Song et al., 1998). Therefore, deletion of this domain eliminates several distinct interactions, thereby complicating the analysis of the fate of the endocytosed mutated GluR2 (Lin et al., 2000).

In addition, similar dynamic colocalization curves obtained from all combinations tested, which indicate reduced colocalization probabilities of stargazin and AMPA receptor subunits, were best fit to similar quadratic functions of duration of ligand incubation, from which the time course of dissociation could be obtained. Maximal dissociation of the complexes was reached by 1266 ± 136 s (n = 14), approximately 21 minutes, at which time ~60% of initial stargazin/AMPAR complexes are dissociated. Interestingly, this time course falls in a similar range as the previously reported time course of AMPA-induced AMPA receptor internalization in cultured neurons (~50–60% internalized when a plateau was reached at ~15 minutes, (Lin et al., 2000)). This similarity in time range between dissociation of stargazin/AMPAR complexes and AMPAR internalization, two processes which might be sequential steps in postsynaptic AMPAR endocytosis, may suggest that the first step of dissociation of stargazin/AMPAR is the crucial determinant of the time course of AMPAR endocytosis. Indeed, one previous report presented evidence that removal of synaptic
AMPARs from extrasynaptic sites (Ashby et al., 2004). Taken together, the time course of postsynaptic AMPA receptor internalization is most likely determined by a slow release of AMPA receptors from stargazin.

Essential control experiments, however, are still required, such as the application of the inactive stereoisomer D-glutamate. Further experiments with other agonists or antagonists of glutamate receptors will provide additional information.

To summarize this chapter, it is shown that AMPA receptors (GluR1, GluR2(R), and GluR2(Q)) dissociate from surface stargazin/AMPAR complexes upon L-glutamate application in live mammalian cells. In general agreement with several previous studies, a model of AMPA receptor endocytosis can be conceived: release of postsynaptic AMPA receptors from stargazin/AMPAR complexes initially anchored by PDZ proteins at postsynaptic densities is likely the first event and the time course-determining step of postsynaptic AMPA receptor endocytosis; the freed AMPA receptors then rapidly diffuse to extrasynaptic sites from where they are rapidly and selectively internalized.
5.2 Assembly and intracellular trafficking of kainate receptors

Kainate receptors may play a key role in modulating synaptic networks depending on their subunit composition, subcellular localization (presynaptic, postsynaptic, and extrasynaptic domains), and membrane delivery. GluR6 and KA2 not only represent low and high affinity subunits, respectively, but are also forming the major subtype of native kainate receptors in the brain. Therefore, GluR6 and KA2 subunits were selected to construct recombinant cDNAs fused with various fluorescent proteins in order to monitor the assembly and intracellular trafficking of kainate receptor complexes in live cells by high lateral resolution confocal imaging and digital analysis. Both spatial and temporal information on receptor assembly and trafficking was collected in these experiments.

5.2.1 GluR6 surface expression is not significantly regulated by Q/R editing

Bearing in mind that the heterologously expressed edited R variants of AMPA receptor subunits (for example, GluR2 (Q586R)) were reported to predominantly retain in the ER while the non-edited Q variants readily traffic to the cell surface (Greger et al., 2002), we considered that RNA editing may play a similar role in kainate receptor trafficking. Although it has been shown that splice variants of the low affinity kainate receptor subunits differing at their intracellular C-terminal domains result in differing amounts of homomeric receptors at the cell surface (Jaskolski et al., 2004; Jaskolski et al., 2005; Ren et al., 2003b; Yan et al., 2004), no previous report, to our knowledge, has focused on the trafficking properties of the Q/R editing isoforms of those subunits. As GluR6(Q) and GluR6(R) subunits form functional homomeric channels in heterologous systems, it is unlikely that the edited R variants (Q588R) are entirely retained in the ER. Indeed, the fluorescently-labeled GluR6(Q) and GluR6(R) subunits can both form functional homomeric receptors at the plasma membrane of HEK 293 cells, as evidenced by the glutamate-evoked currents they produce (Chapter 4.3.1.1 and 4.3.2.1). Confocal 3D scanning demonstrated the robust surface expression of both subunits when expressed alone. In addition, when coexpressed with an ECFP fusion protein serving as ER marker, there was no significant amount of overlapping
yellow fluorescence signals in the ER, as shown in Figure 4.3.17 for GluR6(R)-EYFP fusion proteins. An intracellular aggregation of the R variants in a relatively small compartment close to the nucleus, however, was observed occasionally, which may depend on the time allowed for protein folding and trafficking after transient transfection.

Therefore, RNA editing at the Q/R site of the kainate receptor GluR6 subunit appears not to be a key regulator for receptor surface delivery. Since probably only fully assembled functional receptor complexes are transported to the cell surface, the Q/R site in the pore region of kainate receptors may not be a tetramerization determinant as has been suggested for AMPA receptors (Greger et al., 2003). Given the fact that un-edited calcium-permeable Q variants of GluR2 are almost undetectable in the mammalian central nervous system, whereas around 25% of GluR6 remain un-edited in vivo (8% in hippocampus) (Hollmann, 1999), it is possible that RNA editing at the Q/R sites within the pore regions of AMPA and kainate receptor subunits differentially affects to physiological function and serves different purposes. However, modulation of surface expression of kainate receptor subunits by RNA editing may not be entirely invalid. In order to address this question, further studies will be required, involving various combinations with two other edited codons in the first transmembrane domain (I534V and Y538C) of the GluR6 subunit.

5.2.2 KA2 homomeric assemblies are retained in the ER, fail to deliver to the cell surface, and undergo a dynamic retrograde recycling from the Golgi apparatus to the ER

The low affinity kainate receptor subunits GluR5, GluR6 and GluR7 can form functional homomeric ion channels in recombinant systems. The plasma membrane expression of these subunits is dependent on the splice variants (Jaskolski et al., 2004; Jaskolski et al., 2005; Ren et al., 2003b; Yan et al., 2004). The data presented here indicate that both edited and un-edited variants of GluR6 subunit are predominantly expressed at the cell surface.

However, the high affinity subunits KA1 and KA2 can coassemble with low affinity subunits, forming functional heteromeric channels with distinct pharmacological properties, while they are
unable to form functional homomeric cationic channels. Little was known about these subunits until 2003, when two independent studies reported assembly-dependent trafficking of the KA2 subunit. Both studies were immunocytochemical analyses, using transient transfection of KA2 cDNA into cultured mammalian cells and demonstrating predominant association of KA2 proteins with intracellular membrane compartments, especially the ER (Gallyas Jr et al., 2003; Ren et al., 2003a). The failure of KA2 to form functional homomeric channels may be caused by its failure to be transported to the cell surface.

In the present work, KA2 labeled with fluorescent protein tags, such as EGFP or EYFP, was transiently expressed in adherent cells. Apart from the fluorescence signals obtained from the KA2 fusion proteins, fluorescent markers labeling plasma membrane (FM4-64) or ER (ECFP-ER fusion protein) were used to simultaneously detect these intracellular compartments and correlate them with the intracellular localization of the KA2 subunit. Digital colocalization analysis of the two fluorophores, which is more precise than the commonly used visual-based overlapping, defines the presence of two different molecules residing at the same physical location in a specimen. The apparently well separated clouds formed by pixels with green or red fluorescence signals clustering toward the correlated axes of the 2D cytofluorogram (Figure 4.3.19 D) demonstrate a discrete spatial separation between KA2 and the plasma membrane. In addition, colocalization of KA2-EYFP and ECFP-ER is indicated by the cloud of the pixels presenting both cyan and yellow fluorescence signals clustering toward the middle of the 2D cytofluorogram (Figure 4.3.20 J). Notably, there is almost no pixel with yellow fluorescence signal clustering toward the yellow axis, which means that nearly all of the KA2 subunits are retained in the ER of the cells. Taking advantage of digital analysis, the colocalizing pixels were replotted for visualization of the original spatial information (Figure 4.3.20 D – F), which confirms the localization of those pixels within the ER.

With the colocalization data achieved, however, a prominent question remained unanswered: whether the ER retains KA2 subunits as monomers, or already as oligomers at this early stage of receptor biosynthesis. FRET analysis carried out with live HEK 293 cells coexpressing both ECFP- and EYFP-tagged KA2 subunits support the conclusion that interactions between KA2
subunits do occur within the ER. This is extremely interesting because KA2 subunits are known to be both incapable of cell surface expression and the formation of functional homomeric channels, the latter of which could be a consequence of impaired homomeric assembly. In this study it is shown, for the first time in living cells, that although KA2 subunits are mostly retained within the ER, they are capable of assembling into homo-oligomers. This is shown by direct KA2 protein-protein interaction measured under the same physiological conditions and spatial restrictions as for the interactions between native receptor subunits. This finding is consistent with a previous report suggesting homomeric assembly of KA2 in the ER based on biochemical approaches (Ren et al., 2003a).

Finally, KA2 subunits were also found to colocalize with the Golgi marker which labels the trans-medial region of the Golgi apparatus. Taken together with FRET data, a bidirectional dynamic retention/retrieval pathway between ER and the Golgi apparatus is implied for KA2 homomeric receptor complexes, or at least for assembled oligomers. Although Golgi localization of KA2 homomeric receptor complexes has never been reported before, a recent study demonstrated that the arginine-rich ER retention/retrieval motif in the cytoplasmic tail of KA2 subunit interacts with COPI (coatamer protein complex I), which can be competed out by the KA2 C-terminal association with the 14-3-3ζ chaperone protein (Vivithanaporn et al., 2006). Therefore, KA2 routing to its final destination, whether to the plasma membrane or intracellular compartments, occurs at the Golgi complex via association with a variety of proteins, including chaperone proteins, resulting in ER retention of KA2 homomeric complexes. This mechanism benefits heteromeric assembly of KA2 with other kainate receptor subunits, which in turn increases the surface expression of functional kainate receptors.
5.2.3 Assembly of kainate receptor subunits occurs as early as in the ER

Functional glutamate receptors are composed of multiple subunits, most likely forming tetramers with members of the same receptor subfamily. The hypothesis is that the assembly proceeds in two steps: subunit dimerization, and dimer:dimer association forming dimers of dimers, i.e., tetramers (Ayalon and Stern-Bach, 2001; Gallyas Jr et al., 2003; Laube et al., 1998; Rosenmund et al., 1998; Schorge and Colquhoun, 2003). Given the suggestions that oligomerization may play a key role in the quality control and ER export of many other receptors, it is tempting to test whether the ER also serves as a privileged site for oligomeric assembly of ionotropic glutamate receptors.

Although various techniques, such as electrophysiological measurements, biophysical and biochemical methods, have been used to assay ion channel assembly, few could directly assay the assembly process. Since most ion channels are multimeric assemblies involving several subunits residing at the plasma membrane, it is almost impossible to assemble large biological ion channels in a test tube by in vitro translation methods. To date, functional assays, such as electrophysiological measurements, are the most common methods in studying ionotropic glutamate receptor assembly. Cultured cells are therefore widely used for the proper assembly of ion channels expressed via transfection, as in these cells all required chaperones and processing enzymes are present. Since initial steps of assembly are dynamic processes and take place intracellularly, apparent disadvantages of functional approaches can be the virtual impossibility in isolating the receptor complexes of interest, competing among coexisting subunit assemblies. Other traditionally applied biophysical or biochemical methods in studying protein assemblies, for example, affinity chromatography, coimmunoprecipitation, and two-hybrid techniques, however, do not allow direct access to interactions between the proteins in their natural environment inside the living cell.

By utilizing digital colocalization analysis, and, more importantly, FRET analysis in live HEK 293 cells that demonstrate direct protein-protein interactions and allow to obtain information with nanometer resolution, it was possible in the present study to monitor the assembly of homomeric and heteromeric receptor complexes, at least the putative initial step of homo- and hetero-dimer formation, and to identify the subcellular location of subunit interactions.
Two negative controls were performed at first to estimate the reliability of sensitized emission FRET analysis in our system, with wildtype ECFP plus EYFP, and stargazin-ECFP plus GluR6(Q)-EYFP coexpressing cells. The former combination was chosen to verify whether possible dimer formation between fluorophore variants alone could produce FRET signals. There was no FRET signal detectable in those cells coexpressing both fluorescent proteins. A further control was then carried out with the latter combination of theoretically non-interacting membrane proteins, where both of the fluorescently-tagged membrane proteins are highly concentrated at the cell surface. This was to test whether FRET signals might simply result from random subunit interactions, as a consequence of protein overexpression at high levels in a small compartment of the cell, causing FRET by a ‘random proximity effect’. The FRET and FRET efficiency values of this control pair were both significantly lower than those between two (likely interacting) kainate receptor subunits tested, at 0.67 ± 0.58 (n = 10) and 0.19 ± 0.16% (n = 10), respectively (Chapter 4.3.5.1).

Positive FRET signals were obtained within the ER of cells coexpressing ECFP- and EYFP-tagged GluR6(Q) fusion proteins, revealing that the oligomerization of homomeric GluR6(Q) complexes occurs at an early stage of the biosynthesis pathway. Similar results were obtained for GluR6(R) homomeric oligomerization. Together with the positive FRET signals indicating the presence of KA2 homo-oligomers in the ER, which has been extensively discussed above (Chapter 5.2.2), oligomerization of homomeric kainate receptor complexes is shown to take place in the ER as the initial step of trafficking.

When GluR6(Q)/KA2 and GluR6(R)/KA2 combinations were analyzed, as shown in Chapter 4.3.5.2 and Figure 4.3.36, there were detectable FRET signals between the high and low affinity receptor subunits, indicating that heteromeric oligomerization is occurring in the ER as well.

Evidence was thus provided for the early oligomerization in the ER at the initial stage of the assembly pathways of homomeric and heteromeric kainate receptor complexes. This suggests that the proposed ‘quality control’ function of ER (Ellgaard and Helenius, 2003), apart from proper folding and assembly of polypeptides, may also include the correct assembly of multimeric membrane receptor complexes. Recent studies suggest that chemicals or pharmacological ligands may play a role as chaperones in receptor folding (Morello et al., 2000), a hypothesis that has been
most recently tested for ionotropic glutamate receptors (Mah et al., 2005; Valluru et al., 2005).

Considering the mM range of intracellular glutamate in the CNS, most probably associated with the ER (Berger et al., 1977; Meeker et al., 1989), and the binding affinity of glutamate receptors in the 50 – 200 nM range, it is possible that proper ligand binding is another determinant of trafficking of receptor complexes that are exiting the ER en route to the plasma membrane.

5.2.4 An endoplasmic reticulum – Golgi apparatus – plasma membrane trafficking pathway for oligomeric kainate receptor complexes

Distinct pharmacological and electrophysiological properties of native kainate receptors are correlated with distinct combinations of receptor subunits in different regions of the nervous system. In addition, the functional diversity of kainate receptors is related to their diverse subcellular localization. In the present study, a variety of combinations of kainate receptor subunits was used to investigate receptor formation and trafficking. It was aimed to elucidate possible rules of intracellular transport and proximity of those receptors to specific intracellular compartments, including ER, Golgi apparatus, and plasma membrane.

FRET between ECFP- and EYFP-tagged kainate receptor subunits can provide evidence for subunit interaction and dimer formation in living cells. Förster distance for energy transfer between ECFP and EYFP is 4.9 nm (Patterson et al., 2000), suggesting that significant FRET signals indicate actual physical interaction between the two fusion proteins. In the present study, significant FRET was obtained at the cell surface for GluR6(Q)/GluR6(Q), GluR6(Q)/GluR6(R), and GluR6(R)/KA2 subunit combinations, representing three typical possibilities of kainate receptor complex compositions. Together with electrophysiological functional verification, this demonstrated the formation of homomeric and heteromeric receptor complexes at the plasma membrane of living cells, at least with the recombinant fluorescently-labeled subunits used in this study.

Utilizing the advantage of FRET analysis performed at a high spatial resolution, not only protein-protein interactions at the plasma membrane but also at other intracellular compartments of
living cells could be demonstrated. Intracellular FRET signals were found to be significant for several combinations of subunits. There were FRET signals indicating subunit interactions in homomeric complexes of the low affinity subunit GluR6, both for GluR6(Q)/GluR6(Q) and GluR6(R)/GluR6(R), at the ER and Golgi apparatus. FRET was also detectable for the homomeric combination of KA2 subunits, one of the high affinity subunits, at the ER of HEK 293 cells. Importantly, with GluR6(Q)/KA2 and GluR6(R)/KA2 subunit combinations, significant FRET signals were obtained within the network-like ER compartment of the cells. Furthermore, energy transfer between GluR6(R) and KA2 as well as GluR6(Q) and GluR6(R) fusion proteins were found at a peri-nuclear region with a distinctive structure, suggesting subunit association in the Golgi apparatus in the living cells.

Taken together, several homomeric and heteromeric combinations of kainate receptor subunits have been tested in this work. To our knowledge, these subunits in the present study have been shown for the first time in their natural environment in living cells to assemble into dimers/oligomers in the ER, and to associate with the Golgi apparatus en route to the plasma membrane. Kainate receptors composed of low affinity subunits, with or without high affinity subunits, are therefore probably receptors with the capability of trafficking to the cell surface on their own. Furthermore, it became evident that those receptors undergo assembly even before they reach the plasma membrane. A general sequence of events for intracellular trafficking in the early and late stages of the assembly pathway of oligomeric kainate receptors, as a model for all ionotropic glutamate receptors, can thus be stated as follows: oligomerization of ionotropic glutamate receptors is most likely taking place as early as completion of subunit synthesis in the ER. The physiological function of the ER may thus comprise not only the correct synthesis and folding of the polypeptides, but also the correct assembly of receptor complexes. Those complexes are then either retained in the ER or further transported to the Golgi apparatus via the secretory pathway. The determinants of the ER quality control of the receptor complexes remain unclear, yet, but there is evidence (Fleck, 2006) that this may involve binding of intracellular glutamate and conformational changes of the complexes. Additional sorting of the complexes may then occur at the Golgi apparatus, where the non-functional receptors are returned back to the ER via a dynamic
retrieval pathway (Vivithanaporn et al., 2006), whereas the fully functional receptors are targeted to
the cell surface as their final destination.

5.2.5 Modulation of surface expression of GluR6/KA2 heteromeric receptor
complexes by both constituent subunits

As trafficking of the ionotropic glutamate receptors is likely one of the key factors regulating
synaptic strength, discovering possible rules guiding the surface expression of those receptors is
important for understanding synaptic plasticity and higher brain function. In addition, kainate
receptors, unlike the other two major subfamilies of ionotropic glutamate receptors, namely AMPA
and NMDA receptors, are abundant not only at postsynaptic membranes, but also presynaptic and
even extrasynaptic membranes of the neuronal surface. Therefore, investigation of possible
determinants regulating surface expression of kainate receptors was carried out in the present study.

Consistent with the previous chapters, model kainate receptor complexes composed of
GluR6(Q) or GluR6(R) and KA2 subunits were chosen for exogenous expression in heterologous
system. Formation of electrophysiologically functional ion channels at the plasma membrane of
live HEK 293 cells with our recombinant subunits was verified by several means, including patch
clamp recordings (Chapter 4.3.4, Figure 4.3.29) and FRET analysis (Chapter 4.3.5.1, Figure 4.3.30,
Tables 4.9 and 4.10). Apart from confirming heteromeric receptor complex formation, some
interesting observations were obtained with the cells coexpressing both GluR6 and KA2 fusion
proteins.

Surprisingly, one of the expression features is that the surface expression of GluR6 subunits was
delayed by coexpression with KA2 subunits. GluR6 was observed to be retained in the ER and to
colocalize with KA2 before both subunits are inserted into the plasma membrane. However, a
fairly small fraction of both subunits was eventually found to colocalize at the plasma membrane,
suggesting the formation of heteromeric receptor complexes at the cell surface. In addition, at the
same time there was a significant portion of GluR6 subunits ultimately expressed at the cell surface
but not colocalized with surface KA2 subunits. The distance between those surface GluR6
subunits and KA2 subunits is thus much too far for protein-protein interactions. To compare the time course of GluR6 plasma membrane targeting with or without KA2 coexpression, two controls were considered: the population of cells in the cotransfection dish with both GluR6- and KA2-encoding cDNAs but expressing only GluR6 subunits, and the cells transfected in parallel in a separate dish with a single cDNA encoding fluorescently-labeled GluR6 subunits. The data indicate that assembly of homomeric GluR6(Q) or GluR6(R) complexes is significantly faster than assembly of heteromeric GluR6(Q)/KA2 or GluR6(R)/KA2 complexes. It is also possible that a competitive oligomerization process occurs within the ER during assembly of heteromeric receptor complexes composed of both low and high affinity kainate receptor subunits that results in delayed surface expression of homomeric receptor complexes composed of only low affinity subunits. However, the physiological consequences of this postponed surface insertion of functional homomeric complexes formed with low affinity subunits are unclear. It might be related to the regulation of surface expression of different subsets of kainate receptors composed of different subunits.

Moreover, by digital colocalization analysis, all the KA2 subunits at the plasma membrane were shown to colocalize with GluR6 subunits, which indicates all the surface KA2 subunits were involved in heteromeric interactions with GluR6 subunits. Thus, the interaction with GluR6 subunits is a prerequisite of KA2 surface expression. In addition to the finally surface-inserted KA2 subunits involved in the formation of functional heteromeric receptor complexes, there was a significant amount of KA2 subunits still retained in the ER. Compared to the intracellularly retained GluR6 subunits in the same cell, the amount of intracellular KA2 subunits is much larger. It is therefore likely that not all of the KA2 subunits were involved in heteromeric interactions with GluR6 subunits. Those intracellular KA2 subunits may undergo homomeric assembly into non-functional receptor complexes and are thus retained in the ER.

Although GluR6 and KA2 together form functional heteromeric ion channels, the stoichiometry of the complexes remain unknown. The evidence demonstrated in the present study may imply the preference of homomeric oligomerization of both low and high affinity kainate receptor subunits, over heteromeric oligomerization between low and high affinity subunits. Considering the
hypothesis of assembly of ionotropic glutamate receptors as dimers of dimers in two steps, the data obtained here indicate that the first step of kainate receptor assembly is most probably the homomeric dimerization of either low or high affinity subunits. If heteromeric dimerization of one low affinity and one high affinity subunit were preferred at this step, there should be a predominant formation of heteromeric receptors in those cells coexpressing both subunits, which was, however, not observed. As a consequence for the structure of heteromeric kainate receptor complexes, when one low affinity and one high affinity subunit are stoichiometrically involved in heterotetramer formation, all possible stoichiometries may occur: tetramers consisting of two homodimers, one homodimer consisting of low and one of high affinity subunits, and tetramers consisting of two heterodimers, each containing one low and one high affinity subunit. There are actually two more possible stoichiometries of heterotetrameric kainate receptor complexes, involving one low affinity and one high affinity subunit, where the tetramer is coupled of one homodimer composed of either low or high affinity subunits, and one heterodimer composed of both low and high affinity subunits. The probability of the two latter stoichiometries with low and high affinity subunits not stoichiometrically involved may lie in between the probabilities for the two former stoichiometries with low and high affinity subunits stoichiometrically involved. The surface expression of functional kainate receptors may thus be controlled by the assembly procedure at the initial step of the first dimerization and is therefore modulated by both low and high affinity subunits.
5.3 Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes

Chen and Okayama in their classical paper on transfection of cultured cells (Chen and Okayama, 1987) reported that a peak of high transfection efficiency occurs when an optimal amount of DNA is mixed with mammalian cells under transfection conditions. In the cotransfection experiments investigated here, four fusion proteins, namely GluR6-ECFP, GluR6-EGFP, GluR6-EYFP, and GluR6-DsRed2, were used. Of the four fluorescent protein tags, DsRed2 is a protein very different from the three GFP variants, a fact that may introduce a different expression level (Figure 4.4.1 C). For four different pairs of cDNAs, it is shown that the percentage of cells expressing any fluorescence signal (= cumulative expression efficiency) is not necessarily correlated with the absolute amount of DNA. This suggests that other factors play a critical role in regulating expression efficiencies in simultaneous transfection experiments with multiple cDNAs. One such factor appears to be the cDNA ratio, as the cumulative expression efficiencies were found to vary widely in cotransfections with the same cDNAs at the same total amount of DNA but at different ratios (Figure 4.4.2 B). In addition, not only the cumulative expression efficiency but also individual expression efficiencies vary. Individual expression efficiencies of both proteins in GluR6-EGFP / GluR6-DsRed2 coexpression experiments were much lower than those in EGFP / GluR6-DsRed2 coexpression experiments at identical DNA amounts. This might be explained by the size difference between the small EGFP and the large GluR6-EGFP cDNA that probably alters the expression efficiencies of both proteins. Additionally, the difference in intracellular localization between the soluble EGFP and the membrane-inserted GluR6-EGFP could determine the expression efficiencies or at least alter them. Interestingly however, despite the minimal differences in the coding sequences of GFP variants, there are distinguishable differences in individual and cumulative expression efficiencies of GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-DsRed2, and GluR6-ECFP / GluR6-EYFP combinations at identical DNA amounts. Taken together, the present data suggest that the nature of both of the coexpressed proteins significantly influences the expression efficiencies. The individual or cumulative expression
efficiencies of different proteins and/or different fusion proteins should thus be considered as
highly variable elements that need to be independently assayed and verified for each combination
of coexpressed proteins.

The combined expression of EGFP and GluR6-DsRed2 mimics the commonly employed
method where wild type green fluorescent protein is cotransfected as a marker for the expression of
some target protein. The present study first investigated whether coexpression of both proteins is
indeed 100% predicted for ‘visibly transfected’ cells, as previously claimed (Marshall et al., 1995).
The percentages of cells expressing both fluorescence signals among those expressing the green
signal ‘marker’ were only between 54% and 80%, under various conditions such as differing total
amounts and ratios of the two cDNAs. Meanwhile, the present data indicate that 19 – 30% of cells
expressing GluR6-DsRed2 did not express the ‘green marker’. This means that the criterion
‘visibly transfected or untransfected cells’ does not accurately predict the coexpression state of the
cell. The discrepancy between the present study and earlier reports (Marshall et al., 1995) may be
explained by the small number of cells examined in the study by Marshall et al., compared to the
present study which relies on approximately 1000 cells per condition analyzed. In addition, it is
shown that not even cells expressing EGFP as a marker protein with high fluorescence intensity
necessarily expressed the target receptor subunit at the same time, which is in disagreement with
the postulated reliable correlation between the expression levels of the target subunit and such a
small soluble marker protein. However, the present data do not rule out the possibility to obtain a
relatively high probability of selecting a target protein-expressing cell by picking fluorescent cells
for electrophysiological analysis of that target subunit. Considering current finding of 79.9 ± 3.4%
coexpressing cells among green cells from experiment 1 (1 μg + 5 μg of EGFP and GluR6-
DsRed2), and a wide range (from 54.5 ± 5.4% to 73.7 ± 3.8%) of coexpressing cells among green
cells from experiments 2, 3 and 4 (5 + 1, 1 + 1, and 3 μg + 3 μg of EGFP and GluR6-DsRed2,
respectively), it can be concluded that the common laboratory practice to cotransfect a smaller
amount of fluorescent marker cDNA with a significantly larger amount of target receptor subunit
cDNA is justified. This procedure maximizes the probability of reliably marking target subunit-
expressing cells.
For experiments, however, where coexpression of two target subunits is required, current results are even more sobering as this work-around cannot be employed. The common implicit assumption that any given cell will express all membrane receptor subunits encoded by cDNAs present during transfection was found to be not valid. To exclude possible effects on the transfection or transcription machinery by different molecular weight cDNAs or different properties of the target proteins, analysis was carried out with combinations of two fusion proteins containing the same target protein part (GluR6) but different fluorescent protein parts which were, however, very similar or identical in size. The binary combinations of GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-EYFP, and GluR6-ECFP / GluR6-DsRed2 thus mimic experiments where two receptor subunits are cotransfected for analysis of heteromeric receptor complexes. In the twelve experiments carried out with these combinations, the coexpression efficiencies among transfected cells were occasionally as low as 34%, with the majority scoring below 60% (Figure 4.4.4). Thus, a non-negligable number of transfected cells expressed only a single target protein. This implies a high likelihood of miss-picking cells for analysis of heteromeric receptor assemblies.

The ternary combination of ECFP, GluR6-EYFP, and GluR6-DsRed2 mimics the method where an additional unrelated small fluorescent protein is simultaneously transfected as an expression marker for two target subunits which form heteromeric complexes. A consistently very low efficiency (around 36%) of triple expression among transfected cells was observed, as well as a low efficiency (around 70%) of triple expression among cells coexpressing the fluorescent cotransfection marker (ECFP) and one of the subunits (yellow or red). These data demonstrate the existence of distinct populations of differentially transfected cells following simultaneous transfection. A similar pattern was also observed with other ternary combinations as well as in quadruple transfections of HEK 293 cells. Thus, picking out cells based on the fluorescence signal of one subunit or, similarly, on the presence of an electrophysiological response of one subunit and assuming coexpression of all subunits present during transfection is risky at best. It is not a failsafe procedure and should be employed with extreme caution in the analysis of heteromeric receptor complexes.
Current findings may explain a number of contradictory results found in the literature. For instance, it may explain data from pharmacological investigations of NMDA receptors where HEK 293 cells triple-transfected with NR1, NR2A, and NR2B subunits on the one hand showed similar NMDA and glycine responses as cells cotransfected with NR1 / NR2A while at the same time also showed similar ifenprodil sensitivity as cells cotransfected with NR1 / NR2B (Blevins et al., 1997). It also may explain why the average deactivation time of cells triple-transfected with NR1, NR2A and NR2B was intermediate between cells cotransfected with NR1 / NR2A and NR1 / NR2B (Vicini et al., 1998). It may further explain conflicting reports regarding the functional analysis of the delta2 subunit. It has been reported that cells cotransfected with delta2 plus GluR2 or delta2 plus GluR5 showed glutamate- or kainate-induced currents comparable to cells transfected with GluR2 or GluR5 alone, suggesting lack of interaction (Lomeli et al., 1993). However, using the same approach, Kohda and colleagues demonstrated that delta2 / GluR1 heteromers were not functional, suggesting a dominant negative interaction (Kohda et al., 2003). Apparently, a tight control of coexpression of all the subunit cDNAs present during the transfection would have been necessary to successfully analyze those receptor complexes and obtain unambiguous data.

Therefore it was examined if an excessive amount of one of the cDNAs used in a cotransfection can force coexpression of both subunits and allow prediction of coexpression at least for those cells expressing cDNA transfected with the lesser DNA amount. Four double-combinations at cDNA amounts of 1 + 5, and 5 μg + 1 μg were tested for coexpression efficiencies, revealing that the cells which express the protein encoded by the lower concentrated cDNA not necessarily also express the other protein encoded by the higher concentrated cDNA (Figure 4.4.4 and 4.4.5). In some cases, such as the combination of GluR6-EGFP and GluR6-DsRed2 (Figure 4.4.4 B), excess amounts of one subunit may indeed force coexpression; however, this will then disallow stoichiometric expression studies based on the cDNA ratio applied during cotransfection experiments. The papers by Mansour et al. and Robert et al. are good examples of studies aimed at deriving the stoichiometry of glutamate receptor complexes from differences in functional properties of subunit combinations expressed at varying cDNA ratios. These approaches critically rely on the assumption that subunits get expressed at the exact ratios at which they are present.
during transfection. If this assumption doesn’t hold true, as the present data indicate, conclusions might become questionable.

Figure 5.3.1. Correlation of coexpression efficiencies and individual expression efficiencies of fluorescently-labeled proteins under various conditions of double transfection in HEK 293 cells, calculated for all cells in the observed fields. A – D refer to EGFP / GluR6-DsRed2, GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-EYFP, and GluR6-ECFP / GluR6-DsRed2 combinations, respectively. Data from experiments with different cDNA ratios were pooled for each subunit combination.

The explanation why different populations of cells take up and/or express different combinations of DNA remains unclear. For the calcium phosphate method, linear regression showed that all coexpression efficiencies determined, while never getting close to 100%, have a positive correlation with the expression efficiencies of each single subunit (Figure 5.3.1). However, among those cells expressing any one of the subunits, the coexpression efficiency shows no clear correlation with the expression efficiency of that subunit (Figure 5.3.2). If the common assumption that coexpression occurs in any given transfected cell were correct, regression lines parallel to the x-axis which intersect at 100% with the y-axis would be expected. Notably, in
Figure 5.3.2 A, C and E, the regression lines indeed run almost parallel to the x-axis; however, they intersect the y-axis at values only between 35% and 90%. While this may indicate stable coexpression under various conditions, the coexpression efficiencies never reach the desired 100%. Similar observations were obtained by a biolistic method. Only 75% of those cells expressing any one of the subunits also expressed the second subunit at the same time. With two commercially available lipid-based transfection methods, coexpression reached a high efficiency (83 – 94%). Though the coexpression efficiency still did not reach 100% under any condition tested, it became obvious that a high coexpression efficiency above 80% can be obtained when high transfection efficiencies for single transfections can be accomplished.

In summary, the present data refute the common assumption that when various cDNAs are simultaneously transfected into cells it can be expected that the proteins encoded by the cDNAs are all expressed and present in stoichiometric amounts. Similarly, the common belief that cells express membrane receptor proteins in strict correlation with the amounts of encoding cDNAs used during the transfection cannot be sustained. Accordingly, cotransfection studies aimed at the quantitative analysis of subunit contributions to heteromeric complexes, or at the analysis of subunit stoichiometry, need to be more rigorously controlled.
Figure 5.3.2. Correlation of coexpression efficiencies and individual expression efficiencies of fluorescently-labeled proteins under various conditions of double and ternary transfections in HEK 293 cells, calculated for those cells expressing one particular fluorescence signal. A – E refer to EGFP / GluR6-DsRed2, GluR6-EGFP / GluR6-DsRed2, GluR6-ECFP / GluR6-EYFP, GluR6-ECFP / GluR6-DsRed2, and ECFP / GluR6-EYFP / GluR6-DsRed2 combinations, respectively. Data from experiments with different cDNA ratios were pooled for each subunit combination.
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## Curriculum Vitae

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Publication List

C. Körber, M. Werner, S. Kott, Z.-L. Ma, M. Hollmann (Accepted, 2007)
The transmembrane AMPA receptor-regulatory protein γ4 is a more effective modulator of AMPA receptor function than stargazin (γ2).
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**Publications in preparation:**

As first author:
1. about AMPA receptor trafficking;
2. about kainate receptor trafficking.

As co-author:
3. about expression of kainate receptors in differentiating embryonic stem cells;
4. about composition of glycine receptors;
5. about modulation of neurogenesis and object recognition memory in synRas mice.
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