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AMPA receptor ligand-binding domain: Site-directed mutagenesis study of ligand-receptor interactions

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Academic Dissertation

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Kuusinen A., Arvola M. and Keinänen K. (1995) Molecular dissection of the agonist binding site of an AMPA receptor. *EMBO J. 14,* 6327-6332.

Milla Arvola and Kari Keinänen. (1996) Characterization of ligand-binding domains of GluR-B and GluR-D glutamate receptor subunits expressed as periplasmic proteins in *Eschericia coli*. *J.Biol.Chem.* 271, 15527-15532.

CONTENTS

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
CAMKII	Ca^{2+}-and calmodulin dependent protein kinase II
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CTZ	cyclothiazide
DNQX	6,7-dinitroquinoxaline-2,3-dione
DTNB	5,5´-dithiobis- (2-nitrobenzoic acid)
DTT	4-dithio-DL-threitol
EC_{50}	effective concentration
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GlnBP	glutamine binding protein
GRIP	glutamate receptor-interacting protein
HBP	histidine binding protein
HIBO	homoibotenic acid
iGluR	ionotropic glutamate receptor
KBP	kainate-binding protein
K_i	inhibition constant
K_d	dissociation constant
KSCN	potassium thiocyanate
LAOBP	lysine/arginine/ornithine-binding protein
LIVBP	leucine-isoleucine-valine-binding proteins
LTD	long-term depression of synaptic strength
LTP	long-term potentiation of synaptic strength
mGluR	metabotropic glutamate receptor
NMDA	*N*-methyl-D-aspartate
ODNB	*n*-octyl-5-dithio-2-nitrobenzoic acid
PAGE	polyacrylamide gel electrophoresis
PBP	periplasmic amino acid binding protein
PDZ	PSD-95/blg/ZO-1
PEPA	4-[phenylsulfonylamino)ethylthiol]-2,6-difluoro-phenoxyacetamide
PKA	c-AMP-dependent protein kinase
PKC	protein kinase C
QBP	glutamine binding protein
S1S2	ligand-binding lobes of the glutamate receptor

ABSTRACT

Ionotropic glutamate receptors mediate most of the fast synaptic neurotransmission in mammalian central nervous system. These glutamate-gated receptors are oligomeric cation channels dived into three pharmacological subclasses; AMPA kainate and NMDA receptors. The AMPA receptors are oligomeric assemblies of subunits GluR-A-D. Each subunit has two ~150 residues sized segments, S1 and S2, which form an extracellular binding site for the transmitter (agonist). These ligand-binding segments can be expressed as a soluble fusion protein (S1S2), which is able to bind ligands with the same pharmacological profile as intact membrane associated receptor.

In the present study, we used the S1S2 fusion protein of the GluR-D in order to study the basis of redox modulation phenomena of iGluRs. By using redox agents and protein labelling we showed that a disulfide bridge is formed between Cys-740 and Cys-795 of the S1S2 protein. The breakage of the bridge by mutation increased ligand affinity of the S1S2 protein and caused oligomerization of the protein suggesting that the bridge is important for the stability of unliganded structure of the ligand-binding domain.

We further used the GluR-D S1S2 fusion protein in order to study the structural determinants responsible for agonist and antagonist binding. Since the S1S2 region has weak sequence similarity to several bacterial amino acid binding proteins it has been suggested that the recognition of amino acid ligands is similar in these proteins. In order to test this hypothesis, we used sequence alignment of bacterial amino acid binding proteins with the GluR-D S1S2 to select residues for site-directed mutation. Analysis of the ligand-binding profile of the mutated S1S2 proteins revealed that residues Arg-507 and Glu-727 are necessary for agonist binding. Our molecular model demonstrated that these residues interact with the α-aminocarboxylate core of the amino acid ligand in a similar manner to the corresponding residues in PBPs.

Additional determinants of agonist binding were found by alanine scanning mutagenesis of seven residues in the S2 N-terminal segment. Ligand-binding analysis of mutated proteins revealed that mutations L672A, G675A and T677A selectively affected agonist binding, leaving antagonist binding intact. This data was supported by subsequently published crystal structures of the GluR-B S1S2 in complex with ligands. Our GluR-D S1S2 ligand docking models were based on the above crystal structures. Together these structural models showed that the distal anionic group of the agonist was bound by Tyr-677. In addition, our model showed the direct participation of Leu-672 and Gly-675 in agonist binding. Since antagonists, which are able to prevent receptor activation, do not seem to form interactions with this region, the N-terminal segment of S2 may have particular importance in AMPA receptor activation.

PREFACE

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Helsinki, January 2003

1 INTRODUCTION

1.1 AMPA selective glutamate receptors

1.1.1 Synaptic transmission

Synaptic transmission is a transfer of a signal from one neuron to another at synapses, which are specialized contact sites of two neurons. In most synapses, transmission is chemical and is mediated via neurotransmitter compounds. Neurotransmitters are released by one cell (presynaptic) into the synaptic cleft and bound by the receptor on the subsequent cell (postsynaptic). Glutamate is the most abundant neurotransmitter mediating excitatory signals in the central nervous system (CNS). Glutamate activates two types of glutamate receptors: ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs) (Nakanishi, 1992). Ionotropic glutamate receptors have been subdivided into α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptor subclasses according to their ligand specificity and primary structure. Ionotropic GluRs mediate fast transmission via an intrinsic cation channel, whereas mGluRs are coupled via G proteins and second messenger cascade to ion channels and mediate slower responses to glutamate (Anwyl, 1999) At iGluRs, glutamate binding leads to opening of the receptor channel and to flow of Na^+ and K+ ions down their electrochemical gradients. As a consequence of the conductance changes excitatory postsynaptic potentials (EPSP) are generated, shifting the resting membrane potential (~-40–-90 mV in neurons) towards more positive values. These local depolarizations of membrane potential in turn promote the generation of a new action potential in the postsynaptic cell.

1.1.2 AMPA receptor physiology

Glutamate receptors exist in every part of the mammalian brain and different glutamate receptor subclasses can co-exist in the same cell (reviewed in Dingledine, *et al.,* 1999). Functional properties of glutamatergic neurotransmission seem to be dependent on the iGluRs present in the synapses. The study of native and recombinant glutamate receptors have revealed that glutamate receptor subclasses have differences in time course of receptor activation and desensitization, in neurotransmitter affinities, and in ion permeabilities, which all have their own effect on the time course of signalling (reviewed in Jonas, 2000; Borges and Dingledine, 1998).

The binding of agonist (Clements*, et al.,* 1998) leads to opening of the ion channel. Normally, glutamate is rapidly removed from synaptic cleft, which leads to rapid closure of the channel by deactivation (reviewed in Jonas, 2000). In the continuous presence of glutamate, the channel is closed by desensitization, which is particularly fast and strong for AMPA receptors and approximately three fold slower (~1-6 ms) than deactivation (Table I). AMPA receptor activation occurs with the time constant of sub-milliseconds, whereas deactivation occurs in the time scale of one or two milliseconds (reviewed in Jonas and Sakmann, 1992). However, slower AMPA receptor evoked excitatory postsynaptic currents (EPSCs) have been identified from particular cells (reviewed in Jonas, 2000). NMDA receptors are activated with a time scale of 10-50 milliseconds. The deactivation and desensitization also occurs with a much slower time course (in the time scale of tens or hundreds of milliseconds) than in AMPA receptors (reviewed in Dingledine *et al.,* 1999).

AMPA and NMDA receptors have distinct ion permeability properties. Most AMPA receptors in the CNS only allow the passage of Na^+ and K^+ ions (Mayer and Westbrook, 1987; reviewed in Jonas and Burnashev 1995), but NMDA receptors also allow passage of Ca^{2+} into the cell (Table I). However, Ca^{2+} permeable AMPA receptors have been identified in, for example retinal ganglion neurons and cerebellar Bergman glia cells (Zhang *et al.*, 1995). NMDA receptors are blocked in a voltage-sensitive manner by extracellular Mg^{2+} ions, suggesting that the receptor function is blocked at normal membrane potential (Jahr and Stevens, 1990). The voltage-dependent Mg^{2+} block can be removed by depolarization caused by persistent activity of AMPA receptors (Fig. 1).

Glutamate activates AMPA receptors with lower potency than NMDA receptors (reviewed in Dingledine *et al.,* 1999). The EC_{50} value (the effective agonist concentration that gives half-maximal response of ionic currents) of glutamate is ~500 μM for AMPA receptors, whereas it is ~400 nM - 2 μM in NMDA receptors, depending on receptor subunit composition. NMDA receptor activation is distinct from AMPA receptor activation due to the requirement of a co-agonist glycine together with the agonist L-glutamate (Kleckner and Dingledine, 1988).

Obviously, the functional diversity of AMPA and NMDA receptors is critical for neuronal function, however the precise roles of these receptors are not known. It seems that AMPA receptors, which are rapidly activated and deactivated are the primary mediators of fast neurotransmission, whereas NMDA receptor are detectors of coincidence activation and are functional only during heavy synaptic signalling (Fig. 1). NMDA receptors allow entrance of Ca^{2+} into cell, which promotes activation of second messenger systems and presumably

triggers long lasting changes in cell physiology (reviewed in Seeburg, 1993; Dingledine *et al.*, 1999).

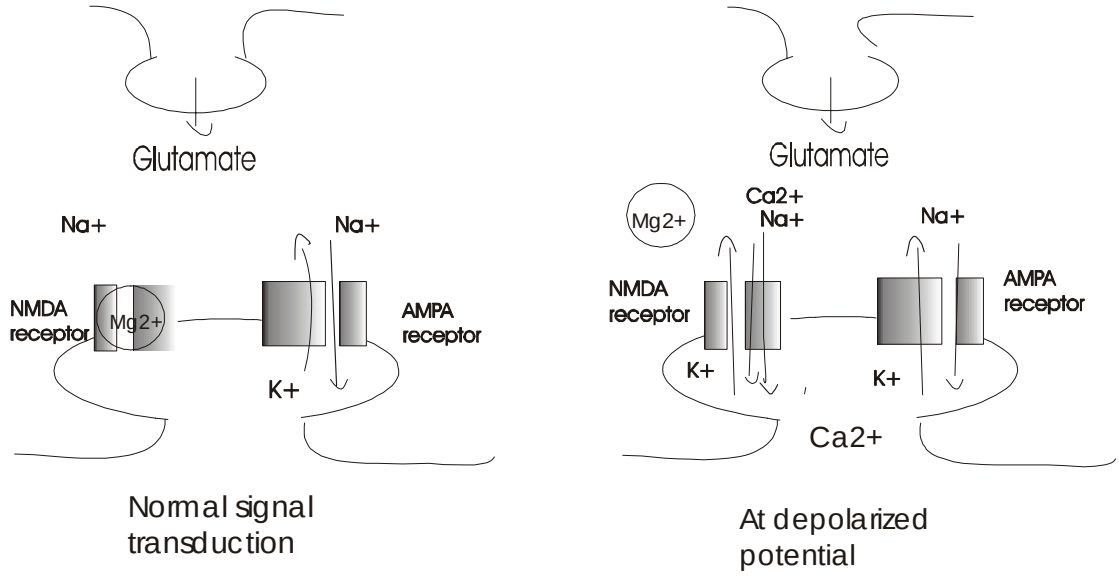

Figure 1. Schematic presentation of the roles of AMPA and NMDA receptor in excitatory synaptic transmission. Most normal synaptic transmission is mediated via AMPA receptors. During normal transmission membrane potential is only slightly depolarized and NMDA receptors are blocked by voltage sensitive Mg^{2+} ions. Frequent AMPA receptor activation leads to depolarization of membrane potential, which removes Mg^{2+} block from NMDA receptors. This leads to elevation of intracellular Ca^{2+} and subsequent activation of intracellular messenger systems (Adapted from Seeburg *et al.*, 2001).

1.1.3 Synaptic plasticity

The definition of synaptic plasticity is long lasting changes in the synaptic properties, which may occur either during development or during adult life. AMPA and NMDA receptors have their own role in synaptic plasticity. The so-called use dependent long-term potentiation of synaptic strength (LTP) has been widely studied, since this phenomenon is thought to be similar to the elementary mechanism underlining learning and memory. LTP has been studied in the hippocampus, a brain region known to be important for the formation of new memories. Three separate phases of LTP can be distinguished; induction, expression and maintenance (reviewed in Abel and Lattal, 2001). One much studied form of LTP can be generated by a brief (1s) high-frequency (100Hz) stimulation of presynaptic hippocampal CA3 pyramidal cells, and recorded from postsynaptic pyramidal neurons in the CA1 region. After this induction, the excitatory post synaptic response (EPSPs) of CA1 cells is increased to constantly maintained level following the stimulus. The induction of LTP has been shown to require NMDA receptor function, transient elevation of intracellular Ca^{2+} levels and subsequent activation of calcium/calmodulin kinase II (CaMKII) (reviewed in Sanes and

Lichtman, 1999). AMPA receptors seem to be responsible for the enhanced synaptic currents in the expression phase of LTP, perhaps due to changes in the number of postsynaptic AMPA receptors (reviewed in Malinow and Malenka, 2002). AMPA receptors are also thought to be involved in another type of use-dependent synaptic plasticity, long-term depression (LTD), which decreases synaptic strength in a use-dependent manner. Interestingly, LTD also involves Ca^{2+} mediated second messenger systems and activation of several kinases. However, the decrease in synaptic transmission (LTD) is thought to be a result of reduction in the AMPA receptor number in the postsynaptic membrane.

1.2 AMPA receptor ligands

1.2.1 Design of ligands

The design of new glutamate receptor ligands aims at development of subtype selective agonists and antagonist to be used for clinical purposes and for basic studies on receptor structure and function. By definition, an agonist is a compound that produces receptor activation, whereas an antagonist is a compound that inhibits the response to an agonist. The groups of AMPA receptor ligands considered to have the most potential use for clinical use are competitive antagonists and allosteric modulators.

Accumulation of glutamate in the synaptic cleft can be toxic to cells. In situations like hypoxia or hypoglycemia an excessive amount of glutamate is released at the same time as active uptake of glutamate (mainly by glia cells) is disturbed. This causes accumulation of glutamate and over activation of glutamate receptors, which eventually leads to neuronal death (Choi, 1988). The glutamate excitotoxicity also causes problems in situations like brain trauma or ischemic stroke induced by artery occlusion. A number of studies show that glutamate excitotoxicity can be prevented by inhibition of receptor activity by using antagonists or negative allosteric modulators (reviewed in Lees, 2000). In addition, animal studies with AMPA receptor antagonists have shown neuroprotection in ischemic stroke models (reviewed in Lees, 2000; Nikam and Kornberg, 2001). Glutamate excitotoxicity also seems to be involved in chronic neurodegenerative diseases like Parkinson´s disease, Huntington´s disease, Alzheimer´s disease and multiple sclerosis. Therefore these situations are considered plausible targets for treatment with AMPA receptor antagonists (Krogsgaard-Larsen *et al.*, 1996; Lees, 2000).

On the other hand, positive modulators of AMPA receptor function (´ampakines´) have been used to promote LTP and improve encoding of memory (Lynch, 1998). They are

considered as potential drugs for the treatment of different forms of dementia and schizophrenia, and some of these have also been studied in clinical trials (reviewed in Lees, 2000).

However, many problems related to the specificity, selectivity and the side effects of the glutamate receptor-targeted drugs will still have to be solved. For the time being no glutamate receptor-targeted drugs are used for clinical purposes (reviewed in Lees, 2000).

1.2.2 Agonists

L-glutamate (Fig. 2) is a natural agonist for all glutamate receptors in CNS. In addition, the endogenous L- aspartate is able to activate NMDA receptors (Patneau and Mayer, 1990). Several other amino acids like ibotenic acid, kainate, quisqualate, and willardiine are synthesized in plants and mushrooms, which act selectively on AMPA and kainate receptors (Krogsgaard-Larsen *et al.*, 1996; Fig. 2). The structures of L -glutamate and the other naturally occurring compounds have been systematically varied in order to design new highly selective glutamate receptor agonists and antagonists. A key feature of all these glutamate receptor agonist structures is that they have an α-aminocarboxylate core and a distal anionic group (Watkins *et al.*, 1990; Fig.2). Since the structure of the agonists seems to be conserved, it is throught that the binding site of the ligand will also have structures that are conserved thoughout the iGluR family. In addition, the AMPA receptor shows strict stereo-selectivity for the agonists, since only L-enantiomers of the amino acid-like ligands can activate the receptor. On the other hand, NMDA receptor can also be activated by NMDA (Fig. 2) and they bind co-agonists glycine or D-serine (see chapter 1.3.5.2).

The structure of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was designed based on naturally occurring ibotenic acid (Fig. 2). The introduction of AMPA facilitated distinction of AMPA and kainate receptors, and the further characterization of AMPA receptor pharmacology (Krogsgaard-Larsen *et al.*, 1980). This highly selective agonist has a nanomolar affinity to AMPA receptor (Sommer *et al.*, 1990). Despite the overall high selectivity, high concentrations of AMPA can activate some kainate receptor subunit combinations (Table I; Herb *et al.*, 1992; Swanson *et al.*, 1996; Schiffer *et al.*, 1997). On the other hand, kainic acid i.e. kainate (Fig. 2.) is a relatively nonselective agonist that has only a micromolar affinity to AMPA receptors (Boutler *et al.*, 1990). However, the ability to induce large non-desensitizing currents at AMPA receptors makes it a potent neurotoxin (Lambolez *et al.*, 1991). At AMPA receptors, the rank order of the agonist potencies is; quisqualate

>AMPA>glutamate>kainate. For a typical kainate receptor, the rank order is; kainate> quisqualate>glutamate>AMPA (reviewed in Hollmann & Heinemann, 1994).

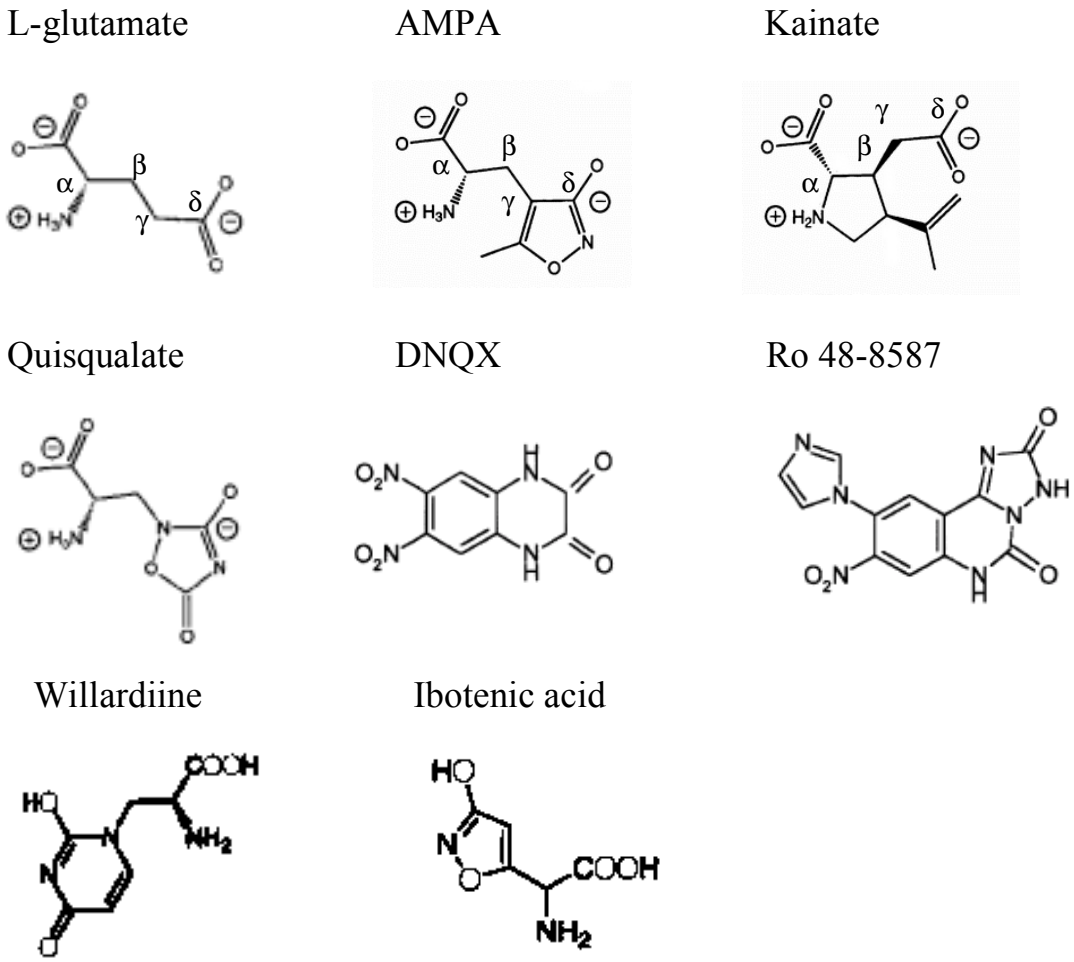

Figure 2. Structures of some naturally occuring and synthetic amino acids showing effect on AMPA receptors. The functional groups involved in agonist binding (α-amino, α-carboxyl and the distal anionic group) are shown on glutamate, AMPA and kainate. (Adapted from Bräuner-Osborne *et al.,* 2000)

The structures of AMPA and willardiine (Fig. 2.) have been used as the lead in attempts to design compounds that could select between AMPA and kainate receptors. A number of AMPA derivatives show a potent agonist and antagonist effects on AMPA receptors (Bräuner-Osborne *et al.*, 2000), and the derivatives of willardiines comprise the second major group of AMPA receptor agonists (Patneau *et al.,* 1992). Willardiine derivatives include a selective AMPA receptor agonist (S)-fluorowillardine as well as selective kainate receptor agonists (S)-iodo- and bromowillardiines (Swanson *et al.*, 1998; Wong *et al.*, 1994; Jane *et al.*, 1997). Ibotenic acid (Fig. 2.) has been the lead compound for designing homoibotenic acid (HIBO) and 2-amino-3-(4-bromo-3-hydroxyisoxazol-5-yl) propionic acid (Br-HIBO) (Krogsgaard-Larsen, *et al.,* 1980). The (S)-enantiomer of HIBO is a potent AMPA

receptor agonist, whereas (R)-HIBO is a weak NMDA receptor agonist (Krogsgaard-Larsen *et al.*, 1996). Interestingly, Br-HIBO has shown discrimination between different AMPA receptor subunits (Nielsen *et al.*, 1998).

1.2.3 Antagonists

A competitive antagonist prevents receptor activation by displacing the agonist compounds. One group of AMPA receptor antagonists is the quinoxaline-2,3-dione compounds, which have high affinity and selectivity (Nikam *et al.,* 1999; reviewed in Nikam and Kornberg, 2001; Lees, 2000). 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Fig. 2) were the first compounds developed in this series and introduction of these antagonists facilitated the discrimination AMPA and kainate receptors from NMDA receptors. However, CNQX and DNQX are almost equally effective at AMPA and kainate receptors. In addition, most quinoxaline-2,3-diones have comparatively high affinity to the glycine-binding site of NMDA receptors (reviewed in Lees, 2000).

One derivative of quinoxalinedione structure is 9-midazol-1-yl-8-nitro-2,3,5,6-tetrahydrotriazolo (Ro 48-8587) which is a relatively new and potent AMPA receptor antagonist (Fig. 2). This compound shows 100-fold selectivity for AMPA over kainate receptors (Mutel *et al.*, 1998). A number of antagonists are acidic amino acids having longer backbone than generally found in glutamate receptor agonists. This group includes a number of tricyclic compounds and derivatives of AMPA, which are unselective AMPA or kainate receptor antagonists (Krogsgaard-Larsen *et al.*, 1991; Wahl *et al.*, 1992; reviewed in Lees, 2000). For example, (R,S)-2- amino-3-[5tertbutyl-3-(carboxymethoxy)-4-isoxazolyl]propionic acid (ATOA) and (R,S)-2-2amino-3-[5tertbutyl-3-(phosphonomethoxyl)-4-isoxazolyl (ATPO) are AMPA receptor inhibitors, but they also act as partial agonists for some kainate receptors (Wahl *et al.*, 1998).

1.2.4 Allosteric modulators

Allosteric modulators are compounds that modulate receptor activation by binding to a site distinct from the agonist site. These are either positive modulators, which enhance receptor activity, or negative modulators, which inhibit receptor activation.

The 2,3-benzodiazepines such as GYKI 52466 and GYKI 53655 are selective inhibitors of AMPA receptor responses with minimal effects on kainate receptors (Bräuner-Osborne *et al.*, 2000). Binding of the GYKI compounds does not compete with the binding of positive allosteric modulators aniracetam and cyclothiazide (Johansen *et al.*, 1995; Partin *et*

al., 1996). Recently, a novel AMPA receptor allosteric antagonist CP-526,427 has been described which competes with the 2,3-benzodiazepine compounds and has a high nanomolar affinity to AMPA receptors (Menniti *et al.*, 2000).

Most of the positive allosteric modulators of AMPA receptor function belong to two structural groups; thiazide derivatives (cyclothiazide [CTZ] and diazoxide) and benzoylpiperidine derivatives (aniracetam, benzoylpyrrolidines), also known as "ampakines". These compounds seem to extend the duration of channel activation by blocking desensitization (Vyklicky *et al.*, 1991). CTZ is a potent and extensively studied positive modulator, which shows remarkable selectivity for so called the flip-splice variants (see chapter 1.3.3.) of the AMPA receptor subunits (Partin *et al.*, 1994; 1995). Aniracetam (a benzoylpyrrolidine) is less potent than CTZ and potentiates the glutamate-induced currents more efficiently in the flop-forms rather than in the flip-forms (Johansen *et al.*, 1995). Further derivatives of aniracetam, such as 4-[phenylsulfonylamino)ethylthiol]-2,6-difluoro-phenoxyacetamide (PEPA) is more potent and selective for the flop-splice variant to varying degrees (Sekiguchi *et al.*, 1997). Some derivatives of aniracetam are studied because of their ability to enhance memory and ability to enter the central nervous system in an active form (reviewed in Lynch, 1998). Potassium thiocyanate (KSCN) is another positive allosteric modulator of AMPA receptor current responses, and it is also known to enhance AMPA binding affinity in radioligand-binding assays (Honore and Drejer, 1988). The affinity effect is likely due to the KSCNs ability to convert receptor into desensitized state (Bowie´and Smart, 1993). It has been suggested that CTZ and KSCN, which have opposite effects on AMPA receptor desensitization, compete in binding to the same site (Donevan and Rogawski, 1998).

Open channel blockers are compounds that inhibit receptor activation by binding directly to the channel, after activation of the receptor has made the binding site available. This group includes natural polyamines and the derivatives found in the venom of arthropods. The argiotoxin and joro toxin selectively blocks the ion flow through AMPA receptors (Herlitze *et al.*, 1993; Iino *et al.*, 1996). The binding of these toxins and of the endogenous cytoplasmic polyamines spermidine and spermine, are dependent on the structure of ion channel forming segment M2 (Herlize *et al.*, 1993; Washburn and Dingledine, 1996; see chapter 1.3.5.3).

1.3 Stucture of the iGluRs

1.3.1 Oligomeric structure of the receptor

Early purification and immunoprecipitation studies with native glutamate receptors showed that, like other ion channels, glutamate receptors are multimeric proteins consisting of several subunits (Chang *et al.*, 1991; Wenthold *et al.*, 1991; 1996; Blackstone *et al.*, 1992). In addition, determination of the hydrodynamic size of the purified preparations by centrifugation through sucrose gradient and studies using chemical cross-linking of subunit structures suggested that the native receptors consist of four or five subunits (Brose *et al.,* 1993; Blackstone *et al.*, 1992; Wenthold *et al.*, 1991; Kuusinen *et al.*, 1999). More recently, an analysis of purified recombinant receptors either using subunit or tag specific antibodies or by electron microscopic imaging, yielded results consistent with pentameric or tetrameric structure (Hawkins *et al.*, 1999; Safferling *et al.,* 2001). Moreover, electrophysiological analysis of receptor combinations of the wild-type GluR-A and subunits carrying a sensitivity marker for a channel blocker suggested a pentameric structure (Ferrer-Montiel and Montal, 1996). Measurements of different single-channel current patterns of mixtures containing wild-type subunits and mutated subunits, which have specific subconductance level, also suggested pentameric structure (Premkumar and Auerbach, 1997). However, most of the recent studies favour tetrameric stoichiometry for AMPA and NMDA receptors. Studies following dose-response curves of activation of mutant and wild-type mixtures of NMDA or GluR-A subunits, have suggested tetrameric structure (Laube *et al.*, 1998; Mano and Teichberg, 1998). Rosenmund *et al.* (1998) used chimeric non-desensitizing AMPA subunit mutants (GluR6/GluR-C) and assumed that the distinct electrophysiological states depended on how many of the receptor binding sites have an agonist bound. They further assumed that occupancy of the two binding sites is necessary for the channel opening and thus suggested that the three measured conductance states implied four sites for agonist binding, one in each subunit of the tetrameric assembly. Another functional analysis of chimeric AMPA receptor subunits (GluR-A/GluR-6) indicated distinguishable responses of the different combinations of heteromeric receptors suggesting that a tetrameric assembly is formed through dimerization of subunit monomers (via N-terminal domain) followed by dimerization of two subunit dimers (Ayalon and Stern-Bach, 2001).

1.3.2 Receptor subunits

The first glutamate receptor subunit was cloned by injection of the rat brain mRNA into *Xenopus* oocytes, and by probing the expression by measuring electrophysiological responses (Hollmann *et al.*, 1989). The first isolated subunit was initially named as GluR-K1 and later shown to form an AMPA-selective receptor and renamed GluR1 or GluR-A (Keinänen *et al.*, 1990; Boutler *et al.*, 1990). The subsequent cloning and characterization of other iGluR subunits supported the previous pharmacological classification of receptors into three subfamilies (Table I). Currently, 14 iGluR subunits are known, which are considered to belong to AMPA, kainate or NMDA receptor subfamily.

The iGluRs form a receptor superfamily of their own, unlike initially suggested. The iGluR subunits are distinct from subunits of nicotinic acetylcholine receptor, γ-aminobutyric acid receptor$_A$, glycine receptor and serotonin receptor by size, the conserved sequence patterns and by transmembrane topology (1.3.4; reviewed in Sprengel and Seeburg, 1993). The subunits of same subfamily (AMPA, kainate and NMDA) have higher amino acid identity with each other than with subunits of a different subfamily (Fig. 3). The amino acid sequence identity between AMPA receptor subunits (GluR-A –D) is 68-78 %, whereas identity to kainate receptor subunits is only ~40 % (Keinänen *et al.*, 1990). A number of expression studies have shown that iGluRs are always assembled from subunits belonging to the same subfamily. Functional AMPA receptors are homomeric or heteromeric assemblies from subunits GluR-A-D (Keinänen *et al.*, 1990; Nakanishi *et al.*, 1990) (Table I). Functional homo- or heteromeric kainate-selective receptors are assembled from GluR5-7 (known as ´low affinity´ subunits), whereas KA1 and KA2 subunits (known as ´high-affinity´ subunits) can assemble into functional channels only when GluR5-7 subunits are co-expressed (Table I) (Bettler *et al.*, 1992; Werner *et al.*, 1991). In turn, functional NMDA receptors are composed of assemblies of the NR1 together with NR2A-D subunits (reviewed in Hollman & Heinemann, 1994a)(Table I). However, the NR1 subunits have been shown to form functional receptors in *Xenopus* oocyte expression, although functional homomeric channels have not been yielded in mammalian cell line expression (Moriyoshi *et al.*, 1991).

Table I. Subunits and characteristics of iGluR channels

Receptor Family	Subunit	Alternative nomenclature	RNA editing	Alternative splicing	Functional characteristics
AMPA	GluR-A, α1	GluR1		Flip/flop	Rapid kinetics, low Ca^{2+} permeability, Glu activates desensitizing current, KA activates non-desensitizing current (Boutler, *et al., 1990;* reviewed in Fletcher and Lodge, 1996)
AMPA	GluR-B, α2	GluR2	Q/R,R/G	Flip/flop, C-sp	
AMPA	GluR-C, α3	GluR3	R/G	Flip/flop	
AMPA	GluR-D, α4	GluR4	R/G	Flip/flop, C-sp	
Kainate	GluR5	β1	Q/R	N-sp, C-sp	Rapid kinetics, Glu and KA activate rapidly desensitizing current (reviewed in Fletcher and Lodge, 1996). AMPA do not activate homomers of GluR6 or GluR7, but heteromeric GluR6/KA2 and GluR7/KA1 or GluR7/KA2 receptors produce slowly desensitizing currents (Egebjerg , *et al.,* 1991; Shiffer, *et al.,* 1997; Herb, *et al.,* 1992).
Kainate	GluR6	β2	Q/R,I/V Y/C	C-sp	
Kainate	GluR7	β3		C-sp	
Kainate	KA-1	γ1			
Kainate	KA-2	γ2			
NMDA	NR1	ζ1		N-sp, C-sp	Slow kinetics, large conductance, little desensitization, high Ca^{2+} permeability, voltage dependent Mg^{2+} block, glycine as co-agonist (reviewed in McBain and Mayer, 1994).
NMDA	NR2A	ε1			
NMDA	NR2B	ε2			
NMDA	NR2C	ε3			
NMDA	NR2D	ε4			
	NR3A				Chatterton *et al.,* 2002
	NR3B				
Orphan	δ1				Do not form functional channels by themselves
Orphan	δ2				

KA = kainic acid, Glu = glutamate, Flip/flop = 38 aminoacid cassette, C-sp = C-terminal splicing, N-sp = N-terminal splicing. AMPA receptor subunits GluR-A-D (Keinänen *et al.,* 1990; Hollmann *et al.,* 1989; Boutler *et al.,* 1990; Nakanishi *et al.,* 1990). GluR5-7 subunits (Bettler *et al.,* 1990; 1992; Egebjerg *et al.,* 1991, Sommer *et al.,* 1992; Lomeli, *et al.,* 1992). KA1-KA2 subunits (Werner *et al.,* 1991; Herb *et al.,* 1992; Sakimura *et al.,* 1992). NR1-NR2 subunits (Moriyoshi, *et al.,* 1991; Merugo, *et al.,* 1992; Monyer, *et al.,* 1992; Ymazaki, *et al.,* 1992. ε1-ε4 subunits (Kutsuwada, *et al.,* 1992; Meguro, *et al.,* 1992; Ikeda, *et al.,* 1992). NR3A-B subunits (Ciabarra *et al.,* 1995; Chatterton *et al.,* 2002). δ1- δ2 subunits (Lomeli *et al.,* 1993).

Two orphan subunits δ1 and δ2, cloned from rat and mouse, have only a low level of amino acid identity to other glutamate receptors (Fig. 3.) and are not able form functional channels (Yamazaki *et al.*, 1992; Lomeli *et al.*, 1993). Even more peculiar are the relatively recently found NR3A-B subunits that also have only low sequence identity to other iGluR subunits (Table I). In co-assembly with NR1 subunits they appear to form a completely new type of excitatory glycine receptor (Chatterton *et al.*, 2002). Glutamate receptors have also been found in the plant *Arabidopsis thaliana* and bacteria *Synechocystis* (Lam *et al.*, 1998; Chen *et al.*, 1999). The bacterial glutamate receptor (GluR0) resembles structurally and functionally both mammalian iGluRs and potassium channels. This receptor binds glutamate, but forms a potassium selective channel (Chen *et al.*, 1999).

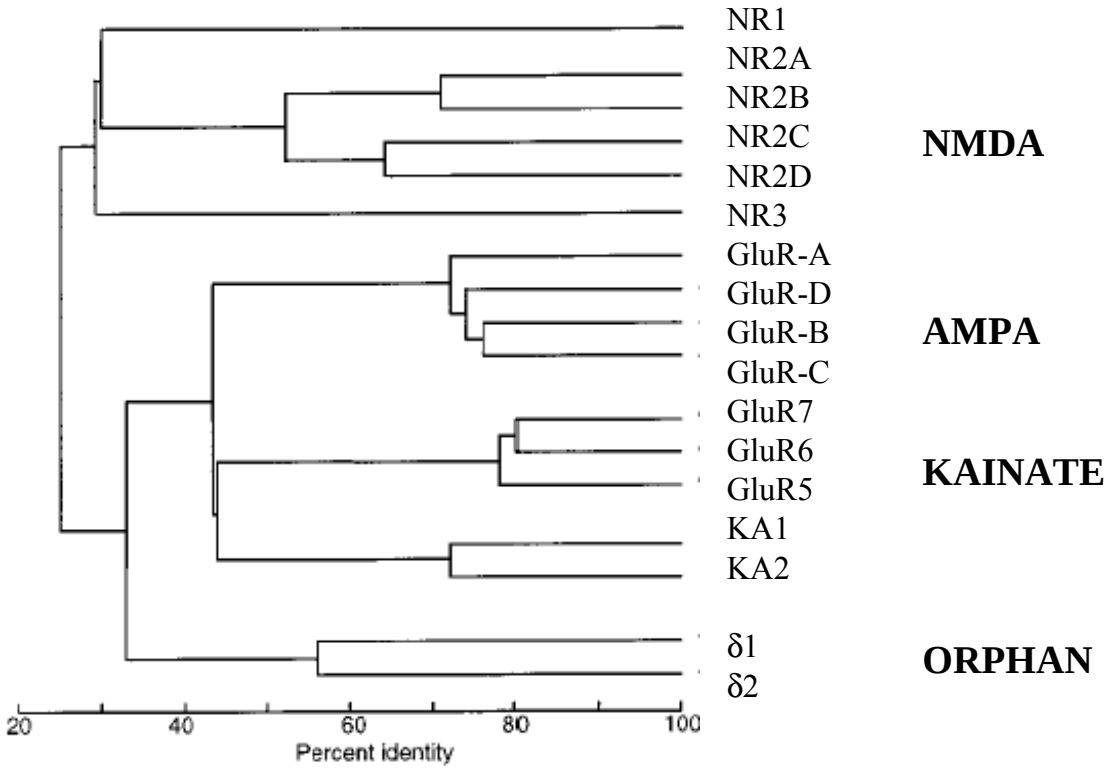

Figure 3. Phylogenetic tree showing the sequence identity between the cloned mammalian glutamate receptor subunits (Adapted from Bräuner-Osborne *et al.*,, 2000).

The sizes of AMPA receptor subunits, GluR-A-D, are 881-889 amino acids when signal peptides are taken into account (Keinänen *et al.*, 1990; Hollmann *et al.*, 1989; Boutler *et al.*, 1990; Nakanishi *et al.*, 1990). The kainate receptors GluR5-7 and the KA1-KA2 subunits and the NMDA receptor subunit NR1 are about same size. In contrast, NMDA receptor NR2 subunits are about 1300 residues long (reviewed in Hollmann & Heinemann,

1994). The sequence of all iGluR subunits contains an N-terminal hydrophobic signal sequence and four hydrophobic segments (19-27 amino acids) that most likely form three membrane-spanning α-helices (M1, M3 and M4) and one membrane re-entrant loop (M2) (Fig. 4). The N-terminal half of the AMPA receptor subunits (~400 amino acids) are much less conserved than the sequence of the C-terminal half of the subunits. The N-terminal segment has several potential consensus sites for N-glycosylation (4-6 in AMPA subunits, see Fig. 4.; Keinänen *et al.*, 1990; Everts *et al.*, 1997), whereas the C-terminal half of the AMPA subunits has several consensus sites for phosphorylation (Roche *et al.*, 1996).

1.3.3 Molecular diversity

A number of expression studies have shown that a large diversity in the functional properties of glutamate receptors can be achieved by simply varying the subunit composition of the receptors (reviewed in Hollmann & Heinemann, 1994). In the native receptors subunit composition can be regulated at gene expression level to meet the demands of the physiological role of the cell. The functional properties of iGluRs are further increased by processing the genome encoded transcription products by alternative splicing or RNA-editing (Table I).

Co-expression studies have shown that the AMPA receptor Ca^{2+} permeability properties are dominated by the GluR-B subunit. Receptors composed of GluR-A, -C and -D subunits are highly permeable to Ca^{2+} and show inward rectification. Incorporation of even one GluR-B subunit in the receptor assembly results in formation of Ca^{2+} impermeable channels with a linear current-voltage (I-V) relationship (Hollmann *et al.,* 1991; Verdoorn *et al.,* 1991; Burnashev *et al.,* 1992; Bowie and Mayer, 1995). The finding that most of the native AMPA receptors have the same characteristics as recombinant receptors containing GluR-B subunits, has supported the suggestion that nearly all native AMPA receptors are assemblies of GluR-B subunit with the GluR-A, -C or -D subunits (Westbrook and Mayer, 1987; Partin *et al.,* 1993; Jonas & Burnashev, 1995). On the other hand, a recent co-expression study of recombinant GluR-A and -B subunits has suggested that the subunits are not assembled randomly (Mansour *et al.,* 2001). By studying conductance properties of the subunits carrying permeation and conductance markers as function of relative expression level, the assembly of two GluR-A subunits together two -B subunits was shown to be more favoured than the other assemblies (Mansour *et al.,* 2001).

All AMPA receptor subunits have alternative segments, which affect the receptor function. The AMPA receptor subunits have two different sites that can be modified by alternative exons; the flip/flop-cassette and the C-terminal splice cassettes (Fig. 4). The flip/flop region (between M3 and M4 membrane-spanning segments) is found in all GluR-A-D subunits (Sommer *et al.,* 1990); two alternative exons encode the 38 amino acid flip/flop-cassettes, which differ by only 9-11 residues. The flip/flop-variants have slight differences in ligand-binding properties (Nakanishi, 1992), different desensitization kinetics (Moscbacher *et al.,* 1994) and different sensitivity to desensitization modulators CTZ (Partin *et al.,* 1995; 2001) and PEPA (Sekiguchi *et al.,* 1997). The second splicing site occurs in the C-terminal domain of the GluR-B and -D subunits, due to which their C-terminal tails contain either 36 or 54 residues (Köhler *et al.,* 1994; Gallo *et al.,* 1992).

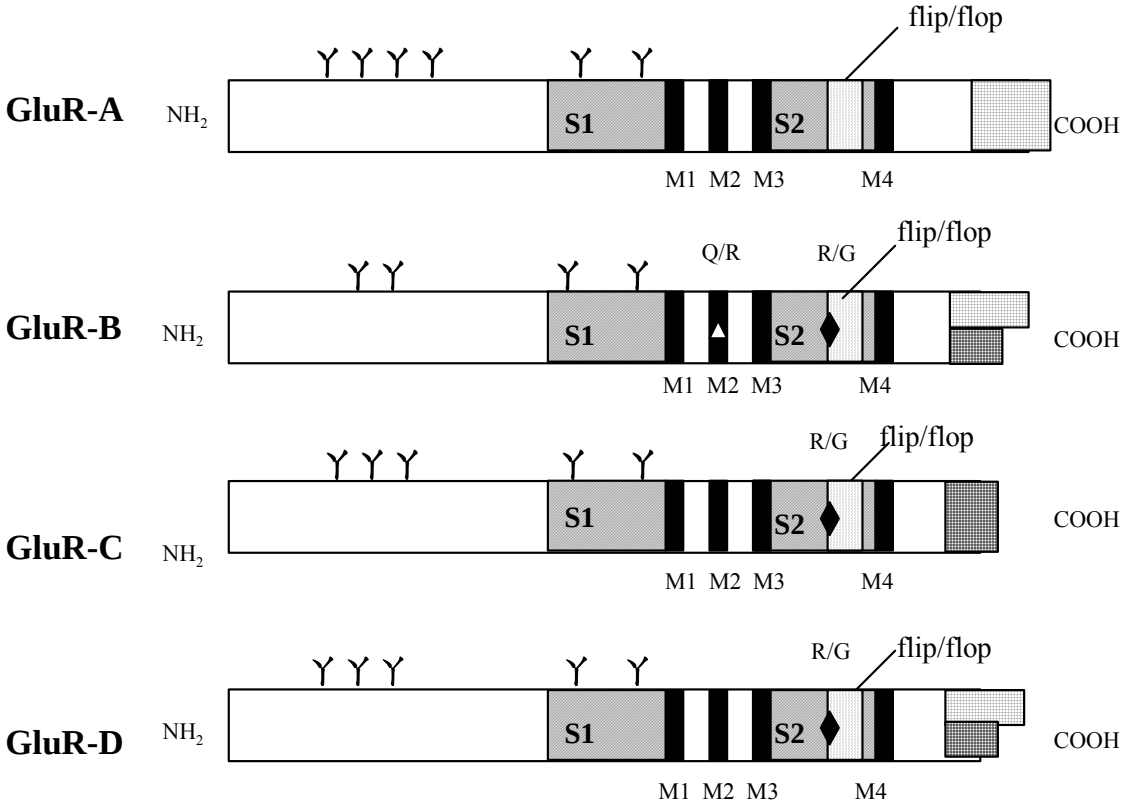

Figure 4. Schematic presentation of AMPA receptor subunits. The size of the GluR-A-D subunits are ~900 amino acids. All subunits contain 4-6 potential sites for N-glycosylation (ϒ) and four hydrophobic membrane segments M1-M4 (black boxes).The ligand binding domain regions S1 and S2 are indicated by shading. Different splice variant forms of subunits are shown. AMPA receptors may have flip/flop and C-terminal (different sized boxes in C-terminus) splice variants. The location of residues altered by RNA-editing are at the Q/R- (△)and the R/G (⧫).

RNA editing is another mechanism, by which the genome encoded sequence of AMPA receptor subunits are altered. The sequence of the pre-RNA is changed by deamination of adenosine to inosine (dsRNA adenosine desaminase), which leads to a change in one amino acid codon in the protein translation (Higuchi *et al.,* 1993; Liu *et al.,* 1999). The AMPA receptor pre-mRNA can be modified independently at two sites (Fig. 4). The first site is found in the second membrane segment (M2) of the GluR-B subunits only (Sommer *et al.,* 1991). Substitution of a glutamine with an arginine (Q/R-site) affects the Ca^{2+} permeability properties of receptors containing GluR-B subunits, as discussed above. The GluR-A, -C and -D subunits occur only in the genomic encoded form (Sommer *et al.,* 1991). The second site affected by RNA editing preceds the flip/flop area of GluR-B-D subunits (Fig. 4). Edition at this so-called R/G-site leads to substitution of an arginine to a glycine and affects the recovery of receptor from desensitization (Lomeli *et al.,* 1994).

1.3.4 Membrane topology

Membrane topology studies have tried to characterize which segments of the receptor proteins are extra- and intracellular and which segments are embedded in the plasma membrane bilayer. Initially, the finding that the subunits contain a N-terminal signal peptide and four suitable length hydrophobic segments led to a suggestion that subunits have four membrane-spanning α-helices like the members of the ligand-gated superfamily (reviewed in Sprengel and Seeburg, 1993). Currently, the revised model suggests three transmembrane (M1, M3 and M4) segments together with one re-entrant membrane segment (M2). Accordingly, the N-terminal domain and region the between M3 and M4 are suggested to be extracellular, whereas the C-terminal domain is thought to be intracellular.

Evidence for the extracellular localization of the N-terminal domain was provided by studies showing the presence of N-glycosylation of endogenous or mutated residues and its effect on receptor size. Also tunicamysin was used in order to show prevention of N-glycosylation (Hollmann *et al.,* 1994; Wood *et al.,* 1995; Kawamoto *et al.,* 1995a,b). In addition, clear indications of N-glycosylation on endogenous and introduced N-glycosylation sites have been observed in the segment between M3 and M4 in the gold fish kainate-binding protein and in the GluR-A (Roche *et al.,* 1994; Hollman *et al.,* 1994b; Wo and Oswald, 1994; 1995a). The intracellular localization of the C-terminal domain has also been indicated, since the C-terminal domain of all AMPA receptor subunits have potential target sites for proteine kinase C (PKC), proteine kinase A (PKA) and Ca^{2+}-and calmodulin dependent protein kinase II (CAMKII), and the receptor activity has been shown to be potentiated by these kinases

(reviewed in Smart, 1997). These findings were in conflict with the originally postulated four membrane-spanning segments based on the hydropathy analysis of subunit amino acid sequence. This controversy was solved by a study, which showed that deletion of the M2 segment did not affect glycosylation of the region between M3 and M4. The data was interpreted to suggest that the relatively hydrophilic M2 segment forms a re-entrant loop instead of membrane-spanning α-helix (Wo and Oswald, 1994; 1995a). Further support was given by a study of the accessibility of the pore lining segments of the AMPA and NMDA receptors to sulfhydryl reagents. The pattern of accessibility and the accessibility of the M2 segment to sufhydryl regents from both sides of the membrane revealed that the stucture of M2 might be similar to the structure of the P-loop of K^+ channels (Kuner *et al.,* 1996; 2001, see chapter 1.3.5.3.).

1.3.5 Structural domains

The sequence alignment of iGluR subunits with other proteins has revealed that the iGluRs have regions with low but significant sequence identity to bacterial proteins (Nakanishi, 1990). Based on these sequence similarities, iGluR subunits can be sectioned into an N-terminal domain (O´Hara, 1993), a ligand-binding domain (Stern-Bach, *et al.,* 1994; Kuryatov *et al.,* 1994; Kuusinen *et al.,* 1995) and a membrane channel (Wood *et al.,* 1995; Chen *et al.,* 1999) (Fig. 5a and 5b). The intracellular C-terminal domain has not been found to have significant sequence identity with any bacterial protein. The modular structure of iGluR subunits suggests that the independent domains are associated with a certain function, which may be similar to the function of the homologous prokaryotic proteins. The multidomain structure of iGluRs, like that of numerous other proteins, may have evolved from distinct ancestral genes by exon shuffling mechanism.

1.3.5.1 N-terminal domain (X-domain)

The amino acid sequence of the N-terminal ~400 residues of iGluR subunits (X-domain) has distant sequence similarity to the N-terminal domain of the mGluRs and to the bacterial leucine (LBP) and the leucine/isoleucine/valine-binding protein (LIVBP) (O´Hara *et al.,* 1993) (Fig. 5a). This ´LIVBP-like´ domain also shows resemblance to the N-terminal domain of guanylate cyclases and atrial natriuretic peptide receptors (O´Hara *et al.,* 1993). Interestingly, the N-terminal domain is entirely absent from the kainate-binding proteins (KBPs) of chick, frog and goldfish, which are otherwise similar to iGluR subunits (Gregor *et al*., 1989; Wo and Oswald, 1994). The recently identified glutamate-activated potassium

channel, ´GluR0´ also lacks the N-ternimal domain (Chen *et al.,* 1999). Since the X-domain is largely variable in amino acid sequence with and between subclasses of iGluRs, it may have subunit specific function.

The X-domain of AMPA and kainate receptor subunits does not seem to contribute to agonist binding properties of the receptor (Everts *et al.,* 1997; Kuusinen *et al.,* 1999), but it has been suggested to control the assembly of the subunits (Kuusinen *et al.,* 1999; Leuschner and Hoch, 1999; Ayalon and Ster-Bach, 2001). In the NMDA receptors, pH sensitivity, glycine-independent desensitization and the regulation of receptor function by compounds like polyamines, histamine, Mg^{2+} and Zn^{2+}, seem to localize or be associated with the X-domain (reviewed in Dingledine *et al.,* 1999; Zheng *et al.,* 2001).

1.3.5.2 Ligand-binding domain (S1S2).

Two separate extracellular regions of ~150 amino acids each, one (S1) preceding the first membrane segment, and the other (S2) locating between the M3 and M4 segments together have weak sequence similarity to the bacterial periplasmic glutamine (QBP) and the lysine/arginine/ornithine binding protein (LAOBP)(Fig. 5a; Nakanishi *et al.,* 1990; Hsiao *et al.,* 1996; O´Hara *et al.,* 1993). These segments also show sesequence similarity to KBPs of of chick, frog and goldfish. Studies with chimeric AMPA/kainate and NMDA receptor subunits have shown that S1 and S2 regions are important determinats of the ligand pharmacology (Stern-Bach *et al,* 1994; Kuryatov *et al.,* 1994). Further mutation studies have supported this view (Paas *et al.,* 1996; Swanson *et al.,* 1997b; Mano *et al.,* 1996). Moreover, expression of these segments as soluble S1S2 fusion protein has shown that these regions form a protein that can bind ligands with the same pharmacology as intact receptors (Kuusinen *et al.,* 1995). Furthermore, crystallization and structure determination of a similar AMPA receptor fusion protein confirmed the ligand-binding role of S1S2 and the structural relationship of iGluRs and PBPs (Armstrong *et al.,* 1998) (see chapter 1.4.1). More recently, functional characterization of the bacterial GluR0 channel (Chen *et al.,* 1999) and subsequent crystallization of the corresponding S1S2 –fusion protein, revealed that the 3-dimensional structure of the ligand-binding domain of iGluR and GluR0 subunits are highly similar (Mayer *et al.,* 2001).

In addition to ligand binding, the S1S2 contributes to AMPA receptor desensitization (see chapter 1.5.2) and redox modulation**.** Variation of the flip/flop segment in the C-terminal part of the S2 has been shown to affect receptor desensitization (Partin *et al.,* 1994; Partin *et al.,* 1996; Sekiguchi *et al.,* 1997). In addition, the so-called R/G RNA editing site, which

precedes the flip/flop region of the AMPA receptor subunits, is also involved in regulation of receptor desensitization (Lomeli *et al.,* 1994).

The S2 segment of all AMPA receptor subunits contains two Cys residues in corresponding to those (Cys-744 and Cys-798) in NMDA receptor NR1 subunit. In the NMDA receptors this conserved cysteine pair has been shown to be responsible for redox modulation, pH sensitivity and binding of Zn^{2+} (Sullivan *et al.,* 1994; reviewed in McBain and Mayer, 1994). However, AMPA receptors have been shown to be insensitive for redox modulation (Aizeman *et al.,* 1989; Terramani *et al.,* 1988). These cysteines have been suggested to be important in stabilization of the structure of the ligand-binding domain of the iGluRs (Sutcliffe *et al.,* 1996; see chapter 1.4.4).

In the NMDA receptors, a number of mutation analyses have shown that the S1S2 region of the NR1 subunit determines the binding of the co-agonist glycine, whereas the same region in the NR2 subunits determines the binding of glutamate (Kuryatov *et al.,* 1994; Hirai *et al.,* 1996; Laube *et al.,* 1997; Ivanovic *et al.,* 1998).

1.3.5.3 Transmembrane channel

The electrophysiological analysis has shown that the RNA editing Q/R-site (see chapter 1.3.3) in the M2 segment determines the Ca^{2+} permeability of the AMPA channels (Hollman *et al.,* 1991; Verdoorn *et al.,* 1991; Hume *et al.,* 1991; Burnashev *et al.,* 1992). This strongly suggests that the M2 segment participates in channel formation. All the AMPA receptor assemblies, which contain one or several GluR-B subunits are impermeable to Ca^{2+} and have linear steady state current-voltage relation, since the subunits of the GluR-B are edited almost 100% and carry the Arg residue at the Q/R-site (Hume *et al.,* 1991; Verdoorn *et al.,* 1991; Burnashev *et al.,* 1992; Dinglenine *et al.,* 1992). Due to the Gln residues at the Q/R-site of the GluR-A, -C and –D subunits, the receptors assembled from these subunits are highly permeable to Ca^{2+} and show inward rectification. The rectifying I-V relationship has been assigned to a block caused by intracellular polyamines, spermine and spermidine, which bind to the Gln at Q/R-site (Bowie and Mayer 1995; Washburn and Dingledine, 1996). Similarly, blockage of Ca^{2+} permeable AMPA receptors by argiotoxin has been assigned to the Gln residue at the Q/R site (Herlitze *et al.,* 1993; Meucci and Miller, 1998).

In addition, the M3 segment of AMPA receptors has been shown to affect gating properties of the receptors. The ´Lurcher mice´ have been found to be associated with spontaneous mutation of Ala to Thr at a highly conserved C-terminal segment of the M3 in the GluRδ2 subunit (Zuo *et al.,* 1997). The corresponding mutation at other AMPA and

kainate receptor subunits makes the receptors continuously active or renders them to be activated by CNQX, normally an antagonist (Zuo *et al.,* 1997; Taverna *et al.,* 2000; Schwarz *et al.,* 2001).

Sequence comparison of the predicted channel regions of the iGluRs and other channels has revealed that these regions have weak similarity to the pore regions of K^+ channels. This suggests that the ion channels of these proteins are composed of a similar module (Wood *et al.,* 1995). The M2 region of the iGluRs was suggested to form a re-entrant loop similar to the P-segment of voltage –gated K+ channels for which the tertiary structure was known from mutation analysis (Wood *et al.,* 1995; Wo and Oswald, 1995a,b). As mentioned, the accessibility profile of Cys-substituted residues of the M2 of the NMDA and AMPA receptors supported the view that the M2 segment forms an α-helical structure followed by random coil (Kuner *et al.,* 1996; 2001). Determination of the crystallographic structure of bacterial potassium channel (KcsA) from *Steptomyces lividans* confirmed that the K^+ channel subunit is formed from one re-entrant loop (P-segment) and two membrane-spanning segments (Doyle *et al.,* 1998). This led to a proposal that the membrane segments M1, M2 and M3 of the iGluR subunits correspond structurally to the channel forming regions of K^+channel subunits (Fig. 5b and 5c). In addition, expression cloning of the bacterial K^+ permeating glutamate receptor, GluR0, revealed that the regions which likely forms the channel of the GluR0 are similar both to the channel regions of other K^+channels and to the potential channel regions of mammalian iGluR subunits (Chen *et al.,* 1999). Thus, it was proposed that the GluR0 receptors have a re-entrant loop and two membrane-spanning segments similar to the K^+ channels, but the orientation of the channel segments would be inverted in the plane of the membrane (Chen *et al.,* 1999; reviewed in Miller, 2000). The iGluR subunits were proposed to contain a similar membrane core structure than K^+channel subunits plus one extra transmembrane segment (M4), which does not exist in the KscA or GluR0 proteins (Fig. 5c).

1.3.5.4 C-terminal domain

The C-terminal domain of the AMPA receptor subunits has no significant sequence similarity to any bacterial or mammalian proteins. The GluR-A subunit has a C-terminal domain of 81 amino acids, whereas the C-terminal domain of the GluR-C subunit is only 50 residues long (Keinänen *et al.,* 1990). The GluR-B and -D subunits have either 68 or 50 amino acids in their C-terminal domains due to alternative splicing (Köhler *et al.,* 1994; Gallo *et al.,* 1992). The C-terminal domain of kainate receptors are of similar size to GluR-A-D

subunits, whereas the C-terminal domain of the NMDA receptor NR2 subunits are significantly larger, ~ 620-640 amino acids (reviewed in Sprengel and Seeburg, 1993).

The C-terminal domain is cytoplasmic and has contacts with proteins of the postsynaptic density (PSD). The C-terminal domain of AMPA receptor subunits has several potential phosphorylation sites for PKA, PKC and CAMKII (Roche *et al.,* 1996), and is potentially subjected to phosphorylation and dephosphorylation. For example, phosphorylation of AMPA receptors by CAMKII has been shown to correlate with increased AMPA receptor activity during induced LTP (Barria *et al.,* 1997). In addition, phosphorylation is thought to regulate the interaction of the C-terminal domain with PSD-proteins, which contains PDZ domains (PSD-95/Dlg/ZO1). Variety of of such intracellular proteins has been shown by immunoprecipitation studies to interact with the C-terminal domain of the AMPA receptors (reviewed in Sheng and Sala, 2001; Malinow and Malenka, 2002). One of the first identified proteins from this group was glutamate receptor-interacting protein (GRIP), which is thought to be involved in AMPA receptor trafficking or in anchoring of the AMPA receptors at synapses (reviewed in Sheng and Sala, 2001). A recent nuclear magnetic resonance (NMR) study suggests that the structure of the C-terminal tail of the GluR-B is not stable in a solution, but is stabilized by interaction with the protein GRIP (Zhang *et al.,* 2001). Another protein containing one PDZ domain, the protein interacting with C-kinase (Pick1), is suggested to be involved in clustering of AMPA receptors (Xia *et al.,* 1999). In addition, AMPA receptors (GluR-A) have been shown to be associated with a SAP97 protein, which belongs to the different PDZ-domain family (PSD-95 family; reviewed in Sheng and Sala, 2001). The C-terminal domain is also known to bind to the N-ethylmaleimide-sensitive fusion protein, which is thought to be involved in membrane fusion events and participate in the insertion of recycled AMPA receptors into the plasma membrane Nishimune *et al.,* 1998; Ostein *et al., 1998).*

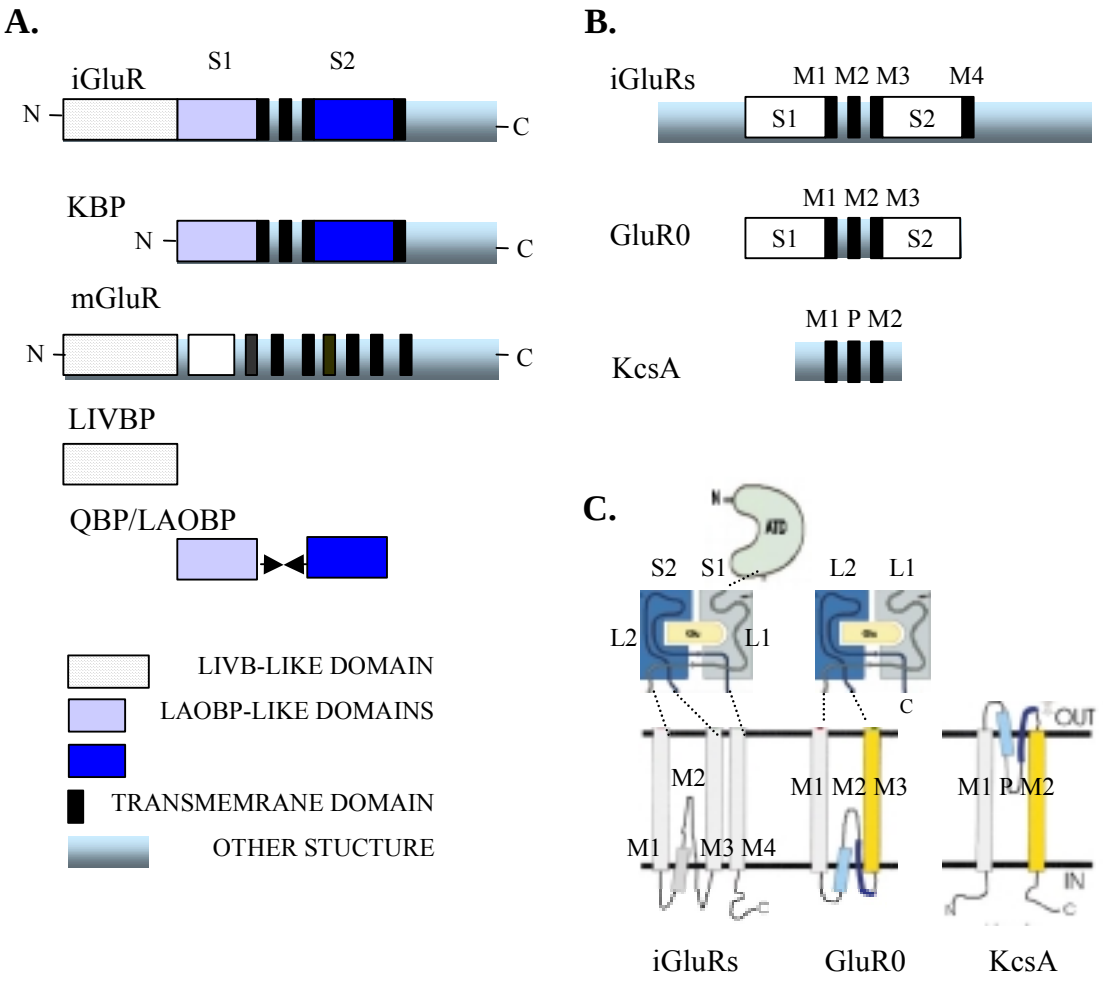

Figure 5. Illustration of the iGluR domain structure. **A**; The iGluR and mGluR extracellular N-terminal domains are related to bacterial periplasmic binding protein leucine/isoleucine/valine binding protein (LIVBP). The extracellular ligand-binding domains are constructed from S1 (light blue) and S2 (blue) segments, that are related to bacterial glutamine binding protein (QBP), lysine/arginine/ornithine binding protein (LAOBP) and to kainate binding proteins (KBP). **B;** Eukaryotic iGluRs have regions, which are related to the bacterial glutamate receptor GluR0 and to the bacterial potassium channel KcsA. The postulated channel regions M1, M2 and M3 of the iGluR subunits have sequence similarity to the M1 M2 and M3 of the GluR0 and to M1, P-segment and M2 of the KcsA. The extracellular ligand-binding domain S1S2 of the iGluR subunits is related by sequence to the ligand binding domain of the GluR0 subunit (Chen *et al.,* 1999). **C;** The predicted membrane topology of the eukaryotic iGluRs, prokaryotic GluR0 and prokaryotic KcsA channel. According to the crystal structure of the ligand-binding domains S1S2 of the iGluRs and GluR0, glutamate (yellow) is bound between the bilobed ligand-binding domain (Armstrong *et al.,* 1998; Armstrong and Gouaux, 2000; Mayer *et al.*, 2001). The schematic presentation of the binding domain shows that the S1 segment forms most of the lobe 1 (L1) and the S2 forms most of the lobe 2 (L2). In the iGluRs the S1 is connected to the N-terminal domain and the M1, whereas the S2 is flanking the M3 and M4 segments. The structure of the channel region of the KcsA is presented schematically based on the crystal structure (Doyle *et al.*, 1998). The KcsA has two transmembrane α-helical segments plus P-segment, which forms a re-entrant loop of an α-helical N-terminal structure followed by a C-terminal random coil structure. The 3-D structures of the iGluRs and GluR0 channel regions are suggested based on their sequence similarity to KcsA. (modified from Miller, 2000).

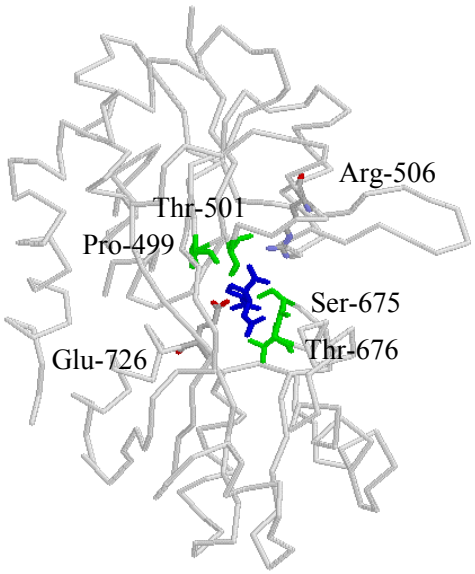

Figure 6. The crystal structure of the GluR-B ligand-binding domain (S1S2) solved by Armstrong, *et al.* (1998). The kainate agonist (in blue color) is bound between two lobes of the S1S2 ligand-binding domain. Residues that interact with all agonists are highlighted with green colour, but the Arg-506 and Glu-726 are colored to show the functional groups of the side-chain groups essential for agonist binding.

1.4 Crystal structure of the S1S2 ligand-binding domain

1.4.1 S1S2 fusion protein as a model for ligand binding and receptor function

The extracellular segments S1 and S2 have been used to construct soluble fusion proteins (S1S2) to be used in studies on ligand-receptor interaction of AMPA, kainate and NMDA receptors (Kuusinen *et al.,* 1995; Lampinen *et al.,* 1998; Keinänen *et al.,* 1998; Ivanovic *et al.,* 1998, Miyazaki *et al.,* 1999; Wells *et al.,* 2001). The segments of S1 and S2 are joined by a linker peptide, replacing the membrane segments M1-M3 of the intact receptor.

(Kuusinen *et al.,* 1995; Keinänen *et al.,* 1998) (Fig. 5b). These S1S2 fusion proteins structurally resemble bacterial amino acid binding PBPs, which also have two globular lobes connected by a flexible hinge peptides. The use of the S1S2 fusion proteins has made it possible to study agonist induced conformational changes of the ligand-binding domains in a variety of biochemical studies (Abele *et al.,* 1998; 1999; Jayaraman *et al.,* 2000). In addition, the affinity and kinetics of agonist binding have been studied by fluorescence stopped-flow techniques using these proteins (Abele *et al.,* 2000). Most importantly, an X-ray analysis of crystals of the GluR-B S1S2 fusion proteins in complex with ligands provided the first detailed three dimensional structure of a neurotransmitter receptor-ligand complex

(Armstrong *et al.,* 1998; 2000). Stable, crystallizable fusion protein was achieved by truncation of the original S1S2 fusion protein into S1S2I and S1S2J proteins, which could be refolded from inclusion bodies of *E. coli* (Chen and Gouaux, 1997; Chen *et al.,* 1998; Armstrong *et al.,* 1998).

1.4.2 General structure of the GluR-B ligand-binding domain

The crystal structures of the GluR-B S1S2I in complex with kainate and the S1S2J protein in complex with glutamate, AMPA and DNQX confirmed for the first time that the ligand-binding domain of iGluR was formed of two globular lobes (lobes 1 and 2) connected by two short stretches of peptides. As was previously predicted by sequence similarity (O´Hara *et al.,* 1993; Kuryatov *et al.,* 1994), this structure is overall very similar to the 3-D structure of the bacterial histidine binding protein (HBP) and LAOBP (reviewed in Adams nd Oxender, 1989 and Oh *et al.,* 1994a; Kang *et al.,* 1991; Oh *et al.,* 1993;). In both the bacterial binding proteins and the GluR-B S1S2 the amino acid ligand is bound in a cleft between the two lobes (Fig. 6). In GluR-B the lobe 1 is composed of the N-terminal part of S1 and a short C-terminal segment of S2, and the lobe 2 is composed mostly of the S2 segment and a short C-terminal segment of the S1 (Fig, 5c; Armstrong *et al.,* 1998). Both lobes are composed of a central core of the β-pleated sheet surrounded by α-helices. The lobe 1 is formed of six α-helices: A, B, C and D from the S1 segment, and J and K-helices, which are formed by the S2 segment. The lobe 2 forms α-helices E, F, G, H and I (Armstrong *et al.,* 1998).

The domain of GluR-B S1S2 with bound agonist is in a closed-cleft conformation, whereas the apo-state (unliganded) conformation has the two binding lobes located more distantly to each other (open-cleft conformation). Consistent with other studies, the structure showed that the closed-cleft conformation is stabilized by multiple hydrogen bonds, whereas the unliganded, open-cleft conformation is less stable (Arinaminpathy *et al.,* 2002; Madden *et al.,* 2001). In the agonist-bound structure, most of the stabilizing hydrogen bonds are formed between the binding lobes and the agonist. In addition, the residues making direct contacts with the agonist are in some cases stabilized by hydrogen bond from residues nearby. Moreover, the agonist-bound structures also are stabilized by hydrogen bonds between the two binding lobes (interdomain bonds), as well as by water molecules, which contribute to the network of interactions. On the other hand, the apo-state, open-cleft conformation, is stabilized by intradomain interactions (Armstrong *et al.,* 1998; Armstrong and Gouaux, 2000).

1.4.3 Direct ligand interactions

Both the iGluRs and PBPs have α-amino and α-carboxyl groups in their amino acid ligands. Therefore, it has been suggested (Kawamoto *et al.,* 1997; Laube *et al.,* 1997, Sutcliffe *et al.,* 1996) that the agonist-receptor contacts in the iGluRs are similar to the ligand interactions seen in the crystal structures of the PBPs (Kang *et al.,* 1991; Oh *et al.,* 1993; Oh *et al.,* 1994a,b; Yao *et al.,* 1994). The crystal structure of the GluR-B S1S2 revealed that the agonist α-amino group is bonded to an oppositely charged residue Glu-726 in the lobe 2 (numbering refer to amino acid residues of the rat GluR-B starting from the first translated residue; EMBL/GenBank accession No. M36419) by hydrogen bonds and electrostatic interactions (Armstrong *et al.,* 1998). The corresponding Glu or Asp residue of the bacterial PBPs or GluR0 interacts with the α-amino group of their ligands (Oh *et al.,* 1993; Oh *et al.,* 1994a,b; Yao *et al.,* 1994; Mayer *et al.,* 2001). The crystal structure of GluR-B S1S2 showed that the negatively charged α-carboxyl group is also bound by electrostatic interactions and hydrogen bonds to the positively charged Arg-506 in the lobe 1. This residue also has a corresponding one in the LAOBP, HBP, glutamine binding protein (GlnBP) and bacterial GluR0 structures (Arg-77, Arg-77, Arg-75 or Arg- 117, respectively; Chen *et al.,* 1999, Mayer *et al.,* 2001). Additionally, in the GluR-B S1S2 structure, the agonist α-carboxyl group is hydrogen bonded to the backbone of Thr-501 and Ser-675, whereas the α-amino group is hydrogen bonded to the side-chain of Thr-501 and to the backbone of Pro-499 (Armstrong *et al.,* 1998; Armstrong and Gouaux, 2000). In turn, the distal anionic group of the GluR-B agonists is hydrogen bonded to the base of an α-helix (helix F) in the lobe 2 involving the side-chain of Thr-676 (AMPA, glutamate and kainate complexes) and the backbone groups of Thr-676 and Ser-675 (glutamate and kainate complexes).

Taken together, in the GluR-B S1S2 structure the side-chains of Thr-501, Arg-506, Thr-676 and Glu-726 have direct interactions with all agonists (Fig. 6). In addition the backbone (carbonyl group) of Pro-499 makes hydrogen bonds to all agonists and Ser-675 backbone (carbonyl group) interacts with glutamate and kainate. Hydrogen bond interactions with antagonist DNQX are made with the side-chains of Arg-506 and the Thr-501 and with the backbone of Thr-501. The agonist binding role of Arg-506 and Glu-726 gets support from a number of previous mutations studies, which have shown that amino acid substitutions at these sites dramatically reduces or abolishes agonist binding or agonist response of the iGluRs (Paas *et al.,* 1996; Mano *et al.,* 1996; Uchino *et al.,* 1992; Wafford *et al.,* 1995; Hirai *et al.,* 1996; Laube *et al.,* 1997; Kawamoto *et al.,* 1997). Moreover, participation of both these

residues in agonist binding has been described in a vibrational spectroscopic study of ligand binding to the GluR-D S1S2 (Jayaraman *et al.,* 2000).

A fundamental finding from the crystal structures of the GluR-B S1S2 protein is that the bilobed structure is always more closed with a full agonist (AMPA and glutamate) or a partial agonist (kainate) than with an antagonist (DNQX) bound. The rigid structure of DNQX seems to prevent the closure of binding domains by directly binding to Thr-707. Kainate seems to restrict the lobe closure due to its isoprenyl group, which has van der Waals contacts with Leu-671. Interestingly, kainate receptors which have high affinity to kainate, always have a small Val residue in place of the Leu-671 of GluR-B. Since mutation of corresponding Leu to a Val or a Thr in the GluR-A subunit has been shown to increase receptor affinity to kainate, it has been suggested that the binding domain might be more closed in the mutant receptor (Mano *et al.,* 1996; Armstrong *et al.,* 2000).

1.4.4 Indirect interactions

All the agonist-bound crystal structures of the S1S2 show that the two binding lobes are so close together that a bond between the side-chains of Thr-707 and Glu-423 (in lobe 1 and 2, respectively) is likely to form. The stabilization of agonist bound structures by this interdomain bond was prevented when the antagonist DNQX was directly bound to Thr-707. Consistent with the suggested stabilization role, mutation of the kainate receptor GluR5 Ser-721 to Asn at a site corresponding to Thr-707 has been shown to reduce affinity to AMPA and 5I-willardiine (Swanson *et al.,* 1997 and 1998). Moreover, mutations of the AMPA or NMDA receptor subunits or of the chicken KBPs at the Gln residue corresponding to Glu-423 of the GluR-B have been shown to decrease agonist sensitivity or to decrease binding affinities of agonists to the receptor (Laube *et al.,* 1997; Mano *et al.,* 1996; Uchino *et al.,* 1992; Paas *et al.,* 1996). The existence of the Thr-707-Glu-423 bond has also been suggested by a model, which was based on a study of GluR-A/GluR-C chimeric mutations. In this study a residue corresponding to Tyr-723 of the GluR-B was suggested to result in the feature that GluR-A_{flop} receptors have higher affinity for Br-HIBO and faster desensitization with Br-HIBO activation than the GluR-C_{flop} receptors (Banke *et al.,* 2001; Mosbacher *et al.,* 1994). This Tyr residue (Tyr-716 in GluR-A) was suggested to be hydrogen bonded via a water molecule to both the Br-HIBO and to the bond corresponding Thr-707–Glu-423 of GluR-B (Banke *et al.,* 2001). On the other hand, molecular models of the GluR-B in complex with kainate and different enantiomers of AMPA, argues that the Thr-707–Glu-423 bond is present only when a functionally inactive (R)-AMPA is bound. The latter study suggests that in the

presence of (S)-AMPA (in the active form) a hydrogen bond would be formed between Glu-423 and Tyr-449 (Szárics *et al.,* 2001).

Two other hydrogen bonds, which are formed only in the fully closed GluR-B S1S2 structures with bound glutamate or AMPA are likely to be important for the maintenance of the ligand-bound conformation and for receptor activation. These bonds are formed between residues Ser-673–Gly-472 and Asp-672–water–Tyr-471 (Armstrong *et al.,* 2000). A disulfide bond Cys-739–Cys-794 between the two binding lobes of the GluR-B subunit has been suggested to stabilize an open apo-state (non-ligand bound) conformation of the binding domain in a similar manner to which a disulfide bond is thought to stabilize the domain structure of the PBPs (Sutcliffe *et al.,* 1996; Oh *et al.,* 1993). This suggestion is supported by studies showing that the agonist affinity is increased by removal of the conserved disulfide bond in both the gold fish KBP (Wo *et al.,* 1996) and in the AMPA receptor subunits (Watase *et al.,* 1997).

1.5 Mechanism of AMPA receptor activation and desensitization

An essential feature of all glutamate receptors is that agonist binding induces activation, which is a conformational change in the receptor structure that leads to an opening of the transmembrane ion channel. Activation is then followed by channel closing either due to inactivation (caused by agonist dissociation) or due to desensitization (caused by continuous presence of the agonist) (reviewed in Jonas, 2000). For example, in high frequency stimulation, glutamate that is normally rapidly removed by uptake to glia cells or by diffusion, may remain in the cleft for prolonged times and cause desensitization of the AMPA receptors. Both activation and desensitization are particularly fast in the AMPA receptors (see chapter 1.1.2) The extent of desensitization of the AMPA receptors depends on the type of agonist. For example, L-glutamate and AMPA induce fast desensitisation (with a time scale of 1-6 milliseconds), whereas kainate does not fully desensitize AMPA receptors. In addition, the desensitized-state of the AMPA receptors show increased affinity to agonists, which is also the case for the well-characterized nicotinic acetylcholine receptor (Hall *et al.,* 1993). Desensitization appers to be a general mechanism of many ligand-gated channels to cut ion flow into the cell in the presence of the ligand (Jones and Westbrook, 1996; Katz and Thresleff, 1957).

1.5.1 Domain closure – mechanism of activation and desensitization

The detailed mechanisms of activation and desensitization of AMPA receptors are still poorly understood. However, it seems that the closure of the ligand-binding domain is required for activation and desensitization (Armstrong and Gouaux, 2000). The crystal structures of the ligand-bound GluR-B S1S2 show that a full agonist induces full closure of the binding domain, whereas a partial agonist induces a smaller domain closure and an antagonist will stabilize the ligand-binding core in an open-cleft conformation. The level of channel activation seems to be dependent on the extent of domain closure, so that large conformational changes are transformed into local arrangements of channel gate regions (Armstrong and Gouaux, 2000). In a similar manner, the activation of bacterial PBPs seems to require domain closure, since the ligand-bound crystal structures of PBPs are more closed that the unliganded structures (Oh *et al.,* 1993; Sun *et al.,* 1998). However, closure of the ligand binding-domain seems to be more dramatic in the PBPs than in the iGluRs, since the ligand-induced closure of the LAOBP and GlnBP structures are 51-56° (Oh *et al.,* 1993; Sun *et al.,* 1998), whereas the kainate- and AMPA-bound structures of the GluR-B ligand-binding domain are closed at 12° and 21° in respect to apo-state structure, respectively (Armstrong and Gouaux, 2000). However, another study used the GluR-D S1S2 construct (with a longer linker between the S1 and S2 as compared to the one used in the crystallization of GluR-B S1S2) and detected almost no closure at all (1.1°) upon agonist binding in the solution scattering measurements (Abele *et al.,* 1999). The GluR-D study suggested that activation of the iGluRs might not require such a dramatic closure of the ligand-binding domain as is required for bacterial PBP activation (Abele *et al.,* 1999). Consistent with that suggestion, a molecular dynamics simulation study of the GluR-B ligand-binding domain found that activation involves only local conformational changes and not dramatic reorganization of binding lobes as suggested by the crystal structure (Arinamipathy *et al.,* 2002). In addition, an infrared spectroscopic study of the GluR-D S1S2 protein (with longer linker) detected local conformational changes in the secondary structure of the ligand-binding domain. Those conformational shifts were always more extensive upon binding of an agonist (Jayaraman, 2000) than upon binding of partial the agonist kainate or antagonist DNQX and CNQX (Madden *et al.,* 2001).

The mechanism of glutamate receptor activation was initially suggested to follow the same mechanism as the activation of bacterial PBPs (Mano *et al.,* 1996). The activation of bacterial PBPs was described to involve domain closure by ´venus a fly-trap´mechanism (Oh

et al., 1993). Mano *et al*., (1996) suggested that activation of the iGluRs would require interaction of the agonist with the lobe 1, whereas the domain closure and entrance into desensitized state would require interaction of the agonist with the lobe 2. However, this theory was in conflict with some other studies. For example, in the crystal structure of the GluR-B S1S2, the lobe 2 participates in kainate binding, although kainate does not allow full desensitization of the AMPA receptors (Armstrong, 1998). Since the kainate-bound structure is not fully closed, as AMPA- and glutamate-bound structures are, it seems that desensitization but not activation requires full closure of the binding domain (Armstrong and Gouaux, 2000). This was supported by a recent study on the activation and desensitization of a mutated GluR-A receptor using small and large willardiine agonists (Kizelsztein *et al.,* 2000). The willardiine docking models (based on the GluR-B crystal structures), suggested that the activated state of the ligand-binding domain would be more open, since it can accommodate large-sized willardiines (which do not desensitize AMPA channels), whereas the desensitized state would be more closed, since it can only accommodate small-sized willardiines (known to be able to desensitize AMPA channels).

Activation of the AMPA receptors has also been studied by examining the order of ligand interactions. Based on analogy with bacterial PBPs, the 3-D structures of the ligand complexes and the fast kinetic measurements of agonist binding, it has been suggested that agonist binding involves two separate steps. The initial binding of an agonist is suggested to involve the lobe 1 and the subsequent interactions with the lobe 2 are thought to involve the closure of the two binding lobes. Two slightly different models have been presented. The first model is based on the kinetics of agonist binding to a mutated GluR-D S1S2 protein as measured by stopped-flow fluorescence methods (Abele *et al.,* 1999) (the numbers of the homologous GluR-B residues are one less than in GluR-D). The initial rapid ´docking´ step was suggested to involve the lobe 1 residues Tyr-472 (directly) and residue Glu-424 indirectly, corresponding to Tyr-471 and Glu-421 of the GluR-B, respectively. The second ´locking´ step, was suggested to involve a three-way hydrogen-bonding formation involving the agonists α-amino group and the residue Glu-727 (lobe 2) and Thr-502 (lobe 1). In addition, the residues Pro-500 and Arg-507 (lobe 1) and residues Thr-677 and Ser-676 (lobe 2) were also suggested to be involved in the agonist binding. The formation of the three-way hydrogen-bonding network was suggested to be essential for receptor activation.

The second model, which was based on the comparison of the apo-state and agonist-bound structures of the GluR-B S1S2 crystals, suggests a slightly different order for the binding contacts (Armstrong and Gouaux, 2000). The initial contacts were suggested to

involve both the α-amino and α-carboxyl groups of an agonist with the residues Arg-506, Thr-501 and Pro-499 (lobe 1) and the residue Glu-726 (lobe 2). Closing of the binding lobes was suggested to occur in the second step, upon direct binding of the agonist γ-carboxyl group to the base of helix F.

As discussed earlier, both of these models have support from mutation studies, particularly since the alteration of the residues corresponding to GluR-B Arg-506, Glu-726, Thr-501 and Glu-423 have been shown to affect receptor activation (Uchino *et al.,* 1992; Wafford *et al.,* 1995; Hirai *et al.,* 1996; Kawamoto *et al.,* 1997; Laube *et al.,* 1997; Mano *et al.,* 1996; Paas *et al.,* 1996).

The crystal structure of the GluR-B S1S2 also reveals that Lys-751 locating in the interdomain β-strand may be involved in control of domain closure and may therefore be important for receptor activation (Armstrong and Gouaux, 2000). Interestingly, in the apo-state of the GluR-B S1S2 the interdomain Glu-726–Lys-751 bond blocks the binding site of the agonist α-amino group, while the interdomain Glu-726–Thr-676 bond blocks the binding site of the agonist γ-carboxyl group. Both these interdomain bonds stabilize the apo-state of the GluR-B binding domain in a way similar to that of the two interdomain bonds in the bacterial GluR0 S1S2 apo-state structure (Mayer *et al.,* 2001). However, in the GluR0 those bonds do not involve the β-strand, nor do they block the binding site for the γ-carboxyl group of an agonist. Due to these differences in the apo-state stabilization, it has been suggested that the control of receptor activation is different in iGluRs and in GluR0 (Mayer *et al.,* 2001). The poor stabilization of the apo-state of the GluR0 and possibly of the PBPs, may explain why both these proteins are found (in the crystal structures) in a closed conformation in the absence of the ligand as well as the open conformation (Flocco *et al.,* 1994). The spontaneous cleft closure of GluR0 may also explain why the GluR0, but not the GluR-B receptors have been found to undergo spontaneous activation in the absence of ligand (Mayer *et al.,* 2001).

1.5.2 The activation-desensitization model

Factors that can affect the kinetics and extent of AMPA receptor desensitization are; subunit composition of the receptor, type of bound agonist, alternative splicing affecting the flip/flop region and the RNA editing of the so-called R/G-site (reviewed in Dingledine *et al.,* 1999; Jonas, 2000). In addition, AMPA receptor desensitization can be modulated by some allosteric compounds (see chapter 1.2.4.). In general, the AMPA receptors carrying the flip-segment in their subunits are desensitized more slowly than their equivalent flop-variants

(Mosbacher *et al.,* 1994). The flip/flop-segment also affects the sensitivity of the receptor to allosteric compounds like CTZ, aniracetam and PEPA (Partin *et al.,* 1994; 1995; Johansen *et al.,* 1995; Sekiguchi *et al.*, 1997). For example, the blockage of AMPA receptor desensitization by CTZ has been shown by mutation analysis to be determined by Ser residue at position 771 of GluR-A flip-variant subunit (Partin *et al.*, 1995; 1996; Johansen *et al.*, 1995). On the other hand, receptors which are composed of GluR-B-D subunits and carry the Gly residue at the R/G-site recover from desensitization faster than the unedited versions (Lomeli *et al.,* 1994). Moreover, a single Leu residue in the S1 region of the GluR-A-D subunits is known to have strong effect on receptor desenzitization. Initially, the participation of this residue was demonstated by the GluR-C mutation L507Y, which was shown to block receptor desensitization completely (Stern-Bach *et al.,* 1998). Another mutation study suggested that residue Tyr-716 in the GluR-A_{flop} subunit (homologous to Tyr-723 of the GluR-B subunit) determines the fast desensitization of the receptor composed of these subunits (Banke *et al.,* 2001; see chapter 1.4.4).

The publication of the crystal stucture of the GluR-B S1S2J provides a new aspect to the study of the desensitization mechanism. It was found that the S1S2 proteins formed dimers with 2-fold symmetry and most of the structural determinants of desensitization as revealed by mutation studies, were found at or near the dimer interface (Armstrong, 1998; 2000). For example, Leu-504 (corresponding to Leu-507 of the GluR-C) locating in the helix D had hydrophobic contacts with residues in helix J of the other dimer. In addition, the flip/flop segment and the R/Q-site (located in helices J and K) were found to be at or near the dimer interface. Therefore, it seems that the interaction of helix D with helix J at a dimer interface participates in the regulation of receptor desensitization (Armstrong *et al.,* 2000, Mayer *et al.,* 2001). A recent mutation study of chimeric GluR-A and GluR6 subunits supports this view and suggests that the helices D, J and K are involved in regulation of desensitization through interface interaction. This study demonstrated that in addition to the residue corresponding to residue Leu-507 of the GluR-C, two previously unidentified residues in the flip/flop area and a new residue in the β-strand 7 contributed to the desensitization effect of CTZ (Partin, 2001).

Strong support for the dimer interacting regulation was given by a recent crystal structure of the GluR-B S1S2J carrying a mutation at Leu-504 residue (Sun *et al,* 2002). The mutant protein formed a similar dimer interface to the one formed in the crystals of the wild-type GluR-B S1S2J. However, the Tyr-504 introduced by mutation was able to form a more tightly stabilized dimer interface by contacts to residues Leu-769 and Lys-773 in the helix J

than the wild-type Leu-504. Since this mutation is also known to block AMPA receptor desensitization (Stern-Bach *et al.,* 1998), it was suggested that an active state is favoured by stabilization of this interface (Sun *et al.,* 2002). Moreover, a crystal structure of another mutant of GluR-B S1S2J (flop-N775S) in complex with CTZ revealed how the Ser residue at the Asn/Ser-site in the flip-segment of the AMPA receptor subunits makes receptor desensitization sensitive for the CTZ blockage. CTZ was bound between Ser-775 from helix J and the residues Pro-515, Phe-516 and Ser-518 from β-strand. In other words, by binding between the dimer interface, CTZ stabilizes the same interface structure that was also stabilized by Tyr in the L504Y S1S2 mutant protein. Therefore, CTZ seems to prevent the receptor to enter a desensitized conformation in a similar manner to the previously mentioned L504Y mutation (Sun *et al.,* 2002).

Finally, the structure of a GluR-B S1S2J mutant flop-N775D revealed that the Asn residue at the Asn/Ser-site is located at the interface of a new type of a subunit-subunit dimer. This second type of dimer interface was suggested to be present when the receptor is in the desensitized state (Sun *et al.,* 2002). This so-called ´later interface´ was shown in the crystal structure to involve the lobe 1 from one subunit and the lobe 2 from the other subunit (Fig. 7).

Based on the previously mentioned two distinct interface structures, a recent activation-desensitization model suggests that the conformational shifts between the activated and the desensitized states are controlled by stabilization of these two interfaces (Sun *et al.,* 2002, reviewed in Madden, 2002). The model suggests that, in the apo-state conformation, the first type of dimer interface exists between lobe 1 of the dimer A and lobe 1 of dimer B. This interface remains unchanged when receptor is activated upon agonist binding, but lobe 1_A is closed in respect to lobe 2_A and the lobes 2_A and 2_B are separated from each other (Fig. 7). Activation also involves the channel opening due to a clockwise screw-axis movement of lobe up and away from the channel 4-fold symmetry axis, causing rotation of TM helices via connecting peptides (Armstrong *et al.,* 2000, Mayer *et al.,* 2001). On the other hand, when the receptor is desensitized the first type of interface between lobe1_A and lobe1_B is reorganized and lobe closure is decoupled from ion channel opening. This leads to the formation of the ´later interface´ between the lobes (1_A and 2_C). This is achieved when lobes 1_A -1_B are separated from each other allowing lobes 2_A and 2_B to come close together. This new conformation allows the closed-cleft conformation around the agonist to stay unchanged, whilst the channel is closed (Sun *et al.,* 2002).

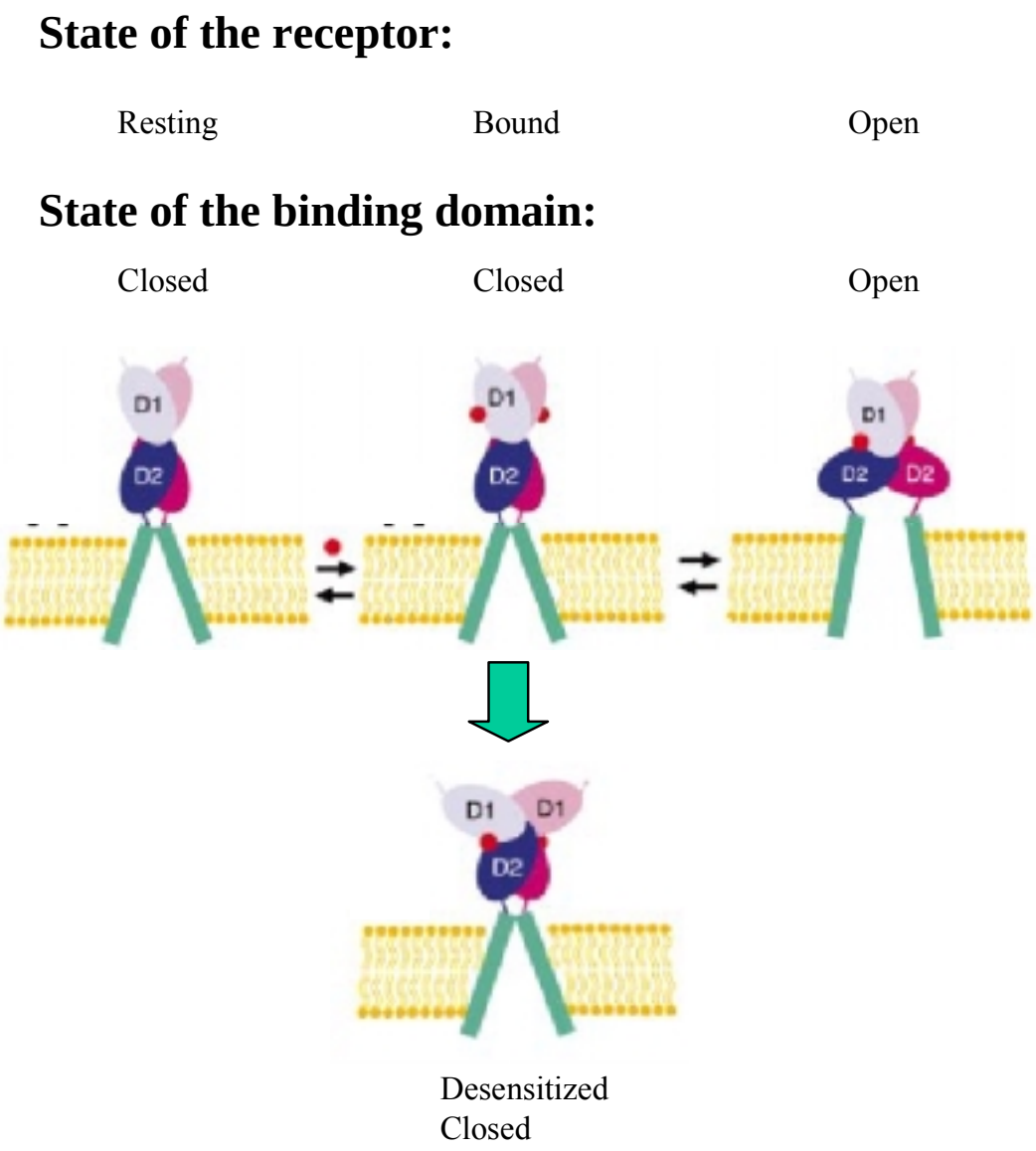

Figure 7. Activation-desensitization model for the iGluRs. Labels D1 and D2 correspond to domain 1 and domain 2 of each subunit. Cylinders illustrate the receptor channel and the circle between the binding lobes corresponds to the bound ligand. Ligand binding induces closure of domain 2 towards domain 1, however the dimer interface between the D1 and D2 of the two subunits is retained when the channel is opened. If activation leads to desensitization, the channel is closed and the D1–D1 interface is interrupted. In the desensitized state a new ´later interface´ is formed between D1 of one subunit and D2 of another subunit. (Adapted from Sun *et al.*, 2002.)

2 AIMS OF THE PRESENT STUDY

The present study addressed the following questions:

1. Is the ligand-binding domain of the GluR-D AMPA receptors structurally and functionally related to bacterial amino acid binding proteins?

2. What are the roles of the conserved cysteines in the ligand-binding domain of the AMPA receptors?

3. What are the structural determinants responsible for the specific binding of agonists and antagonists to the GluR-D AMPA receptors?

3 MATERIALS AND METHODS

Method	Original publication where methods and materials are described in
Anion exchange chomatography*	I
Edman degradation*	I
Homology modelling*	II
Immunoaffinity chromatography	I
Ligand docking*	III
Ligand minimization*	III
Mass spectroscopy*	I
N-terminal sequencing*	I
Radioligand-binding assay -Equilibrium dialysis*	I
Radioligand-binding assay -Filter binding	I-III
Receptor minimization*	III
Recombinant baculovirus expression	I-III
Protein labelling with thiol-specific reagents*	I
SDS-PAGE	II-III
SH-group titration*	I
Site-directed mutagenesis	I-III
Structural modelling*	III
V8- protease digestion*	I
Western blotting	II-III

* Methods that the author of the thesis has not utilized

4 RESULTS

4.1 Expression of the soluble S1S2 ligand-binding domain

The AMPA receptor ligand interactions were studied in the present work by using the GluR-D S1S2 fusion protein as a surrogate for the intact GluR-D receptor (I, II and III). As described previously, the protein was constructed by joining the ligand-binding regions (S1: residues 404-546 and S2: 649-813; numbering refer to amino acid residues of the rat GluR-D starting from the first translated residue; EMBL/GenBank accession No. M36421) of the GluR-D with a hydrophilic linker peptide (Kuusinen *et al.,* 1995). Such fusion proteins of the GluR-D or -B lack the membrane-spanning segments and the N- and C-terminal domains of the receptor, but they can be expressed as soluble secreted proteins both in the baculovirus system and in the *E.coli* periplasm (Kuusinen *et al.,* 1995; Arvola and Keinänen, 1996). Compared to the intact receptors the expression and purification of the S1S2 protein is less complicated due to its solubility. Moreover, the pharmacological profile of the S1S2 fusion protein is almost identical to that of the corresponding intact receptor (Kuusinen *et al.,* 1995).

The wild-type S1S2 protein and the S1S2 constructs carrying site-directed single amino acid mutations were expressed in *Trihcoplusia ni* High Five cells (I, II and III). The proteins carrying the N-terminal Flag and C-terminal myc epitopes were detected in the culture supernatant by Western blotting with the anti-Flag M1 antibody. Usually detection showed one 42-kDa band or in some cases a doublet of 40-kDa and 42-kDa species. Taking into account the possible presence of N-glycosylation on the two consensus sites at the N-terminus of the S1, the sizes of the detected bands were close to the calculated size of 39 kDa of the S1S2 polypeptides. The 40- and 42-kDa bands likely represent different degrees of glycosylation of the S1S2 protein. The finding that the protein was soluble and secreted to the culture medium strongly suggested that the protein was correctly folded and the signal peptide was correctly processed. In most cases (II and III), the intensities of the mutant protein bands were similar to wild-type S1S2 proteins, suggesting that the mutations did not affect the level of expression. However, the single Cys mutants (C740S and C795S), and the douple Cys mutant (C740S/C795S) had a significantly lower expression level than the wild-type S1S2 (I: see Table III). The single mutants formed covalent oligomers which after treatment with DTT or β-mercaptoethanol appeared as monomers on SDS-PAGE. The douple mutant seemed to form noncovalent aggregates, which did not dissapper after treatment with the recucing agents, but were captured by 300-kDa cut-off filter.

4.2 Cysteine residues

The amino acid sequence of the ligand-binding domain of all ionotropic glutamate receptor subunits contains three conserved cysteines. In the NMDA receptors two of these cysteines are known to be involved in modulation of receptor function by reducing agents (Sullivan *et al.,* 1994). Such modulation is thought to involve the reversible formation and breakage of a disulfide bridge by oxidation and reduction. It is thought that formation of a disulfide bridge between two lobes of the ligand-binding domain would stabilize the unbound ligand conformation of the binding domain in all iGluRs (Sutcliffe *et al.,* 1996).

In order to study if the disulfide bridge is formed in the ligand-binding domain of the GluR-D, we analyzed the accessibility of the cysteines of the S1S2 wild-type protein to thiol-specific reagents (I). In addition, we studied how disruption of such a disulfide bond would affect the binding affinity of the ligands by analyzing the ligand-binding properties of the cysteine-mutated S1S2 proteins (I).

The potential existence of a disulfide bond was studied by reacting the SH groups of the proteins with hydrophobic 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) under non-reducing and reducing, denaturing conditions. From the reaction done under non-reducing conditions one molecule of DTNB/mol of S1S2 protein was detected in Ellman reaction, whereas three molecules of DTNB/mol of S1S2 protein were detected if the protein was reduced with 1,4-dithio-DL-threitol (DTT) before reaction with DTNB. These results suggest that the S1S2 has one disulfide bridge and one free cysteine. In addition, the accessibility of the free cysteine to DTNB or to the more hydrophobic *n*-octyl-5-dithio-2-nitrobenzoic acid (ODNB) was studied in native conditions. Under non-reducing, native conditions 0.1 cysteines/molecule of S1S2 were detected by using DTNB, whereas 0.9 cysteines/S1S2 were detected with ODNB, suggesting that the free cysteine is partially buried in a hydrophobic environment. The accessibility of the disulfide bridge to the reducing agents was also studied under native conditions by reacting the S1S2 with DTT prior to reaction with DTNB. Only 1.2 cysteines/S1S2 was detected, suggesting that the disulfide bridge is only 10% accessible to reduction by DTT in the native protein structure.

The position of the disulfide bridge was confirmed by labelling the S1S2 protein with *N* –(1-pyrenyl)maleimide in two separate procedures. Firstly, only free cysteines were labelled; secondly, only the disulfide-bridge-forming cysteines were labelled. The separation of the V8-protease digested peptide mixture yielded one nonapeptide fragment from the mixture containing free cysteines labelled. Detection by matrix-assisted laser desorption

ionization mass spectroscopy (MALDI-MS) and N-terminal sequencing revealed that the peptide fragment had one Cys corresponding to Cys-447 of the intact GluR-D. (I: see Fig. 1). From the mixture with the labelled disulfide bridge-forming cysteines two major fractions were separated. Two cysteines were detected from these fractions at positions corresponding to Cys-740 and Cys-795 of the intact GluR-D.

The effect of the disulfide bond on ligand binding was analyzed by a radioligand-binding assay of the S1S2 proteins carrying mutations C740S or C795S. In the filter-binding assay the wild-type S1S2 bound [^{3}H]AMPA with a dissociation constant (K_d) value of 13.3 nM, whereas the affinities of the mutants C740S and C795S were increased nearly 2-fold (K_d 7.2 nM and 7.8 nM, respectively). Equilibrium dialysis analysis also showed that the binding affinity for [^{3}H]glutamate was increased 2.4-fold in C740S; the affinity of the C795S mutant was not determined (I: see Table III.) However, the kainate affinities were slightly reduced (~1.7-fold) in both mutants as measured by competition filter-binding assay. Like the single mutants, the double mutant C740S/C795S showed an increased affinity (~2.5-fold) for [^{3}H]AMPA in a filter-binding assay (unpublished result: Lampinen, *et al*). The binding affinity for [^{3}H]glutamate could not be measured by equilibrium dialysis due to shortage of the pure S1S2 mutant protein. These results suggest that the receptor agonist affinities were reduced by formation of the disulfide-bond between Cys-740 and Cys-795.

4.3 Charged residues

Based on the similarities between the amino acid sequences of the iGluR ligand-binding domain (S1S2) and the bacterial PBPs, it has been predicted that the mechanism of ligand binding could be similar in these proteins (Sutcliffe *et al.,* 1996; Kawamoto *et al.,* 1997; Laube *et al.,* 1997). We tested the hypothesis that the major binding pocket interactions with the ligand are conserved from bacterial proteins to glutamate receptors (II). Specifically, we selected candidate residues for agonist binding from sequence alignments with the LAOBP and HBP, and subjected these residues to point mutations. The key interaction in the LAOBP and HBP are made by the charged Arg-77 and Asp-161 residues with the oppositely charged α-carboxyl and α-amino group of their amino acid ligand (Oh *et al.,* 1993; Oh *et al.,* 1994b; Yao *et al.,* 1994). Due to the substantially low sequence homology, particularly of the S2 segment, to PBPs, manual adjustments of sequence alignment were used to maximize the similarities within short sequences. We also controlled for the correctness of our sequence alignment by mutating and analyzing several charged groups which could potentially align with the ligand-interacting residues of the LAOBP and HBP.

The Arg-507 residue, which is conserved among all iGluR subunits, aligned well with Arg-77 of the LAOBP and HBP. However, three other positively charged residues were also selected for mutation to test their possible interaction with either the α-carboxyl or γ-carboxylate group of glutamate (II: see Fig. 1). The wild-type S1S2 protein had an affinity for [^{3}H]AMPA of K_d 37 nM, whereas the conservative mutation of R507K resulted in a complete loss of specific [^{3}H]AMPA binding in the saturation binding assay (II: see Table I). In our structural model of the GluR-D S1S2, the side-chain of Arg-507 formed hydrogen and ionic bonds with the α-carboxylate oxygens of the agonist (glutamate) in a similar manner to the bonds which are formed by Arg-77 of the LAOBP and HBP (II: see Fig. 4). None of the other mutations done to the other positively charged residues (Lys-431, Lys-432 and Lys-471) significantly altered the binding of [^{3}H]AMPA to the mutant proteins. They also bound glutamate, kainate and DNQX with affinities similar to the wild-type protein. Only the mutation L471H slightly reduced binding affinities of [^{3}H]AMPA and glutamate (K_d 90 nM and K_i [inhibition constant] 0.7 μM, respectively). However mutation of the same residue to Arg or Ala did not change the affinity for [^{3}H]AMPA (II: see Table I).

Three negatively charged residues were also mutated to test their possible role in agonist binding. The Glu-727 aligned (with some adjustments) with Asp-161 of the LAOBP and HBP (Oh *et al.,* 1993; Oh *et al.*, 1994b; Yao *et al.*, 1994). This Glu residue is in all AMPA and kainate receptor subunits, whereas NMDA receptor subunits have negatively charged Asp residue (II: see Fig. 1). When the Glu-727 was mutated to a neutral aliphatic (Ala) or neutral polar residue (Gln, Ser) a complete loss of [^{3}H]AMPA binding resulted (II: see Table I). However, substitution with the negatively charged (Asp) residue resulted in a slight (5-10-fold) increase in [^{3}H]AMPA affinity. Interestingly, the binding affinities for glutamate and kainate were decreased (10- and 1000-fold, respectively), whereas the affinity for the antagonist DNQX was increased (40-fold) for the E727D mutation. These findings suggest that the Glu-727 has an important role in the agonist binding and selectivity. In our model structure of the ligand-binding domain, Glu-727 formed hydrogen bonds and ionic interactions to the agonist α-amino group in the similar manner to which Asp-161 forms key interactions with the ligand in the LAOBP and HBP structures (II: see Fig. 4). The second negatively charged residue, Glu-710 also aligned near the Asp-161 of the PBPs and was mutated as a control. When Glu-710 was mutated into Asp or Ala, no significant changes in agonist affinities were detected. The third residue Glu-424, previously shown to contribute to agonist affinities of the iGluRs (Sutcliffe *et al.,* 1996; Kyryatov *et al.*, 1994), aligned with

residue Tyr-14 of the LAOBP , which contacts the distal group of the amino acid side chain. Of the mutants created, E424D, E424Q and E424A, only the first two showed a slightly (~5-fold) reduced affinity for glutamate.

In parallel with the charged residues, we mutated residue the Tyr-472, since the corresponding residue of the chicken kainate-binding protein has been shown to contribute to antagonist binding (Paas *et al.*, 1996). The mutant Y472F showed reduced affinity for [^{3}H]AMPA (~ 10-fold), whereas the affinities for the other agonists were similar to those of the wild-type protein. The affinity for DNQX was also reduced (~4-fold).

4.4 N-terminus of the S2 segment

The iGluR N-terminal segment of the S1S2 is homologous to a region in some PBPs which interacts with amino acid ligands (Kang *et al.*, 1991; Oh *et al.*, 1993; Oh *et al.,* 1994a; b; Yao *et al.*, 1994). Since the binding partners for the glutamate α-carboxyl and α-amino groups were already predicted (II), we subjected seven amino acids (L672-K678) from this region to mutations, in order to find additional determinants of agonist and antagonist binding (III). All residues were substituted with a small neutral alanine residue in order to abolish specific interactions of the side chain residue but not to disturb the main chain folding. This region was later shown by the crystal structure of the GluR-B S1S2 ligand complexes (Armstrong *et al.*, 1998; Armstrong and Gouaux, 2000) to correspond with residues that precede or form an α-helical structure (helix F). Those structures were also used to construct docking models of the GluR-D S1S2 with bound glutamate, kainate, AMPA and DNQX to interpret our binding data. The mutant proteins were analyzed in a similar manner to the earlier mutants. In addition, we now used a new antagonist [^{3}H] Ro-48 8587, which was described to have nanomolar affinity to rat brain membranes (Mutel *et al.*, 1998). Thus we could study the affinities of agonists and antagonists independently from the proteins ability to bind [^{3}H]AMPA.

Firstly, the binding of agonists were studied using [^{3}H]AMPA and competitive displacement by unlabelled ligands. This revealed that the mutations L672A, G675A and T677A led to a complete loss of [^{3}H]AMPA binding. At 100nM [^{3}H]AMPA concentration, shown to be saturable for the wild-type S1S2 , no specific binding could be detected with these mutants (III: see Fig. 2). On the other hand, the three mutants D673A, S676A and K678A showed only a slightly decreased affinity for [^{3}H]AMPA (K_i 65.1 nM, 92.6 nM and 103 nM, respectively) and the mutant S674A had an affinity (K_i 32.6 nM) similar to the

affinity of the wild-type S1S2 protein (K_i 24.8 nM). The binding affinity for glutamate was also slightly decreased in the mutants D673A and K678A

Secondly, the agonist affinities of all mutants were studied by competing the [^{3}H] Ro-48 8587 binding with unlabelled ligands. In addition to the reduced [^{3}H]AMPA binding affinity, the mutant L672A showed a remarkably reduced affinities for glutamate (~80-fold) and kainate (~25-fold). In the ligand-docking models, the selective loss of AMPA, kainate and glutamate affinity was shown to result from a loss of hydrophobic side-chain packing (III: see Fig. 4). In GluR-B a tyrosine residue (Tyr-723) is at the same position as Phe-724 of the GluR-D, and affects the orientation of residue Leu-671 which corresponds to Leu-672 in the GluR-D. The residue is found in two different orientations in the GluR-B S1S2 structure, one observed in the AMPA- and glutamate-bound complex and the other in the kainate-bound complex. In our GluR-D model, Leu-672 is suggested to be in a similar orientation to Leu in the stucture of GluR-B S1S2-kainate complex. In that orientation, Phe-724 is packed against Leu-672 enabling the formation of hydrophobic contacts between the side-chain of Leu-672 and the hydrophobic parts of the agonist (IV: see Fig. 5). Thus, the affinity loss for AMPA is likely to be caused by a breakage of the hydrophobic contacts between AMPA methylene group and an aromatic part of the isoxazol ring with Leu-672. In the same way, the mutation to Ala would eliminate the binding of two methylene groups of glutamate with the side-chain of Leu-672. On the other hand, a bulky isoprenyl group of kainate would have favourable interactions with the small alanine side chain, consistent with the observed moderate decrease in the kainate-binding affinity of L672A.

Although the mutation G675A led to a complete loss of [^{3}H]AMPA binding, the [^{3}H] Ro-48 8587 competition assays showed that this mutation led only to a slightly reduced binding affinity for kainate, whereas the affinity for glutamate was unchanged. Based on the AMPA docking model, the selective loss of AMPA affinity is due to one water molecule near Gly-675 (´w1´in Fig. 4d of IV), which is present only in the AMPA-complex. Hydrogen bonding of the water molecule to a negatively charged oxygen atom (at 3-position of the isoxazole ring) and to an α-carboxylate of AMPA would be lost when this water molecule is displaced by the introduction of the methyl group of Ala in the place of hydrogen of Gly.

The mutant T677A, which did not bind [^{3}H]AMPA, had a reduced affinity for all agonists tested in the [^{3}H] Ro-48 8587 competition assay. The glutamate affinity was reduced ~4000-fold and the kainate affinity ~15-fold (III: see Table I and II). In our agonist docking models, the side-chain, OH-group of Thr-677 forms hydrogen bonds with these agonists. Mutation T677A would lead to elimination of the contacts that the Thr residue makes with the

negatively charged oxygen attached to the isoxazole ring of AMPA and with the distal carboxylate groups of glutamate and kainate. Thus, resulting in the observed reduction in the binding affinities of these agonists. The large loss in AMPA affinity is logical as the hydroxyl group of the isoxazol ring makes the only contacts of AMPA with the helix F region. However, whilst glutamate has fewer points of contact with the binding pocket as compared to AMPA, it does interact with the helix F region. Thus explaining the strongly reduced binding of the T677A mutant for glutamate.

Consistent with the observed minor changes in [^{3}H]AMPA binding for the D673A, S674A and K678A mutants, the [^{3}H] Ro-48 8587 competition assay showed that the affinities for glutamate and kainate were unchanged or only slightly changed (III: see Table II). Our ligand docking models show that the side-chains of Asp-673, Ser-674 and Lys-678 point away from the binding cavity (III: see Fig. 4) The slightly changed binding affinities for agonists in the S676A mutant (III: Table I and II) were in agreement with the model where Ser-676 participates in agonist binding only via the main-chain amide nitrogen, which forms a hydrogen bond with the agonist distal anionic group (III: see Fig. 4).

After examining the agonist binding properties, we determined the binding affinities for DNQX and [^{3}H]Ro-48 8587 antagonists by inhibiting the [^{3}H]Ro-48 8587 binding with unlabelled ligands. The antagonist [^{3}H]Ro-48 8587 had a high-affinity to the wild-type GluR-D S1S2 (K_d 15.6 nM) similar to the previously reported nanomolar affinity to rat brain membranes (Mutel *et al.*, 1998). Strikingly, none of the Leu-672–Lys-678 mutants showed significantly altered affinity for [^{3}H]Ro-48 8587. The affinity for the another antagonist, DNQX, was also unaffected in all the mutants tested. The unchanged affinities for antagonists DNQX and Ro-48 8587 were consistent with the docking model of the GluR-D S1S2 with bound DNQX and Ro-48 8587 (IV: see Fig. 6). In this model, DNQX does not make any close contacts with residues Leu-672–Lys-678 in helix F region. Similarly, Ro-48 8587 does not make any side-chain contacts with the helix F region, although the antagonist imidazolyl group may be hydrogen bonded to some main chain residues.

5 DISCUSSION

In this study we have experimentally tested the hypothesis that AMPA receptors and PBPs may use similar mechanisms for binding their respective ligands. We performed site-directed mutation analysis by using a S1S2 fusion protein as a surrogate for the intact receptor (Kuusinen *et a.*, 1995, Arvola and Keinänen, 1996). In principle, mutations may affect ligand interactions by at least two distinct mechanisms. Firstly, a mutation may eliminate structures which directly participate in ligand binding. Secondly, a mutation may eliminate structures which are important for the folding and stability of the ligand-binding domain. A complete loss of ligand binding may reflect either of these two cases, whereas mutations that affect the binding of only specific ligands are more likely due to the former case. We studied the binding of different types of ligands and used molecular modelling in order to distinguish between the two mechanisms mentioned above. The results from the first part of this work predicted that the binding of ligands is indirectly affected by a disulfide bridge, which stabilizes the structure of the unliganded binding domain (apo-state) by connecting the two lobes of the domain. The results from the second and third parts of the present work predicted residues which directly form hydrogen bonds and ionic contacts with agonist or antagonist compounds. After publication of the first and second part of this work, the first crystal structure of the iGluR ligand-binding domain became available (Armstrong *et al.*, 1998). Although, the mutations described in the III publication were designed before the structure was published, the analyses of the binding data were done with the help of the new models based on the newly available structural data.

5.1 Disulfide bridge

All subunits of the different iGluR subclasses carry three cysteine residues in their ligand-binding domain S1S2. By using biochemical analysis we demonstrated that two of these Cys residues in S2 segment form a disulfide bridge. By mutation analyses we showed that this disulfide bridge is able to modulate agonist binding affinities of the GluR-D S1S2 protein.

Analysis of the GluR-D S1S2 protein by redox agents and thiol specific reagents revealed that the disulfide bond is formed between Cys-740 and Cys-795, whereas the Cys-447 is free. The bridge forming cysteines are at equivalent positions as Cys-726 and Cys-780 in the NR1, which has previously been shown to be sensitive to the reducing agent DTT (Sullivian *et al.,* 1994). DTT has also been show to modulate NMDA receptor function by

increasing receptor currents and by decreasing the EC_{50} value for agonists (Aizeman *et al.,* 1989; Köhler *et al.,* 1994). A number of studies on the NMDA receptors have shown that receptor function can be affected by several other oxidizing or reducing agents or by the subunit composition (Aizeman *et al.,* 1989; Gilbert *et al.,* 1991; reviewed in Dingledine *et al.,* 1998). These finding have led to a suggestion that control of the redox-oxidation state of the NMDA receptors may serve as neuroprotective mechanism in pathological situations (reviewed in McBain and Mayer, 1994, Lipton *et al.,* 1993). In our study, subjection of the GluR-D S1S2 to the reducing agent DTT identified for the first time that the actual target for DTT modulation is the disulfide bridge forming cysteines. Moreover, subjection of the GluR-D S1S2 to DTT under native conditions revealed that the disulfide bridge is relatively inaccessible to redox modulation by DTT. This may explain the previous observation that AMPA receptors are not sensitive to potentiation by DTT (Aizeman *et al.,* 1989; Terramani *et al.,* 1988). It has been suggested that the insensitivity for DTT modulation depends on subunit composition, which would determine if a disulfide bridge could be formed (Gozlan and Ben-Ari, 1995). However, our result suggest that AMPA receptors do have a disulfide bridge, but it is not accessible for particular reducing agents since it is buried in a hydrophobic environment. This means that the subunit composition might instead regulate the accessibility of the disulfide bridges to reducing agents. In that case the regulation of the redox state of the receptor could be controlled by regulation of subunit composition alone and the maintenance of appropriate redox state would not require active modulation *in vivo* as has been suggested (Gozlan and Ben-Ari, 1995). The finding that free cysteines were accessible to DTNB and ODNB to different extents revealed that the accessibility of the disulfide bridge and perhaps the modulation of redox state of different receptors are also dependent on the chemical nature of the reducing/oxidizing agent.

The analysis of the single mutants C740S and C795S and the double mutant C740S/C795S by ligand-binding assays revealed that a disruption of the disulfide bridge resulted in an increase in the binding affinity of AMPA and in some cases an increase in the binding affinity of glutamate. These results were consistent with the previous mutation analysis, which showed that the mutation of C722S in the GluR-C led to an increased affinity for glutamate and to decreased channel conductivity (Watase *et al.,* 1997). In addition, the usage of the soluble ligand-binding domain of the GluR-D in our studies revealed that changes in the EC_{50} or agonist affinities of intact receptors are due to changes in the free energy of ligand binding and not due to changes in the free energy of gating. In other words, the effect that DTT modulation has on ligand-binding affinity of the receptor can be mediated

by the ligand-binding domain S1S2 alone. We also found that the cysteine-mutated S1S2 proteins were expressed at lower levels than the wild-type S1S2 proteins, and the double-cysteine mutants formed aggregates, suggesting that the disulfide bridge is also important for the correct folding and the general stability and functionality of the ligand-binding domain. This finding is in agreement with a previous model of the GluR6 subunit, which suggested that the disulfide bridge stabilized the apo-state conformation (Sutcliffe *et al.,* 1996). That model was based on the predicted analogy to bacterial PBPs. They also have three cysteines although at positions distinct from the cysteines in the S1S2 structure. In the unliganded structures of several PBPs a disulfide bridge is formed between cysteines at two loop structures, which move apart from each other when ligand binding induces closing of the binding domain (Sutcliffe *et al.,* 1996; Kang *et al.,* 1991; Oh *et al.,* 1993; Hsiao *et al.,* 1996; Gozlan and Ben-Ari, 1996; Yao *et al.,* 1994). After publication of our results, the crystal structure of the GluR-B S1S2 showed that the Cys residues at corresponding sites to Cys-740 and Cys-795 of the GluR-D are at such distance that a disulfide bridge can be formed only if agonist is not bound (Armstrong *et al.,* 1998; Armstrong and Gouaux, 2000). By connecting the two binding lobes of the ligand-binding domain the disulfide bond would favour the unliganded conformation, as strain would be introduced in the bound conformation. Thus, the structure also supports our finding that elimination of the bridge leads to increased agonist affinities, whereas the formation of a disulfide bridge leads to reduced agonist affinities.

Despite the evidence showing formation of a disulfide bridge between the two lobes of the AMPA receptor ligand-binding domain, the involvement of this bond in redox modulation of native receptors is still unclear. The disulfide bond may be important for stabilization of the native structure in general, and that role alone might be enough to explain the conservation of this bond throughout the iGluR family.

5.2 Charged residues in agonist binding

By using sequence alignment of the GluR-D S1S2 with the HBP and LAOBP, followed by mutation analysis, we identified candidate residues which may interact with the shared α-aminocarboxylate core of the amino acid ligands of these proteins. Nonconservative mutations R507K and E727A,Q,S resulted in a complete loss of detectable specific binding even at saturable (100 nM) concentration of [^{3}H]AMPA. In our sequence alignment Arg-507 aligned with Arg-77 of the LAOBP and HPB, which in their crystal structure makes ionic and hydrogen bond interactions with the α-carboxyl group of L-lysine and histidine ligands, respectively (LAOBP: Oh *et al.*, 1993; Oh *et al.*, 1994b; HBP: Yao *et al.*, 1994). Our

molecular model suggested that in the GluR-D ligand-binding pocket the cationic side-chain group of the Arg-507 is at a suitable distance from the anionic α-carboxyl group of the agonist so that ionic and hydrogen bond interactions are formed between these groups. This interaction is logical since the Arg residue is conserved throughout the iGluR family and all iGluR agonists have a carboxyl moiety attached to α-carbon of amino acid compound. Previously, a number of point mutations have been done on subunits of vertebrate iGluRs in order to study the effect of mutation on receptor activation or on ligand-binding affinity (see Table II). The studies where GluR-A subunit residues equivalent to Arg-507 of GluR-D were replaced by Glu, Gln or Lys resulting in a totally abolished agonist binding to the receptor, led to a suggestion that this residue is essential for agonist binding either directly or indirectly (Uchino *et al.*, 1992, see Table II: Kawamoto *et al.*, 1997). In addition, in NMDA receptors residues equivalent to Arg-507 of GluR-D has been shown to be essential for binding of glycine to NR1 subunits or binding of agonist to NR2B subunit (Kuryatov *et al.,* 1994; Wafford *et al.*, 1995; Hirai *et al.*, 1996, Laube *et al.*, 1997). Based on these analyses, some earlier models already suggested that residues corresponding to Arg-507 of GluR-D would have interaction with the α-carboxyl of the agonist (Kawamoto *et al.*, 1997; Laube *et al.*, 1997); whilst another model suggested that this Arg would interact with the γ-carboxyl group of the agonist. However, in the latter model the γ-carboxyl of the agonist is in a different orientation than the ligands bound to the LAOBP and HBP, thus making the α-carboxyl group a more reliable binding partner for Arg-507 (Sutcliffe *et al.*, 1996).

In the case of Glu-727 of the GluR-D, a comparison of the binding results from mutants E727D,A,Q,S showed that the negative charge at the position 727 is essential for the binding of agonist. The disruption of the negative charge by displacement of the Glu residue with residues Ala or Gln completely abolished the detectable [^{3}H]AMPA binding, whereas displacement with a negatively charged Asp led to an increased affinity for AMPA and a decreased affinity for glutamate and kainate. Our molecular model demonstrated how the anionic side chain group of Glu-727 (carboxylate group) is involved in the formation of salt bridges and hydrogen bonds with the agonist α-amino group. We suggest that the mutation E727D causes slight changes in the positions of residues near the agonist and results in slightly better contacts with AMPA compound and worse contacts with glutamate and kainate. These results indicate for the first time that Glu-727 of the GluR-D corresponds to the Asp-161 of the HBP and LAOBP and that also the α-amino group of the ligand is bound by similar mechanism in both these proteins. This strongly supports the sequence homology-

based prediction of the structural relationship between PBPs and the ligand-binding domains of iGluRs (Nakanishi *et al.*, 1990; O´Hara *et al.,* 1993). Although the residues corresponding to Glu-727 were not previously identified in binding of α-amino group of the agonist, some previous studies on equivalent residues suggests an essential role for this residue. Supporting our finding, a disruption of a negatively charged Glu residue (equivalent to Glu-727 of the GluR-D) by substitution with Gln or Ala was found to abolish kainate binding in the chicken KBP (Paas *et al.*, 1996). In addition, substitution of the equivalent Asp residue of the NR1 subunit with Gly, Ala or Glu strongly reduced the glycine sensitivity of the heteromeric NMDA receptor (Williams *et al.*, 1996).

Our prepictions that Arg-507 and Glu-727 are essential in the stabilization of the α-carboxyl and α-amino groups of the agonists were subsequently confirmed by the determination of crystal structure of the GluR-B S1S2 in complex with ligands (Armstrong *et al.*, 1998; Armstrong and Gouaux, 2000). The crystal structure also shows that the α-carboxyl and α-amino groups of the bound glutamate, AMPA or kainate form hydrogen bonds with side-chain or main-chain atoms of Thr-502, Ser-676 and with the main-chain of Pro-500. The interaction of glutamate with Pro-500 was also predicted in our model. Furthermore, a recent infrared spectroscopy measurements of the GluR-D S1S2 in the presence or absence of kainate or glutamate provided support for the proposal that Arg-507 and Thr-502 form hydrogen bonds to the α-carboxylate of the bound agonist, and that Glu-727 together with the backbone group of Pro-500 form hydrogen bonds to the α-amino groups of the agonist (Jayaraman *et al.*, 2000). The crystal structure of the GluR-B S1S2 with bound ligands were also consistent with our results which showed that mutation of charged residues Lys-431, Lys-432, Lys-471 and Glu-710 of GluR-D S1S2 did not affect the binding of agonist of antagonist. As we suggested, the crystal structure showed that respective residues of GluR-B were not in direct contact with bound agonists or antagonists (Armstrong *et al.*, 1998; Armstrong and Gouaux, 2000). However, some previous mutations of the GluR-A or -B (Li *et al.*, 1995), NR1 or NR2 (Wafford *et al.*, 1995, Laube *et al.*, 1997; Anson *et al.*, 1998) subunits on residues corresponding to Lys-471 of the GluR-D have shown to decrease agonist binding affinities.

We also analyzed the role of Glu-424 of the GluR-D in ligand binding, since in some previous predictions the residues corresponding to Glu-424 has been suggested to be the counterpart for the α-amino group of agonist (Paas *et al.,* 1996; Sutcliffe *et al.,* 1996; Laube et al., 1997). Mutations at this position in the GluR-A subunit (Mano *et al.*, 1996; Uchino *et*

al., 1992), in the NR1 and NR2 subunits (Kuryatov *et al.*, 1994; Laube *et al.*, 1997) and in the KBPs of goldfish and chicken (Wo and Oswald, 1996; Paas *et al.,* 1996) have been shown to reduce sensitivity to agonist (Table II). In addition, one recent study suggests that the residue the Glu-416 of GluR-A subunit (corresponding to Glu-424 of the GluR-D) participates directly in binding of willardiine agonists (Kizelsztein *et al.*, 2000). However, our results strongly suggests that Glu-424 does not form any important polar contacts with AMPA, glutamate or kainate, since our mutant E424A showed unchanged binding affinity for [^{3}H]AMPA and glutamate, and only slightly reduced affinity for kainate. In contrast to our results the crystal structure of the GluR-B S1S2 with bound agonists demonstrates that residue Glu-423 (corresponding to Glu-424 of the GluR-D) stabilizes the agonist-bound domain structure by forming an interdomain hydrogen bond E424-T707 (Armstrong *et al.*, 1998). However, we suggest that if such an interdomain hydrogen bond would be present in the GluR-D our results from mutant E424A should show significant decrease in agonist affinities. Yet, another indirect role for Glu-424 is suggested by a recent fluorescence titration studies with the GluR-D S1S2 proteins carrying mutations at position Glu-424 or Tyr-472 (see Table II: Abele *et al.*, 2000). In that study the residue Glu-424 is suggested to interact via a water molecule with the side-chain OH group of Tyr-472. The aromatic ring of Tyr-472 has direct hydrophobic interactions with agonists. The presence of a hydrogen bond between Glu-424 and Tyr-472 is not supported by our results, since substitution of Tyr-472 with Phe in the GluR-D eliminated the side-chain OH-group of Tyr, however the mutation did not lead to any changes in glutamate or kainate affinity.

5.3 Agonist vs. antagonist binding

We identified three individual mutations (L672A, G675A and T677A) which selectively altered agonist binding but had unaltered antagonist affinities. Moreover, a further mutation (Y472F), which strongly reduced the affinity for the antagonist DNQX, whilst leaving most of the agonist affinities unaltered, was also identified.

Replacement of Tyr-472 of the GluR-D S1S2 with Phe showed that DNQX and AMPA binding is affected by this residue. Affinities for AMPA and DNQX were reduced, whilst the affinities for kainate and glutamate were unchanged. Our molecular model predicted that the aromatic ring of Tyr-472 has favourable stacking interactions with the aromatic ring of DNQX. We further suggested that the hydroxyl group attached to the aromatic ring of Tyr-472 is important for the favourable orientation of that ring, since the elimination of the OH-group caused DNQX affinity to be reduced. Consistent with our

results, the subsequently published crystal structure of the GluR-B S1S2 in complex with DNQX showed that the aromatic ring of Tyr-471 (corresponding to Tyr-472 of the GluR-D) has favourable hydrophobic π-stacking interactions with the quinoxalinedione rings of DNQX in GluR-B (Armstrong and Gouaux, 2000). In addition, the crystal structure shows that Tyr-471 makes only weak hydrophobic interactions with the isoprenyl group of kainate, which is consistent with the unaltered affinity for kainate in our Y472F mutant. Previously described mutation experiments have also shown that the Tyr residue at this position is involved in antagonist binding, since mutation of Tyr to Ile in the chicken KBP (Paas *et al.*, 1996) or Tyr to Ser in the gold fish KBP (Wo and Oswald, 1996) led to a decreased interaction with CNQX.

The alanine scanning mutation of N-terminal S2 residues (Leu-672–Lys-678) showed that Leu-672, Gly-675, and Thr-677 participate selectively in agonists binding, but are not involved in binding of DNQX or Ro-48 8587 antagonists. The binding data was interpreted by constructing a ligand-docking model of the GluR-D S1S2 on the basis of the newly published crystal structure of the GluR-B S1S2 in complex with ligands. These new crystal structures also showed that the residues corresponding to the GluR-D Leu-672–Ser-674 form a loop structure and the residues corresponding to Gly-675–Lys-678 forms the N-terminal part of helix F in the GluR-B S1S2 structure (Armstrong *et al.*, 1998; Armstrong and Gouaux, 2000). The completely abolished affinity of T677A mutant for [^{3}H]AMPA and reduced affinities for all agonists as tested by [^{3}H]Ro-48 8587 competition assay were explained by the ligand docking models of the GluR-D S1S2. The docking models were almost identical to the template structures of the GluR-B S1S2 ligand complexes, since the amino acid sequence of the GluR-D and GluR-B subunits differ by only one residue within the S1S2 region. Both the GluR-D S1S2 model and the GluR-B S1S2 structure show that the side-chain OH-group of Thr-677 is hydrogen bonded to the γ-carbon substituents of kainate (carboxymethyl group) and glutamate (γ-carboxylate) and to the distal anionic group of AMPA (the hydroxyl group in the isoxazole ring of AMPA). Thus, the strongly reduced agonist affinities of the T677A mutant is well explained by the loss of hydrogen bonding between the distal anionic groups of the agonists and Thr-677. Consistent with our binding data, Thr-to-Ala mutations at this position have previously been shown to abolish kainate affinity for the chicken KBP (Paas *et al.,* 1996) or to reduce glutamate affinity for NMDA receptors (Anson *et al.,* 1998).

The strongly reduced affinity for all agonists in the L672A mutant was explained by the loss of hydrophobic contacts in our ligand docking models of the GluR-D S1S2. These

agonist docking models are similar to the crystal structure of the GluR-B S1S2 in complex with kainate, whereas in glutamate complex the conformation of the side chain residue of Leu-672 is different. However, in the GluR-B S1S2 structure, Leu-671 clashes with the isoprenyl group of kainate preventing full closure of the ligand-binding domain. Whereas the Phe-724 in GluR-D (at position corresponding Tyr-723 of the GluR-B) packs against Leu-672 and ideally orientates it to form hydrophobic interactions with AMPA, glutamate and kainate. It has been shown that Leu-688 to Val or Thr mutation leads to an increased affinity for kainate and Leu-688 to Ala mutation to a decreased sensitivity to glutamate desensitization (Mano *et al.*, 1996) Based on these early findings and the crystal structures, it was suggested that the ligand-binding domain of kainate receptors is fully closed upon kainate binding due to the small Val residue at a position corresponding to Leu-672 in the GluR-D (Armstrong and Gouaux, 2000). This closure was suggested to explain the desensitization of kainate receptors by kainate activation, whereas AMPA receptors are not desensitized by kainate (Armstrong and Gouaux, 2000). However, our binding data suggests that even if the Ala side-chain introduced by mutation (L672A) would have favourable interactions with the isoprenyl group of kainate in the GluR-D S1S2, the loss of hydrophobic contacts with the Leu side-chain would probably favour the overall less stable closed-state structure.

The Ala substitution of residue Gly-675 had a strikingly specific effect on the AMPA binding. The [^{3}H]AMPA binding was totally abolished, whereas affinity for kainate was only moderately reduced and affinity for glutamate was unchanged. This specific effect on the AMPA binding was explained with the ligand-docking models, which indicate that a water molecule near the Gly-675 is only present in the AMPA complex. In the AMPA complex that water forms hydrogen bonds with the negatively charged hydroxyl group in the isoxazole ring of AMPA, the α-carboxylate of AMPA and the main-chain NH-group of Thr-677. Thus, the mutation of Gly-675 to Ala introduces an additional methyl side-chain group, which displaces the water molecule important for AMPA binding. Consistent with our results, a previous substitution of the Gly residue at this position with Ala in the NR2 subunit has been shown to remarkably reduce glutamate affinity of the NMDA receptor (Anson *et al.*, 1998).

Although the residues mentioned above specifically affected agonist binding, none of mutations at Leu-672–Lys-678 analyzed by ligand-binding assay with Ro-48 8587 or DNQX, affected antagonist binding. The DNQX docking model of the GluR-D S1S2 is identical to crystal structure of the GluR-B S1S2 and both show that the helix F region is not in contact with DNQX. Similarly, the docking of Ro-48 8587 showed no direct interactions with helix F. This suggests that the helix F region may not be involved in antagonist binding at all. Such a

selective participation of the helix F in agonist binding may have importance in receptor activation. In general, activation is thought to initially involve either large or more local conformational changes within the ligand-binding domains, which are then converted to conformational shifts of the channel forming segments (Abele *et al.,* 1999; Madden, 2002; Arinaminpathy *et al.,* 2002). The opening of the channel in receptor activation could be achieved by transmitting the conformational changes within the helix F region (Domain 2) to the M3 and M1 segments, since these membrane segments are directly linked to the helix F region and to C-terminus of the S1, respectively.

On the other hand, in bacterial PBPs the ligand-binding role of the region corresponding to the helix F region is different. Although, the LAOBP and HBP (Oh *et al.*, 1993; 1994a,b) have direct ligand interactions at the helix F region with residues corresponding to Leu-672 and Thr-677 of GluR-D, not all PBPs have ligand interactions with this region (Kang *et al.,* 1991; Oh *et al.,* 1993; 1994a,b; Yao *et al.,* 1994; Hsiao *et al.,* 1996, Sun *et al.,* 1998). The agonists of the iGluRs have always an anionic group attached to the γ-carbon, whereas the amino acid ligands of PBPs have largely variable side-chain residues. Due to this, both the binding mechanisms of side chain groups of the amino acid ligands of PBPs and the location of the binding site for these groups can vary greatly in PBPs (Mayer *et al.,* 2001). Thus, the involvement of helix F may be important for iGluR activation, but the activation of bacterial PBPs (which are not connected directly to a channel) may not require contacts with the helix F region.

5.4 Conclusions

In the present work, key residues involved in agonist binding were identified by combining analysis of ligand-binding properties of the mutated GluR-D S1S2 protein with molecular modelling of the binding-domain. Furthermore, mutation studies and protein chemical analysis demonstrated the presence of a disulfide bridge, which appears to be important for the stability of the ligand-binding domain structure. Overall, the present results provide support for a structural and functional relationship between bacterial amino acid binding proteins and the ligand-binding domain of ionotropic glutamate receptors. Despite the distinct functional roles of these proteins, they share a similar mechanism of ligand-binding and structural stabilization of the binding domain by a disulfide bond.

The present work also demonstrates clear differences in the binding modes of agonists as compared to antagonists to the GluR-D. In particular, the residues in the helix F region have an important role in agonist vs. antagonist discrimination. Since the helix F region is adjacent to the channel forming segments, the identification of the agonist interactions within this region may have relevance in understanding the activation mechanism of the receptor.

Table II. Residues important for ligand binding by ionotropic glutamate receptors

	Mutation[b]	Location/function[c] (crystal structure)	GluR-B Equivalent[d]	Effects of mutations on activity[e]	Refs
AMPA receptors					
GluR-A	E398Q	Pocket/lobe-lobe contacts	E423	13.5×↑ in Qius EC_{50}; 15×less densitization in response to Qius	10
	Y446F	Pocket/KA binding	Y471	Less desensitization in response to Glu and Quis (not fully determined)	
	L646T	Pocket/van der Waals contact with KA	L671	20×↓in KA EC_{50}	
	L646A		L671	More than 200×↓desensitization in response to Quis	
	S650V	Glu, KA and AMPA binding	S675	11.5×↑ in Quis EC_{50} ;more than 200×↓in desensitization in res ponse to Quis	
GluR-A	E398K	Pocket/lobe-lobe contacts	E423	100 000×↑, 45×↑, 27×↑, 21×↑ in Glu, Quis, KA and AMPA EC_{50}, respectively	2,11
	D443K	Outside the pocket	D468	5×↑, 5×↑, 4×↑, 3×↑ in Glu, AMPA, Quis and KA EC_{50}	
	K445Q	Outside the pocket	K470	51×↑, 3×↑, 0×↑in AMPA, Glu and KA EC_{50}	
	K445E		K470	22×↑, 17×↑,6×↑, 0×↑in Glu, Quis, AMPA and KA EC_{50}	
	R481Q,K	DNQX, KA, Glu and AMPA binding		Complete loss of channel activity and AMPA affinity	
GluR-A	L497Y	Far from the pocket	L505	Complete block of desensitization	12
GluR-B	K449E	Outside the pocket	K470	3.5×↑ in Glu and KA EC_{50}; no change in AMPA EC_{50};	2
GluR-C	T504A	DNQX, KA, Glu and AMPA binding	T501	134×↑in Glu EC_{50}	12
	L507Y	Far from the pocket	L504	Complete loss of desensitization	
	L507S,T		L504	No change in desensitization response to Glu	
GluR-C_{flop}	F728Y	Pocket	Y723	7×↑ in KA affinity, 33×↓ in (R,S)-BrHIBO affinity; and ~WT affinity in Glu	16
	F728Y, G769R		Y723,G764	12×↑in KA affinity; 28×↓ in (R,S)-BrHIBO affinity and ~WT affinity in Glu	
GluR-A_{flop}	Y716F	Pocket	Y723	14×↓ in KA affinity, 23×↑in (R,S)-BrHIBO affinity;	16
	Y716F, R757G		Y723,G764	19×↓ in KA affinity, 20×↑in (R,S)-BrHIBO affinity	
GluR-A	A636T		Not in 3-d str (A643 in M3)	200×↓ and 85×↓ in Glu and KA EC_{50}	17
GluR-A_o	E398Q	Pocket/lobe-lobe contacts	E423	88×↑, 10×↑, and 2×↓ in 5H-willardiine, 5F-willardiine and 5I-willardiine EC_{50}; and ~no change in 5Br-W and KA EC_{50}	15
	Y446F	Pocket/KA binding	Y471	10×↑, 9×↑, 15×↑ and 3.6×↑ in H-W, F-W, Br-W and I-W EC_{50} and no change in KA EC_{50}	
	L646A	Pocket/van der Waals contact with KA	L671	247×↑, 67×↑,245×↑ and 2×↑ in H-W, F-W, Br-W and I-W EC_{50} , respectively and no change in KA EC_{50}	

	Mutation[b]	Location/function[c] (crystal structure)	GluR-B Equivalent[d]	Effects of mutations on activity[e]	Refs
	S650A	Glu, KA and AMPA binding	S675	~5×↑, ~5×↑ and 2×↑ in H-W, Br-W and I-W EC_{50} and ~no change in F-W and KA EC_{50}	
GluR-D [h]	E424D	Pocket/lobe-lobe contacts	E423	5×↓ and 2.5×↓ in Glu and KA affinities; and ~WT affinity in AMPA	14
	E424Q			28×↓ and 24×↓ in Glu and KA affinities and ~WT affinity in AMPA	
	K471H	Outside the pocket	K470	5×↓ and 4×↓ in Glu and AMPA affinitities and ~WT affinity to KA	
	K471A		K470	3×↓ in AMPA affinity and ~WT affinities to Glu and KA	
	K471R		K470	No change in AMPA, Glu and KA affinities	
	Y472F	Pocket/KA binding	Y471	15×↓, 12×↓ and 2.3×↓ in AMPA, Glu and KA affinities	
	R507K	DNQX, KA, Glu and AMPA binding	R506	23000×↓ and 865×↓ in AMPA and Glu affinities	
	E727D	Glu, KA and AMPA binding	E726	2400×↓ and 56×↓ in KA and Glu affinities; ~WT affinity to AMPA	
	E727A			25000×↓ and 41600×↓ in AMPA and Glu affinities	
	E727Q			AMPA no binding; Glu NB; and KA not determined	
GluR-D [h]	E424D	Pocket/lobe-lobe contacts	E423	7×↓Glu affinity, ~ no change in AMPA and KA affinity	13
	E424Q		E423	6×↓and 4×↓in Glu and KA affinities; AMPA affinity as WT	
	E424E	Pocket/lobe-lobe contacts	E423	6×↓ in KA affinity; no change Glu and AMPA affinities	
	K471R,A	Outside the pocket	K470	~No change in Glu, AMPA and KA affinities	
	K471H			4×↓and 2.5×↓ in Glu and AMPA affinities; KA affinity as WT	
	Y472F	Pocket/KA binding	Y471	10×↓ and 4×↓ in AMPA and DNQX affinities; ~no change in Glu and KA affinities	
	R507K	DNQX, KA, Glu and AMPA binding	R506	Complete loss of AMPA affinity	
	L672A	Pocket	L671	1750×↓, 77×↓ and 25×↓ in AMPA, Glu and KA affinities; DNQX and Ro 48-8587 affinities ~WT	
	D673A	Pocket	D672	2.6×↓, 2-6×↓ and 2.2×↓ in AMPA, Glu and KA affinities; 1.6×↑ in Ro 48-8587 affinity; DNQX affinity ~WT	
	S674A	Pocket	S673	1.3×↓ and 1.5×↓ in AMPA and Ro 48-8587 affinities; no change in Glu, KA and DNQX affinities	
	G675A	Pocket	G674	225×↓ and 6×↓ in AMPA and KA affinities; Glu, DNQX and Ro 48-8587 affinities ~WT	

	Mutation[b]	Location/function[c] (crystal structure)	GluR-B Equivalent[d]	Effects of mutations on activity[e]	Refs
	S676A	Main chain contacts with KA ,Glu, AMPA	S675	3.7×↓, 2-2.8×↓ and 1.6×↓ in AMPA, Glu and Ro 48-8587 affinities; KA and DNQX affinities ~WT	
	T677A	Side chain contacts with KA, Glu and AMPA	T676	25000×↓, 3800×↓ and 13×↓ in AMPA, Glu and KA affinities; DNQX and Ro 48-8587 affinities ~WT	
	K678A	Outside the pocket	K677	4×↓, 2.4×↓ in AMPA and KA affinities; Glu, DNQX and Ro 48-8587 affinities ~WT affinities	
	E727D	Glu, KA and AMPA binding	E726	840×↓ and 9×↓ in KA and Glu affinities; 8×↑ and 38×↑ in AMPA and DNQX affinities	
	E727Q,S,A		E726	Complete loss of AMPA affinity	
NMDA receptors					
NR1	Q387K	Pocket/lobe–lobe contacts	E423	14 000×↑in Gly EC_{50}; 13×↑in Glu EC_{50}	2,1
	F390T,W,A,S	Cleft	Y426	35–63×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	Y392A	Far from the pocket	M428	12×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	D481N	Outside the pocket	D468	7×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	K465E		K470	420×↑in Gly EC_{50}; ~5×↑in Glu EC_{50}	
	K483Q		K470	125×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	K483A		K470	3×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	F466A,H	Pocket/KA binding	Y471	6300×↑and 2100×↑in Gly EC_{50}; 10×↑and 3×↑in Glu EC_{50}	
	F466Y		Y471	~No change in Gly EC_{50}; ~no change in Glu EC_{50}	
	F515A	Near (before) hinge1[g]	F516	Complete loss of response to agonists	
	V666A	Pocket	L671	13×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	S669G	Pocket	G674	25×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
	D732E, G,N,A	Pocket/KA binding	E726	4200–36 400×↑in Gly EC_{50}; ~no change in Glu	
	F735A,S	Near (before) hinge2[g]	L748	16×↑and 23×↑in Gly EC_{50}; ~no change in	
	F736A,S	Near (before) hinge2	D749	28×↑in Gly EC_{50}; ~no change in Glu EC_{50}	
NR1	A714T	Pocket/lobe–lobe contacts	T707	4×↓in Gly EC_{50}	3
	A714C,F,E		T707	2–9×↑in Gly EC_{50}	
	A714K,Y,V		T707	14–16×↑in Gly EC_{50}	
	A714I,Q,R,L		T707	34–62×↑in Gly EC_{50}; A714L, no change in Glu	

	Mutation[b]	Location/function[c] (crystal structure)	GluR-B Equivalent[d]	Effects of mutations on activity[e]	Refs
NR2B	E387A	Pocket/lobe–lobe contacts	E423	237×↑in Glu EC_{50}; no change in Gly EC_{50}	1
	F390S	Cleft	Y426	47×↑in Glu EC_{50}; no change in Gly EC_{50}	
	K459E	Outside the pocket	K470	180×↑in Glu EC_{50}; no change in Gly EC_{50}	
	H460F	Pocket/KA binding	Y471	9×↑in Glu EC_{50}; no change in Gly EC_{50}	
	S486A	Pocket/KA binding	P499	43×↑in Glu EC_{50}; no change in Gly EC_{50}	
	R493K	Pocket/KA binding	R506	Complete loss of response to agonists	
	V660A	Pocket	L671	18×↑in Glu EC_{50}; no change in Gly EC_{50}	
	S664G	Pocket/KA binding	S675	118×↑in Glu EC_{50}; no change in Gly EC_{50}	
	V709A	Pocket	M729	27×↑in Glu EC_{50}; no change in Gly EC_{50}	
NR2A	N463A	Outside the pocket	D468	6×↑in Glu EC_{50}	4
	K465E	Outside the pocket	K470	10×↑in Glu EC_{50}	
	H466A	Pocket/KA binding	Y471	216×↑in Glu EC_{50}	
	H466F		Y471	13×↑in Glu EC_{50}	
	T665A	Outside the pocket	T670	7×↑in Glu EC_{50}	
	V666A	Pocket	L671	12×↑in Glu EC_{50}	
	G669A	Pocket	G674	320×↑in Glu EC_{50}	
	T671A	Pocket/KA binding	T676	1030×↑in Glu EC_{50}	
Kainate receptors					
CKBP	E33V	Pocket/lobe–lobe contacts	E423	112×↓in KA affinity; no change in Glu affinity	5
	Y36F	Cleft	Y426	30×↓in Glu affinity; 5×↓in KA affinity	
	Y36I		Y426	Complete loss of KA affinity	
	K72A,S	Outside the pocket	K470	No change in KA and Glu affinities	
	Y73I	Pocket/KA binding	Y471	90×↓in Glu affinity; 10×↓in KA affinity; >250-fold ↓in CNQX affinity	
	P100A	Pocket/KA binding	P499	10×↓in CNQX affinity; no change in KA and Glu affinities	
	T102A	Pocket/KA binding	T501	100×↓in Glu affinity; 58×↓in KA affinity	
	R107S	Pocket/KA binding	R506	Complete loss of KA affinity	
	S266A	Pocket	G674	5.5×↓in KA affinity; no change in Glu affinity	
	S267A	Pocket/KA binding	S675	5×↓in KA affinity; no change in Glu affinity	
	T268A	Pocket/KA binding	T676	Complete loss of KA affinity	
	Y299A	Pocket/lobe–lobe contacts	T707	5.8×↓in KA affinity; no change in Glu affinity	
	E316A	Pocket/KA binding	E726	Complete loss of KA affinity	
	F342I	Hinge 2–Pocket	Y753	Complete loss of KA affinity	
GFKAR[b]	Q12E	Pocket/lobe–lobe contacts	E423	5×↑in Glu affinity; ~no change in KA affinity	6
	A51K	Outside the pocket	K470	13×↓in Glu affinity; 2.6×↓in KA affinity	

	Mutation[b]	Location/function[c] (crystal structure)	GluR-B Equivalent[d]	Effects of mutations on activity[e]	Refs
	Y52S	Pocket/KA binding	Y471	>100-fold ↓in KA affinity; .40-fold↓in CNQX affinity	
	Y52F		Y471	Slight ↓in KA affinity; no change in Glu affinity	
	C305S	S–S bridge (see text)	C739	3.5×↑in KA affinity Loss of binding modulation} 12×↑in KA affinity {under redox conditions	
	C358S	S–S bridge (see text)	C794	5×↑in Glu affinity; ~no change in KA affinity	
GluR5	S721N	Pocket/lobe–lobe contacts	T707	Drastic loss of sensitivity to AMPA and iodowillardiine	7,8
GluR6	A689S	Pocket/KA binding	S675	Slower desensitization rate in response to KA	7,8
	N721S	Pocket/lobe–lobe contacts	T707	Becomes responsive to AMPA and iodowillardiine	

The table is modified from Paas *et al.,* (2000) Abbreviations: AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX: 6,7-dinitroquinoxaline-2,3-dione; GFKAR: gold fish kainite binding receptor Glu: glutamic acid; KA: kainic acid; Quis: quisqualate; W: willardiine.

[a] The effects of mutations were examined using homo-oligomeric receptor assemblies with the exception of the NMDA receptors, and GluR-B (which was assembled with GluR-A) and study of GluR-A/GluR-C chimeras by Banke et al, 2001 (ref, 16). [b] In some cases the amino acid numbers include the signal peptide. [c] 'Location' corresponds to the place of the native amino acid in the crystal structures. The term 'pocket' means that the amino acid lines the water-accessible surface of the ligand-binding pocket. The term 'cleft' means that the residue is projecting into the interlobe cleft but does not necessarily contribute to the water-accessible surface of the pocket. The specific chemical interactions between ligands and receptor subunits are provided in Ref. Armstrong, N. *et al* (1998) (the crystal structure). [d] The numbering of GluR-B S1S2 residues include the signal peptide; the equivalence is according to the sequence alignment presented by Armstrong et al. 1998. [e] Activity is determined by measurements of: (1) flow of ionic currents through open channels (EC50 is the agonist concentration that gives half-maximal response); (2) desensitization (closure of channels) following agonist-elicited responses; and (3) ligand-binding affinity by monitoring the binding of radiolabelled ligands to the receptor and/or inhibition of radiolabelled ligand binding by unlabelled ligands. [f] K70 is located in loop 2 and its side-chain projects out of the ligand-binding pocket. However, it is very close to D672 and S673, which belong to the lower lobe, and thus, its substitution might interfere with the degree of lobe closure necessary for tight accommodation of Glu and AMPA. [g] Hinges 1 and 2 of GluR-B-S1S2 are formed by β-strands 7 and 12, respectively (Armstrong *et al.* 1998). [h] Water-soluble S1S2 fragment of GluR-D was used in this study (Lampinen *et al.* 1998). 1. Laube *et.al.,* 1997. 2. Dingledine *et al.,* 1999. 3. Wood *et al.,* 1997. 4. Anson *et al.,* 1998. 5. Paas *et al.,* 1996. 6. Wo and Oswald, 1996. 7. Swanson, *et al.,* 1997. 8. Swanson *et al.,* 1998. 10. Mano *et al.,* 1996. 11. Kawamoto *et al.,* 1997. 12. Stern-Bach *et al.,* 1998. 13. Lampinen *et al.,* 1998. 14. Abele *et al.,* 2000. 15. Kizelstein *et al.,* 2000. 16. Banke *et al.,* 2001. 17. Taverna *et al.,* 2000.

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