Structural and functional architecture of AMPA-type glutamate receptors and their auxiliary proteins

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In brief

AMPA-type glutamate receptors are major mediators of excitatory synaptic transmission and plasticity. In this review Greger et al. summarize latest insights into receptor architecture, their assembly, and functional association with auxiliary subunits. They discuss how the unique and versatile receptor structure is beginning to explain their role in information processing at synapses.

Abstract

AMPA receptors (AMPARs) are tetrameric ion channels that together with other ionotropic glutamate receptors (iGluRs), the NMDA- and kainate receptors, mediate a majority of excitatory neurotransmission in the central nervous system. Whereas NMDA receptors gate channels with slow kinetics, responsible primarily for generating long-term synaptic potentiation and depression, AMPARs are the main fast transduction elements at synapses and are critical for the expression of plasticity. The kinetic and conductance properties of AMPARs are laid down during their biogenesis, and are regulated by post-transcriptional RNA editing, splice variation, post-translational modification and subunit composition. Furthermore, AMPAR assembly, trafficking and functional heterogeneity depends on a large repertoire of auxiliary subunits – a feature that is particularly striking for this type of iGluR. Here, we discuss how the subunit structure, stoichiometry and auxiliary subunits generate a heterogeneous plethora of receptors, each tailored to fulfill a vital role in fast synaptic signaling and plasticity.

Introduction

Glutamate receptors are the primary mediators of excitatory synaptic transmission in the brain (Traynelis et al., 2010). AMPA receptors (AMPARs), together with other ionotropic glutamate receptor (iGluR) family members, N-methyl-D-apartate receptors and kainate receptors (NMDARs and KARs), are cation permeable receptor tetramers. iGluRs are localized at excitatory central synapses and primed for activation by presynaptically released L-glutamate. The exceptionally rapid kinetics of the AMPARs, on the sub-millisecond time scale, ensures fast depolarisation of the postsynaptic membrane, allowing high fidelity propagation of impulses between nerve cells. The AMPARs also act as one of the gatekeepers of NMDAR-dependent synaptic plasticity by relieving their voltage-dependent channel block by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984), allowing the postsynaptic Ca^{2+} entry that initiates changes in synaptic strength (Bliss and Collingridge, 1993; Huganir and Nicoll, 2013; Kessels and Malinow, 2009). At some synapses, AMPARs can also mediate calcium influx directly, triggering various forms of postsynaptic plasticity (Cull-Candy et al., 2006; Liu and Zukin, 2007).

AMPARs are the prime elements that undergo change during synaptic plasticity, through alterations in their number (by endo- or exocytosis and lateral diffusion), subunit composition, protein partner interactions, or phosphorylation state (Huganir and Nicoll, 2013; Newpher and Ehlers, 2008; Opazo and Choquet, 2011; Shepherd and Huganir, 2007). Identifying how the various building blocks determine AMPAR function at central synapses, and dissecting the mechanisms controlling their biogenesis is key to understanding a fundamental function of the brain – information transfer and storage. On the darker side, AMPARs are important players in triggering the cell damage associated with a large number of neurological conditions and brain trauma (Bowie, 2008).

AMPAR assemblies are complex signalling machines that function as homo- or heterotetramers built from combinations of four core subunits, GluA1 to -4. As each subunit differs in its contribution to channel kinetics, ion selectivity and receptor

trafficking properties, heteromerisation adds considerable functional diversity. Alternative mRNA processing of GluA subunits widens the receptor complement further, producing distinct AMPAR isoforms (Seeburg and Hartner, 2003). Of particular consequence is an RNA editing event, which modifies the pore region of the GluA2 subunit, influencing calcium permeability and channel block by endogenous polyamines. While many AMPARs appear to be heteromeric GluA2-containing assemblies with low calcium permeability ('calcium impermeable'; CI-AMPAR), GluA2 lacking, calcium permeable AMPARs (CP-AMPARs), form a significant and important minority that plays key roles in synaptic signalling, plasticity and disease (Cull-Candy et al., 2006; Liu and Zukin, 2007; Luscher and Malenka, 2011).

It is not merely the pore-forming subunits that control the functional properties of AMPARs. Many auxiliary subunits determine basic features of receptor gating, channel conductance, pharmacology and expression at synapses (Haering et al., 2014; Jackson and Nicoll, 2011; Straub and Tomita, 2012). The expression pattern, composition and stoichiometry of these auxiliary proteins within the AMPAR complex appear to vary greatly between neuron types and between synapses of individual neurons. Together with the differential expression of AMPAR core subunits (GluA1-4), the auxiliary subunits add considerably to the variation in AMPAR properties, and hence to the possible diversity of information transfer at different synapses and circuits within the brain.

Here we will consider the building plan of the AMPAR – their architecture, assembly and interaction with auxiliary subunits. We will examine how this plan produces a plethora of receptors with distinct functional properties, and the significance of this variety for the key task of information processing in the brain.

The architecture of AMPA-type iGluRs

When compared with other types of ligand-gated ion channels, such as purinergic (P2X)(Kawate et al., 2009) and Cys-loop receptors (Miller and Aricescu, 2014; Unwin, 2005), the eukaryotic tetrameric iGluRs display a unique modular architecture. Each subunit consists of four distinct domain layers – an extracellular N-terminal domain

(NTD; also referred to as the amino-terminal domain or ATD), a ligand-binding domain (LBD), a membrane-embedded transmembrane domain (TMD) that forms the ion channel, and a cytoplasmic C-terminal domain (CTD) (**Figure 1**).

The extracellular region (ECR) encompasses the vast majority of the receptor (~ 85% of its mass). Arranged as an intertwined dimer of dimers, the ECR displays 2-fold symmetry, whereas the ion channel exhibits four-fold symmetry (Sobolevsky et al., 2009) (**Figure 1B**). These three domain layers (NTD/LBD/TMD) are connected by peptide linkers, which render the receptor highly flexible. Together, these architectural features underlie the complex and versatile gating behavior of iGluRs (e.g. (Robert and Howe, 2003)).

Sequence analyses indicate that eukaryotic iGluRs are the product of gene fusion events, and that the ion channel and LBD, both highly conserved throughout vertebrates (**Figure 1A,** see also **Figure 5A**), form a core unit that is shared with prokaryotes. However, prokaryotic iGluRs, such as GluR0 (Chen et al., 1999), lack the sequence-diverse NTD and CTD as well as the transmembrane segment M4. Below, we discuss these domain layers individually, before considering their influence on the receptor as a whole.

The C-terminal domain (CTD)

The cytoplasmic CTD, which varies in length between subunits and splice variants, has been a focus of intense efforts to understand subunit-specific AMPAR trafficking, a process that drives synaptic plasticity (Shepherd and Huganir, 2007). The C-tails participate in subunit-specific protein interactions that can be regulated by phosphorylation and have been implicated in multiple stages of AMPAR trafficking. Nevertheless, the precise role of the C-tail in receptor trafficking that underlies the expression of long-term potentiation (LTP) still remains to be clarified (Boehm et al., 2006; Granger et al., 2013; Hosokawa et al., 2015; Kim et al., 2005). Beyond trafficking, phosphorylation of the CTD has been shown to influence channel conductance alter peak channel open probability and impact on auxiliary subunit enhancement of channel conductance (Derkach et al., 1999) (Kristensen et al., 2011).

The transmembrane domain (TMD)

The TMD is formed of four helical elements: M1-4 (**Figure 1A, E**). The early appreciation that this region evolved from a precursor related to K^+ channels (Wo and Oswald, 1995), where a re-entrant loop forms the ion-selective constriction of the pore (Doyle et al., 1998), offered initial insights into the TMD architecture of iGluRs. However, while the K^+ channel's re-entrant loop dips into the membrane from the extracellular side, that of iGluRs (M2) enters from the cytoplasmic side, meaning that the channel is inverted compared with K^+ channels. This similarity between K^+ channels and AMPA- and kainate receptors is further emphasized by the fact that spermine blocks ion flow in both channel types (Bowie and Mayer, 1995; Fakler et al., 1994; Kamboj et al., 1995), although the underlying mechanism is complex, and is influenced by auxiliary subunits (see below).

The M2 segment forms a narrow constriction at the base of the channel pore, with the Q/R RNA editing site in GluA2 at the apex of this pore loop (**Figures 1A, E**). The editing induced switch from glutamine (Q) to arginine (R) renders GluA2-containing AMPARs far less permeable to calcium ions and resistant to polyamine block. Editing at this site is highly efficient (close to 100%) and is crucial for survival of the organism (Higuchi et al., 2000). The M2 loop is followed by the M3 transmembrane helix (**Figure 1B**). The channel gate, the most highly conserved region of iGluRs, is formed by crossover of the four M3 helices and constrains the pore at the extracellular side of the ion conduction path. The M3 helices are connected by linkers to the LBDs for activation gating. Mutations in M3 influence agonist efficacy, modulate the function of partial agonists, and impact on desensitization kinetics (Moore et al., 2013; Taverna et al., 2000). Recent crystal structures show that non-competitive antagonists such as GYKI and the anti-epileptic drug perampanel dock in the outer perimeter of the M3 helical bundle to antagonize the channel (Yelshanskaya et al., 2016). M4, and the trailing CTD are evolutionarily more recent additions and provide further versatility to metazoan iGluRs where they are essential in subunit assembly, trafficking and controlling the functional properties of the

predominantly heteromeric AMPARs found in higher eukaryotes (Herguedas et al., 2013; Salussolia et al., 2013) (see also **Figure 5A**).

The ligand-binding domain (LBD)

Whereas agonist-binding sites are formed at the interface *between* subunits in PTX channels and Cys-loop receptors, in iGluRs the agonist docks in dedicated ligand-binding domains (LBDs) (Armstrong and Gouaux, 2000; Stern-Bach et al., 1994) (**Figure 1B, D**).

The structure of the LBD (excised from the receptor) has been determined in various states, including unliganded (apo), agonist-bound, and antagonist-bound (Armstrong and Gouaux, 2000). This has provided atomic level details of ligand coordination (Mayer, 2006; Pohlsgaard et al., 2011) and generated gating models that have been explored further in intact AMPAR structures (Durr et al., 2014; Meyerson et al., 2014; Sobolevsky et al., 2009). The LBD is bilobate and belongs to the periplasmic-binding protein (PBP) superfamily that captures its ligand in an interlobe cleft, triggering closure of the clamshell-like structure (Quiocho and Ledvina, 1996) (**Figure 1A, D and 2A**). Since the LBDs of adjacent subunits dimerize back-to-back via their upper (D1) lobes, closure of the clamshell around glutamate causes separation of the lower (D2) lobes, which transmits mechanical force to open the channel gate followed by rapid desensitization (Mayer, 2006).

The key role of the LBD dimer interface in gating was recognized in early studies where dimer-stabilizing mutations were found to attenuate AMPAR desensitization (Stern-Bach et al., 1998; Sun et al., 2002). The base of the D1 interface forms an acceptor site for positive allosteric modulators (**Figure 1D**), which also stabilize the interface (Jin et al., 2005; Partin, 2015; Sun et al., 2002), a feature that has generated considerable interest because of its potential in the development of drugs to alleviate cognitive impairment and neurodegeneration (Partin, 2015).

mRNA processing events also target the D1 interface, resulting in functionally relevant variations in the LBD sequence. Alternative splicing of exons 14 and 15 (the

flip/flop cassette mapping to the D1 dimer interface) (Sommer et al., 1990) and RNA editing at the R/G site (Lomeli et al., 1994) (**Figure 1A, D**) impact on AMPAR gating kinetics (Mosbacher et al., 1994), subunit assembly (Greger et al., 2006) and trafficking from the ER (Coleman et al., 2006; Penn et al., 2008). This splicing event is regulated by neuronal network activity, and can therefore influence the assembly of kinetically different AMPARs as part of a homeostatic control mechanism (Penn et al., 2012). Flip/flop splicing also alters binding-site residues for allosteric modulators rendering some of these ligands (such as the widely used cyclothiazide) splice-isoform specific (Partin, 2015), and also influences interaction with auxiliary subunits (Cais et al., 2014; Kott et al., 2007; Semenov et al., 2012).

Subunit associations within the LBD layer appear to be relatively weak (Sun et al., 2002), permitting the rearrangements required for rapid gating transitions (Plested and Mayer, 2009). Early work implied that desensitization is associated with rearrangements at the D1 dimer interface (Armstrong et al., 2006; Sun et al., 2002) and recent structures revealed that a complete rupture of the LBD layer can accompany desensitization in intact GluA2 AMPARs, resulting in a transition from 2-fold to 4-fold symmetry and ultimately a decoupling of the agonist-activated LBD from the channel gate (Meyerson et al., 2014). GluA2 receptors trapped in various states exhibit transient, state-dependent inter-dimer interfaces (Durr et al., 2014; Meyerson et al., 2014; Yelshanskaya et al., 2014), where the LBD layer forms a 'gating ring' and expansion of this central opening appears to accompany activation across iGluR subfamilies (Mayer, 2016). In addition, interaction of auxiliary subunits with the LBD is expected to alter the conformational range displayed by this layer (see below) and thereby affect gating kinetics.

The LBD in AMPAR gating
Functional analysis, combined with a better understanding of LBD structure, has also revealed a clearer picture of other important features of the gating behavior of AMPAR channels. Both native and recombinant AMPARs open to several sub-conductance levels, in addition to their maximum conductance (Swanson et al., 1997); each appearing to correspond to varying degrees of ligand occupancy

(Rosenmund et al., 1998) (**Figure 2**). Thus, incremental steps in channel sub-conductance are thought to correspond to independently gated subunits (**Figure 2A**). In keeping with the view that AMPAR subunits can be separately gated, single-channel conductance is dependent on agonist concentration both in cerebellar (Smith et al., 2000) and hippocampal neurons (Gebhardt and Cull-Candy, 2006), a feature that appears unique to AMPAR channels (**Figure 2C**). Three temporally distinct conductance steps were initially observed (2 sub-conductances, and a main conductance) in a study using a fast application technique to switch from antagonist to agonist, with the smallest level apparently corresponding to closing of two LBDs (Rosenmund et al., 1998). However, others have identified 3 sub-conductances plus a main level under steady-state conditions (Kristensen et al., 2011; Prieto and Wollmuth, 2010; Zhang et al., 2008); summarized in (Shelley et al., 2012). Whether four stepwise (sequential) increases in conductance can be detected in the presence of AMPAR auxiliary subunits would now be of great interest, as it would signify whether or not the activation of a single subunit is indeed sufficient to gate the initial opening of a neuronal AMPAR. Emerging evidence suggests that this is indeed the case (Coombs et al., 2017).

It has been shown that CaMKII phosphorylation of heteromeric GluA1/2 AMPARs, an event implicated in expression of LTP at hippocampal CA1 synapses (Derkach et al., 1999; Kristensen et al., 2011), increases the efficiency with which each AMPAR subunit can activate. Thus, use of phosphomimetic mutants increased the average channel conductance by altering the relative percentage of time spent at each of the open levels. Surprisingly, this alteration in GluA1/2 gating appears entirely dependent on the presence of auxiliary subunits (Kristensen et al., 2011). A similar mechanism has recently been ascribed to the synaptic plasticity of GluA2/3 receptors in cerebellar Purkinje cells where gating of individual subunits in a tetramer is subject to regulation by cAMP (Gutierrez-Castellanos et al., 2017), suggesting this may represent a more widely utilized means of postsynaptic AMPAR plasticity.

In functional terms, glutamate binding at the LBD has conflicting requirements. For high frequency transmission, the LBD needs to open readily to allow trapped

glutamate to dissociate rapidly. By contrast, efficient transmission requires synchronous opening of AMPAR channels and hence rapid closing of the LBD. Destabilizing the closed-cleft conformation, by disrupting cross-cleft interactions, speeds channel deactivation and causes a reduction in the open probability of glutamate bound channels (Zhang et al., 2008). It has been suggested that the extent of LBD closure is the major determinant of agonist efficacy, with antagonists stabilizing a more open conformation, and partial and full agonists promoting increasing degrees of domain closure (Armstrong and Gouaux, 2000; Jin et al., 2003). However, the reduced efficacy caused by disrupting cross-cleft interactions does not appear to result from a reduced cleft closure. Interestingly, weakening cross-cleft interactions also increases prevalence of openings to lower sub-conductance levels and delays channel activation, underscoring that agonist efficacy is dependent on the stability of the closed-cleft conformation (Zhang et al., 2008).

There is compelling evidence from work examining *mode changes* in AMPAR channel gating that the number of occupied LBDs determines the apparent agonist affinity of the receptor (Prieto and Wollmuth, 2010). Thus, the lowest sub-conductance level (of a recombinant AMPAR) can arise from singly liganded AMPARs, existing predominantly in a low apparent affinity/ low open probability mode. Once a second LBD is occupied and affinity increases, other LBDs rapidly become occupied, entering the channel into a high open probability mode (Prieto and Wollmuth, 2010). This would suggest that glutamate binding to an LBD in one subunit influences binding/gating of its partner subunit – either *via* the dimer interface or possibly *via* an effect mediated by the ion channel which is allosterically coupled to the LBD.

Molecular dynamics simulations of the LBD have also examined whether an increased domain closure, or other structural changes (such as a relative twist between D1 and D2; (Holm et al., 2005)), fully describe increased ligand efficacy (Lau and Roux, 2011). These studies have shown that there is not a single low energy closed conformation. Rather, there appears to be a range of conformations with different extents of closure, with the minima differing for full and partial agonists. This fits well with observations from structural and functional studies identifying

conformational intermediates, with differences in domain closure for each of the four LBDs (Herguedas et al., 2016; Lau et al., 2013). Further, it has been proposed that partial agonists can stabilize the presence of non-conducting conformational intermediates (Salazar et al., 2017) (Ahmed et al., 2011), contributing to the low efficacy of partial agonists in activating channel openings.

Analysis of free energy landscapes has also revealed striking differences in the behavior of AMPA- and NMDAR LBDs (Yao et al., 2013). While the LBD of certain NMDAR subtypes can close 'fully' in the absence of agonist binding, for AMPARs this occurs only with agonist bound – suggesting an 'induced fit' mechanism. It is tempting to propose that this could be essential for high fidelity AMPAR synaptic signalling. Thus, closing of AMPAR LBDs in the absence of agonist would generate detrimental background 'noise', given that closing of a single LBD is sufficient to partially open the channel (**Figure 2C**).

It is worth emphasizing that despite the considerable amount of progress and valuable functional data gleaned from functional studies, there are still many surprising gaps as to how elementary features of channel behavior relate to AMPAR structure. In particular, it remains unclear (1) which changes in pore structure underlie the different sub-conductance levels; (2) how binding of glutamate to individual subunits triggers such changes; and (3) whether there are other functional differences between sub-states (beyond conductance), for example in ion permeability or polyamine block.

The N-terminal domain (NTD)
Similarly to the LBD, a two-fold symmetric architecture is also seen for the distal NTD (alternatively called amino-terminal domain, ATD), a segment that is unique to eukaryotic iGluRs. This bulky domain, encoded by 9 of the 16 exons, encompasses ~50% of an AMPAR subunit and drives receptor assembly (Herguedas et al., 2013), and plays a role in AMPAR anchoring at synapses (Watson et al., 2017). By analogy with NMDARs, further roles could include allosteric regulation of channel gating (Gielen et al., 2009; Yuan et al., 2009), although this remains to be established.

Like the LBD, the NTD also folds into a bilobate structure. Hence, the extracellular region of metazoan iGluRs is composed of eight clamshell-like domains (**Figure 1A. B**). This flexible, modular assembly might be capable of versatile allosteric regulation as ascribed to the NTDs of NMDARs (Zhu and Paoletti, 2015). Clamshell motions of AMPAR NTDs have indeed been inferred from coarse-grained simulations, yet no known ligand has been identified (Dutta et al., 2012; Sukumaran et al., 2011). While NTD-deleted AMPARs are fully functional, they exhibit subtly altered desensitization kinetics, underlining that this domain can influence the motions of the LBDs below (Moykkynen et al., 2014).

Structurally, NTD dimers resemble the ligand-binding cores of type-C G-protein coupled receptors (GPCRs), such as metabotropic glutamate receptors (mGluRs) and GABA$_B$ receptors. Ligand binding in these GPCRs initiates downstream signaling cascades, where clamshell cleft closure is associated with rearrangement of the dimer (Krieger et al., 2015; Kunishima et al., 2000; Tsuchiya et al., 2002). In contrast with the LBDs, the NTDs of AMPARs and KARs form tight dimers (Kumar et al., 2011; Rossmann et al., 2011; Zhao et al., 2012) (**Figure 1C**), a feature which is expected to restrict NTD mobility. An exception to this is GluA3, which has uniquely flexible NTD lower lobes (Sukumaran et al., 2011), that could potentially expose modulator binding sites, as has been described for mGluRs (Tsuchiya et al., 2002) and NMDARs (Mony et al., 2011). The functional relevance of the unique GluA3 NTD arrangement is currently unknown and while it appears to promote assembly into heteromers (Rossmann et al., 2011), it raises intriguing questions about possible pharmacological differences between GluA3 and other AMPAR subunits. This may be of particular relevance in light of the recently elucidated role of GluA3 in Alzheimer's disease (Reinders et al., 2016).

Dynamics of the AMPAR ECR
In NMDARs, the NTD and LBD layers are closely associated, and thus well poised to allow allosteric coupling between the NTD and the LBD (Karakas and Furukawa, 2014; Lee et al., 2014) (**Figure 3**), as delineated in recent cryo-EM structures (Tajima

et al., 2016; Zhu et al., 2016). However, in homomeric GluA2 structures, the two layers are much more loosely associated and the receptor adopts a characteristic 'Y'-shaped structure, with the arms of the letter Y formed by the two NTD dimers that project away from the LBD layer (Mayer, 2016) (**Figure 3**). This architecture has been observed in resting and pre-activated states, but not in the desensitized state where the NTD and LBD layers of the receptor adopt a wide spectrum of conformations (Durr et al., 2014; Meyerson et al., 2014). The looser association between NTD and LBD prevailing in homomeric AMPARs and KARs would seem less competent to transmit allosteric signals from the NTD than those of NMDARs. However, a recent cryo-EM structure of a GluA2/3 heteromer shows that AMPARs can adopt a vertically compressed, 'NMDAR-like' conformation (**Figure 3**), with the NTD layer trapped in a compact 'O-shaped' arrangement by a cysteine cross-link (Herguedas et al., 2016). A similar organization has also been seen in isolated GluA2/3 and GluA2/4 NTD crystal structures and in coarse-grained simulations (Dutta et al., 2015; Herguedas et al., 2016). This closely packed conformation could facilitate an influence of the NTD on the rest of the receptor, either by the binding of potential allosteric ligands to the NTD, or by its interaction with synaptic protein partners (discussed below). Assessing the energetics of major conformational intermediates by a combination of cryo-EM and coarse grain simulations will lead to a better understanding of the AMPAR functional spectrum.

Subunit assembly

In the brain, the majority of AMPARs exist as heterotetramers, a feature that greatly extends the number of functional receptor subtypes. However, while AMPARs are *preferential* heteromers assembled from GluA1-GluA4 in various combinations, they can also form functional homomers. Many receptors contain the GluA2 subunit, which restricts Ca^{2+} permeability and confers a low single-channel conductance (Isaac et al., 2007; Swanson et al., 1997). Neurons express varying levels of GluA2 lacking CP-AMPARs that are important in synaptic plasticity, and various neurological conditions (Cull-Candy et al., 2006; Liu and Zukin, 2007; Luscher and Malenka, 2011; Traynelis et al., 2010). Below we discuss the assembly process, the understanding of which has been aided by the rapid advance in AMPAR structural biology.

Mechanisms of assembly

As is the case for most transmembrane proteins, AMPAR subunit composition is established in the endoplasmic reticulum (ER). Rules underlying the assembly process have been identified for the pore-forming tetramer, with individual subunit domains fulfilling specific roles at various stages in biogenesis (reviewed in (Gan et al., 2015; Greger et al., 2007; Herguedas et al., 2013)). Although auxiliary subunits likely play a critical role in this process (e.g.(Brockie et al., 2013; Harmel et al., 2012), their precise contribution is currently poorly understood. In common with K^+ channels (Deutsch, 2002), AMPAR subunits form dimers that subsequently assemble into tetramers. The formation of a dimer is driven by the NTD, and atomic details of this strategic interface are available for homo- and for hetero-dimers across all iGluR subfamilies (Elegheert et al., 2016; Herguedas et al., 2016; Karakas et al., 2011; Kumar et al., 2011).

NTD dimer affinities of AMPAR subunits range from low-nanomolar to low-micromolar (Rossmann et al., 2011; Zhao et al., 2016a), with the specificity of assembly imparted by the more sequence-diverse lower lobes. It remains to be clarified at what stage during the assembly process heteromerization occurs. Due to the high affinity for NTD self-assembly, individual subunits emerging from polyribosomes during translation are expected to initially form homodimers, which could reshuffle into heterodimers when encountering a 'non-like' dimer in the ER membrane (Herguedas et al., 2013). This 'reshuffling' involves inter-dimer interfaces in the LBD and TMD, and is ultimately driven by affinity differences between the NTDs. The GluA3 NTDs provide a good example, having an affinity for GluA2 that is more than an order of magnitude higher than for self-assembly, thus preferentially favoring the assembly of GluA2/3 heterodimers (Rossmann et al., 2011; Zhao et al., 2016a). Similarly, the NTD of GluA1 has an affinity for GluA2 that is >200 fold stronger than for other GluA1 partners, favoring GluA1/2 heterodimers. The effect of these affinity differences manifest in CA1 pyramidal neurons, where expression of GluA1, GluA2 and GluA3 results in receptors that are almost exclusively GluA2

containing heterotetramers (Lu et al., 2009) with relatively low levels of GluA2 lacking CP-AMPARs (Mattison et al., 2014). The bias for the formation of GluA2 containing receptors is compounded by Q/R editing, which impedes GluA2 homotetramerization, resulting in an ER pool of GluA2 dimers that is available for incorporation into the majority of AMPARs (Greger et al., 2002). Such stringent assembly rules likely allow for spatio-temporal control over the production of CP-AMPARs.

While dimerization is driven by the NTD, tetramerization is mediated by contact points in all domain layers. In the NTD layer, the interdimer interface (of GluA2 homomers) is small (~ 300 Å) (Sobolevsky et al., 2009) and tetramers have not been observed in isolated NTDs, highlighting their relatively weak association. Nevertheless, it is worth pointing out that this interface is consistently seen in many crystal structures of isolated AMPAR and KAR NTDs (Kumar and Mayer, 2012). The different and more compact NTD tetrameric interface seen in GluA2/3 and 2/4 heterotetramers (Herguedas et al., 2016) highlights the dynamic nature of this region (discussed below and **Figure 5B**).

In the LBD layer, the two LBDs within a dimer are separated such that dimerization via the D1 interface occurs *between* dimers in a receptor tetramer, thus giving rise to a 'domain swap' between the ECR layers (**Figure 1B**). This unique feature (observed across the iGluR subfamilies) is important for receptor assembly, as tightening of the D1 interface *within* a dimer disfavors tetramer formation (Shanks et al., 2010). Developmental and activity-dependent RNA processing in the D1 interface (**Figure 1A**) (Lomeli et al., 1994; Monyer et al., 1991; Penn et al., 2012) affect AMPAR assembly and forward trafficking from the ER (Coleman et al., 2006; Penn et al., 2008). 'Flop' receptors show dramatically reduced receptor forward trafficking from the ER compared with flip variants, a deficit that can be alleviated by the chaperone-like activities of auxiliary subunits (Coleman et al., 2006; Harmel et al., 2012).

Finally, at the TMD, the M4 helix imparts stability on the tetrameric complex (Gan et al., 2016; Gan et al., 2015; Salussolia et al., 2013), intercalating between M1 and M3

of neighboring subunits and thereby locking together the TMDs of individual subunits (**Figure 1E**).

Subunit positioning in AMPAR tetramers

iGluR tetramers harbor two pairs of diagonally opposed subunits, referred to as A/C and B/D (Sobolevsky et al., 2009). Each pair is conformationally distinct: in the A/C pair, the LBD and the region immediately above the M3-gate are located closer to the channel pore ('pore-proximal'), whereas the B/D chains are pore-distal (Sobolevsky et al., 2009) (**Figure 1B and 3**). This architecture is expected to have significant functional implications. The linkers that connect M3 to the LBD, and couple glutamate binding to channel opening, exhibit greater structural change between the resting and activated states in the B/D chains (Chen et al., 2014; Durr et al., 2014). In NMDARs, the pore-distal (glutamate-binding) GluN2 subunits exert a greater pulling force on the M3 gate helix and are more sensitive to mutation than the proximal, glycine-binding GluN1 subunits (Kazi et al., 2014). Similarly, in the heteromeric GluK2/5 KARs, subunit positioning appears to be fixed, with GluK2 subunits locating pore-distal and GluK5's pore-proximal, with subunit-specific ligand preferences and affinities (Herb et al., 1992; Kumar et al., 2011). Therefore, subunit placement in these iGluRs, into either the A/C or the B/D slot, will inevitably influence gating properties.

Whether such placement rules apply to the preferentially heteromeric AMPARs is not fully established. Early studies proposed that identical subunits sit diagonally opposite each other, in either A/C or B/D positions (Mansour et al., 2001), which could be confirmed in recent structures of heteromeric NTDs and in a full length GluA2/3 heteromer (Herguedas et al., 2016) (**Figure 3**). This arrangement requires tetramerization from pairs of hetero-, rather than homo-dimers (Rossmann et al., 2011; Zhao et al., 2016a). In both GluA2/3 and GluA2/4 NTD structures, GluA2 occupied a position that could be traced to the pore-proximal slot in an intact GluA2/GluA3 heteromer. Interestingly however, crosslinking experiments suggest that this positioning may not be strictly adhered to, as GluA2 can be located *either*

proximal *or* distal to the pore axis in both GluA2/3 and GluA2/4 AMPARs (Herguedas et al., 2016). In apparent contrast, a recent study reported positional preference in GluA1/2 heteromers, where the GluA1 subunits are located pore-proximal, a feature driven by sequence elements in the signal peptide (He et al., 2016).

Promiscuous assembly behavior would generate a far greater diversity in the possible heteromeric AMPAR combinations. For example, reported differences in glutamate affinity between AMPAR paralogs (Traynelis et al., 2010) would translate into varying agonist efficacies for a given subunit combination. And a greater heterogeneity in the auxiliary subunit interaction region would also be highly likely (see below). Hence, further work is required to fully clarify the spatial subunit organization in AMPAR tetramers.

Auxiliary subunits of the AMPAR

Main families of auxiliary subunits

So far we have considered the assembly and architecture of the GluA pore-forming tetramer. However, unlike NMDA- and kainate receptors, AMPARs uniquely assemble with a *wide variety* of auxiliary subunits. Some of these proteins associate with the core GluA subunits early in biogenesis and remain an integral part the receptor. These auxiliary subunits affect AMPAR trafficking and expression at synapses, as well as regulating many of their functional properties, thereby dramatically increasing receptor diversity in the CNS (Jackson and Nicoll, 2011).

The first AMPAR auxiliary subunit to be identified, stargazin (or γ-2), was discovered from experiments on the ataxic *stargazer* mouse, a naturally occurring mutant which lacks the prototypical auxiliary subunit stargazin and consequently lacks excitatory transmission at cerebellar mossy fiber-to granule cell synapses (Chen et al., 2000; Hashimoto et al., 1999; Letts et al., 1998). Stargazin belongs to the transmembrane AMPAR regulatory protein (TARP) family, which have been classified into type-1 (γ-2, γ-3, -4 and -8) and type-2 (γ-5 and -7) TARPs, based on differences in their amino acid sequence and functional properties (Jackson and Nicoll, 2011) (**Figure 4A**).

Proteomic screens have identified three additional auxiliary subunit families that also modulate AMPARs (**Figure 4A**). Cornichons (CNIHs) are three-transmembrane proteins involved in cargo sorting from the ER in metazoans (Schwenk et al., 2009). The cystine-knot protein, CKAMP-44, together with three other family members (also called Shisas) (Farrow et al., 2015; von Engelhardt et al., 2010) have only a single membrane spanning region. In addition, GSG1L (germ cell specific gene 1-like protein) is a four transmembrane spanning protein with a secondary structure reminiscent of TARPs (Schwenk et al., 2012; Shanks et al., 2012), which acts as an auxiliary subunit that appears to negatively regulate AMPARs (Gu et al., 2016; McGee et al., 2015).

It has been suggested that the 'core' of the AMPAR consists of the GluA tetramer together with up to four other constituents enlisted from amongst the TARPs, cornichons and GSG1L proteins (Schwenk et al., 2012; Shanks et al., 2012). These are thought to be surrounded by a more variable periphery in a layered arrangement Hence, the overall assembly may contain a large number of constituents from a pool of > 30 different proteins (see section below). The large array of peripheral interactors are either transmembrane, cytoplasmic or secreted proteins that include CKAMPs, proline-rich transmembrane proteins (PRRT) 1 and 2, neuritin, the noelins 1-3, the leucine-rich repeat transmembrane (LRRT) protein 4, the carnitine O-palmitoyltransferase 1 (CPT1C) or the C9orf4/FRRS1l protein. However, the role of many of these peripheral proteins remains elusive (Schwenk et al., 2012).

Below we discuss the stoichiometry of the main AMPAR auxiliary subunit families and the architecture of the AMPAR-TARP complex, for which structural information has recently been described (Twomey et al., 2016; Zhao et al., 2016b), before outlining how these subunits dictate the basic biophysical and pharmacological properties of AMPARs.

Stoichiometry of AMPAR-auxiliary subunit complexes
Modulation of AMPAR signaling is affected both by the type and the stoichiometry of the associated auxiliary subunits. It is known that a variety of different auxiliary

subunits can co-assemble with the core AMPAR tetramer, each contributing a unique facet to the receptor's functional properties (Herring et al., 2013; Kato et al., 2010; Khodosevich et al., 2014; Schwenk et al., 2012). The expression pattern of these proteins can be confined to specific brain regions or circuits (Lein et al., 2007; Schwenk et al., 2014; Schwenk et al., 2009; Shanks et al., 2012; Tomita et al., 2003; von Engelhardt et al., 2010). This is best understood for TARPs, whose stoichiometry appears to be variable and to depend on TARP expression level. For example, there is evidence of a 'dose-dependent' TARP modulation of AMPARs in cerebellar granule cells in wild type *vs* heterozygous stargazer mice, where both amplitude and decay time of mEPSCs was reduced in the heterozygous mutant. Conversely, in wild-type granule cells the over expression of γ-2 dramatically slowed the mEPSC decay and increased kainate efficacy (a hallmark of TARP action; **Figure 4C**), suggesting synaptic AMPARs in these cells are not usually fully saturated by TARPs (Milstein et al., 2007).

Biochemical evidence suggests that individual AMPARs can contain between one and four TARPs, each interacting independently with the receptor in a non-cooperative fashion (Kim et al., 2010), again implying that TARP expression level can determine stoichiometry. Furthermore, the properties of recombinant AMPARs appear functionally distinct depending on whether they contain zero, two or four TARPs (Shi et al., 2009). By fixing TARP stoichiometry, using AMPAR-TARP fusion constructs (by tethering the AMPAR C-tail with the TARP N-terminus), Shi et al (2009) were able to demonstrate that the efficacy of kainate on AMPARs containing two γ-2 molecules is approximately half that of kainate activating receptors that contain four TARP molecules, thereby providing a convenient read-out of TARP stoichiometry.

The GluA/TARP stoichiometry has also been examined with single-molecule counting methods. Hastie et al. showed that while up to four γ-2 or γ-3 molecules (type-1a TARPs) can co-assemble with individual AMPAR complexes, a maximum of only two γ-4s (type-1b) can do so (Hastie et al., 2013), implying that TARP subtypes may have different binding sites in the AMPAR complex. It would clearly be of interest to know whether single-molecule counting methods detect a difference between the

stoichiometry of γ-8 and γ-4 (the type-1b TARPs), as functional data have suggested that in γ-8 containing hippocampal neurons TARP stoichiometry can be either two or four per AMPAR assembly depending on the neuronal subtype (Shi et al., 2009).

Another potential signature of TARP stoichiometry is 'resensitization', a slow increase of the steady state current upon prolonged L-glutamate application, which appeared unique to γ-4, -7, and -8 AMPAR complexes (Kato et al., 2010). This feature was suggested to indicate the presence of four TARPs per AMPAR and was absent in AMPARs associated with two TARPs (in recombinant settings) as well as in native AMPARs in certain hippocampal neurons (Gill et al., 2011). As these neurons also showed a high expression of CNIH-2, it was inferred that CNIH-2 regulates TARP stoichiometry such that each receptor would contain just two γ-8s plus two CNIH-2s (Gill et al., 2011). A recent study suggests that resensitization reflects the presence of a 'superactive' high open probability state of the AMPAR-TARP complex, and is therefore not unique to γ-4, -7, and -8, being also present with other TARPs, including γ-2 (Carbone and Plested, 2016). However, this does not preclude the possibility of it being indicative of the presence of fully TARPed AMPARs.

A somewhat different perspective of the interplay between TARPs and CNIH has also been suggested. Herring et al. proposed that only GluA1 can simultaneously bind to CNIH-2 and γ-8, and that TARP γ-8 itself prevents the functional association of CNIHs with other (non-GluA1) AMPAR subunits (Herring et al., 2013). This mechanism would suggest that the GluA1/2 heteromers present in CA1 hippocampal cells contain four γ-8s and one or two cornichons, while that GluA2/3 heteromers contain only γ-8s (four molecules). CNIHs cause profound slowing of GluA1/2 AMPAR kinetics in HEK cells (Schwenk et al., 2009) (**Figure 4B**). However, as this property is not observed in CA1 pyramidal cells it is appears to be obviated by association with γ-8 (Herring et al., 2013). These results point to a primary role for CNIHs in AMPAR forward trafficking, consistent with their function as an ER cargo adapter (Harmel et al., 2012), and offer a mechanism for selective surface trafficking of GluA1 containing AMPARs. Nevertheless, a study in hilar neurons in the dentate gyrus has revealed a

profound kinetic contribution of CNIH-2 to the AMPAR EPSC (Boudkkazi et al., 2014). It also remains to be determined how CNIH-2 affects the stoichiometry of TARPs other than γ-8. While it is apparent that CNIH-2 more markedly affects the properties of type-1b (γ-4, γ-8) than type-1a (γ-2, γ-3) TARPs (Gill et al., 2012), it remains unknown whether CNIH-2 also modifies AMPARs containing type-2 TARPs (γ-7 and γ-5).

A picture of the intricate interaction between various auxiliary proteins and different GluA core subunits is emerging, with an impressive degree of functional diversity. Elucidating the functional significance of AMPAR composition at individual synapses in a given circuit remains a major future challenge in understanding information storage and transfer in the brain.

Structure of the AMPAR-TARP complex

Recent cryo-EM structures of GluA2 associated with one, two or four γ-2 molecules per receptor (Twomey et al., 2016; Zhao et al., 2016b), firmly establishing that four TARPs can associate with an AMPAR (**Figure 4F and G**). The structures of TARP-associated GluA2, in an antagonist-bound state, revealed the TARP binding sites on the TMD sector, where the main contact points are formed by the GluA2 transmembrane helices M1 and M4, and the γ-2 helices TM3 and TM4 (**Figure 4G**). This overall organization has been confirmed recently by functional experiments, where segments of the TARP-insensitive GluK2 KAR subunit were introduced into GluA3 (Ben-Yaacov et al., 2017). Interactions between positively charged residues in the LBD lower lobe (D2) and negatively charged residues in the first extracellular loop of γ-2 have been inferred from functional studies and this LBD region has been highlighted as a key element in TARP modulation (Dawe et al., 2016). However, no direct interaction between the γ-2 extracellular segments and the LBD were apparent in the current structures, where the electron density for the two extracellular TARP loops is limiting.

Also of note, the receptor structure with two TARPs bound shows interaction of the γ-2 first extracellular loop with the pore-proximal B/D subunits (**Figure 1B**), perhaps highlighting preferential TARP binding sites on the tetramer (Twomey et al., 2016). With four TARPs bound, closer contacts of the TARP extracellular region with the B/D subunits is also apparent, further highlighting the non-equivalence between the four pore-forming subunits. Although these structures provide a key template for further analysis of AMPAR modulation, more detailed information on TARP-LBD or even TARP-NTD interactions, which may occur in activated or desensitized states (Cais et al., 2014; Nakagawa et al., 2005), are still to be determined. In addition, atomic resolution information of the transmembrane sector will be crucial; this region has recently been shown to provide a binding-site for modulatory drugs that selectively block γ-8 containing AMPARs (Kato et al., 2016; Maher et al., 2016), which present potentially powerful new pharmacological tools.

Regulation of channel function by auxiliary subunits
TARPs associate with a majority of AMPARs in the CNS enhancing charge transfer through synaptic AMPARs channels by slowing the time course and increasing the size of the synaptic current (Jackson and Nicoll, 2011). Specifically, TARPs enhance agonist efficacy and potency (Priel et al., 2005; Turetsky et al., 2005), increase weighted mean single-channel conductance (Soto et al., 2007; Tomita et al., 2005a), slowing receptor desensitization (type-1 TARPs; see below) by affecting activation gating, and slow deactivation kinetics (Cho et al., 2007; Milstein et al., 2007). They also markedly modify the block of GluA2-lacking (CP-) AMPARs by extracellular and endogenous intracellular polyamines (Jackson et al., 2011; Soto et al., 2009) (**Figure 4B-E**).

Differences in the ability of TARPs to modulate desensitization and deactivation provided evidence for the presence of two distinct functional groupings within the type I TARPs (**Figure 4A**) (Cho et al., 2007; Milstein et al., 2007). While all TARPs share the ability to slow desensitization and deactivation, and increase the steady-state current, γ-4 and γ-8 (type 1b TARPs) achieve this to a much greater extent than

γ-2 and γ-3 (type 1a). TARPs have further surprising influences on receptor pharmacology, greatly increasing the efficacy of the partial agonist kainate (**Figure 4C**), converting the antagonist CNQX into a partial agonist of AMPARs assemblies that contain type-1 (Menuz et al., 2007) but not type-2 TARPs (Bats et al., 2007) and attenuating the block of CP-AMPARs by intracellular spermine (Soto et al., 2007; Soto et al., 2009). We discuss next four ways in which auxiliary subunits modify channel function.

i) *Auxiliary subunits increase weighted mean channel conductance*

TARPs, CNIHs and CKAMP family members all increase the macroscopic average (mean) channel conductance of AMPARs. TARPs are the most effective, typically increasing it by >40%, although the increase depends on TARP subtype, with γ- 8, enhancing it to a greater extent than γ-2, -3, or -4 (Coombs et al., 2012; Jackson et al., 2011; Shi et al., 2010; Soto et al., 2009; Tomita et al., 2005a) (**Figure 4E**). By contrast CKAMP family members produce only a modest change (Khodosevich et al., 2014).

Two possible mechanisms have been considered to explain how TARPs might give rise to an increased mean channel conductance: they could modify the relative proportion of higher sub-conductance states visited, or they could increase the amplitude of all sub-conductance states including the maximum level. The former mechanism would suggest that TARPs influence channel conductance by LBD-mediated interactions (Dawe et al., 2016), while the latter might favor a modification of the ion channel/permeation pathway.

Although there is evidence for an increase in the proportion of higher sub-conductance states visited (Tomita et al., 2005a; Zhang et al., 2014), in another study an increase in amplitude of the individual sub-conductance levels has been observed for different TARP/AMPAR subunit combinations (Shelley et al., 2012). A key test to distinguish between these two possibilities is whether TARPs can produce an increase in the channel's maximum conductance state. While γ-2 does not significantly alter the size of individual sub-conductance levels or the maximum

conductance of GluA4 homomers (Tomita et al., 2005a), γ-4 enhanced the amplitude of all sub-conductances of GluA1 homomers - including the maximum conductance state (Shelley et al., 2012). These different observations could suggest that interplay between multiple mechanisms underlies the mean channel conductance increase, depending on the AMPAR/TARP subunit combinations and the TARP interaction.

Two regions in the AMPAR/TARP assembly have been implicated in influencing the increase in mean channel conductance. Deletion of the TARP γ-2 C-tail greatly enhances its ability to increase the mean channel conductance; and a conserved residue in the AMPAR pore-lining region, close to the Q/R site (Q/R+4), which is important for polyamine block (Panchenko et al., 1999), also determines the ability of TARPs to increase mean channel conductance (Soto et al., 2014). While these are useful insights, they are just the beginning of the required full mechanistic understanding of TARP action.

(ii) *Auxiliary subunits modify polyamine and PhTx block of CP-AMPARs*
TARPs, and to a lesser extent CNIHs, markedly attenuate the voltage-dependent block of CP-AMPARs by shifting the apparent affinity of AMPARs for endogenous intracellular polyamines (Soto et al., 2007) (**Figure 4D**). By contrast, TARPs *enhance* the extracellular channel block by philanthotoxin analogues, in a manner that is dependent on TARP subtype and on whether full or partial agonists are used, suggesting a conductance state-dependence of inhibition by philanthotoxin (Jackson et al., 2011). Both TARPs and CNIHs also increase the relative calcium permeability of GluA2-lacking AMPAR channels (Copits and Swanson, 2012; Kott et al., 2009). This could indicate a direct action on ion selectivity, or that ion selectivity varies with channel sub-conductance state. While γ-2 increases channel permeability of several cations, it does not appear to alter the estimated AMPAR pore size, suggesting that changes in pore diameter do not cause the relief of polyamine block by TARPs (Soto et al., 2014). Furthermore, unlike the relief of block of CP-KARs by the auxiliary

subunits Neto1 and 2 (Fisher and Mott, 2012), positively charged residues in the intracellular C-tail of the TARPs do not play a role in relief of block (Soto et al., 2014).

(iii) *GSG1L: an auxiliary subunit that negatively regulates AMPARs*

While auxiliary subunits mostly increase charge transfer through the AMPAR channel, not *all* auxiliary subunits follow this pattern. Like the TARPs, GSG1L slows the deactivation and desensitization of recombinant AMPARs (Schwenk et al., 2012; Shanks et al., 2012). However, in other respects GSG1L appears to be a somewhat 'anomalous' auxiliary subunit. Not only does it slow AMPAR recovery from desensitization (Schwenk et al., 2012; Shanks et al., 2012), a feature it shares with γ-4 and γ-8 (Cais et al., 2014), but it also reduces the mean single-channel conductance, diminishes the calcium permeability of recombinant CP-AMPARs, and greatly suppresses outward current flow through CP-AMPAR channels (McGee et al., 2015). This together with the fact that endogenous GSG1L has a suppressive action on synaptic currents in hippocampal neurons (Gu et al., 2016) suggests it acts as a negative regulator of AMPARs .

(iv) *Differential regulation of CP- versus CI-AMPARs by auxiliary subunits*

Several studies have identified roles for specific TARPs in the selective expression, assembly and trafficking of CP-AMPARs. γ-5 selectively modulates AMPARs composed of subunits that have a long C-tail (GluA1 and GluA4), which generally constitute CP-AMPARs, increasing their mean single-channel conductance by roughly 50% and reducing polyamine block (Soto et al., 2009). In keeping with this view, cerebellar Bergmann glia, which express a high level of γ-5 (Fukaya et al., 2005), possess only CP-AMPARs and exhibit properties that resemble recombinant γ-5 associated AMPARs. Furthermore γ-5 fails to rescue transmission in *stargazer* cerebellar granule cells (Tomita et al., 2005a), which express only CI-AMPARs. However, there is also evidence that γ-5 will selectively regulate only Q/R edited GluA2-containing AMPARs (Kato et al., 2008). Unlike other TARPs, γ-5 decreases peak channel open probability (Soto et al., 2009), which could complicate interpretation of whole-cell recordings. It is thus clear that differential regulation of CP- and CI- AMPARs by γ-5 deserves further examination.

Cerebellar stellate cells express only γ-2 and -7, associated with a mix of CP- and CI-AMPARs (Liu and Cull-Candy, 2000). In *stargazer* mice (lacking γ-2) these cells show a marked decrease in EPSC amplitude and increase in inward rectification (Bats et al., 2012; Jackson and Nicoll, 2011), which likely reflects an increase in the relative proportion of CP-AMPARs (but see (Jackson and Nicoll, 2011)). Strikingly, evidence suggests that synaptic currents arise from TARPless receptors, whereas extrasynaptic AMPARs are associated with γ-7 (Bats et al., 2012). In addition, knock down or knockout of the remaining TARP (γ-7) is able to rescue transmission (Bats et al., 2012), upon which both CP- and CI-AMPARs localize at synapses (Studniarczyk et al., 2013) demonstrating that AMPARs can localize at synapses in the absence of TARPs. This evidence suggests that γ-7 selectively enhances the expression of synaptic CP-AMPARs, while suppressing CI-AMPARs.

This 'differential regulation' model is not entirely clear, as recent evidence shows that mEPSC amplitude and frequency was neither further diminished nor rescued (as per Bats et al., 2012), in stellate cells from γ-2/γ-7 double knockout mice, leading to the proposal that γ-7 plays little role in AMPAR regulation in these cells (Yamazaki et al., 2015). Despite this, immunogold labeling has shown large losses in GluA2 and GluA3 in γ-2-knockout mice, with further reductions seen in the double knockout (γ-2 and -7) at all asymmetrical synapses (Yamazaki et al., 2010). Further, a reduction in GluA4 was identified at mossy fiber–granule cell synapses in γ-7-KO mice, which, together with functional evidence suggests γ-2 and γ-7 cooperate to promote synaptic expression of AMPA receptors in cerebellar cells (Studniarczyk et al., 2013; Yamazaki et al., 2010).

These examples highlight the potential for the differential regulation of CP- and CI-AMPARs through subunit-specific auxiliary subunit action and interaction, and begin to explain the requirement for such a diverse range of AMPAR interacting proteins.

AMPAR architecture at synapses

The rapid diffusion of AMPARs between extrasynaptic and synaptic sites (Choquet and Triller, 2013) is thought to be important both in short term plasticity (Heine et al., 2008), and during long term plasticity where the recruitment and diffusional trapping of additional AMPARs enables synaptic strength to be rapidly altered (Huganir and Nicoll, 2013; Kessels and Malinow, 2009). Recent super-resolution imaging has suggested that efficient signal transmission depends on the accumulation of AMPARs in subsynaptic nanoclusters directly opposite presynaptic release sites (Nair et al., 2013; Tang et al., 2016), as previously hypothesized (Raghavachari and Lisman, 2004).

Synaptic localization can be determined by non-specific interactions such macromolecular crowding in protein-enriched synaptic regions (Li et al., 2016), or by altered diffusion of receptors in different lipid environments (Dotti et al., 2014). Specific clustering of AMPARs is likely achieved by a combination of auxiliary subunit association with PSD-95, CTD interactions with the post-synaptic density (PSD) and by ECR interactions with a variety of transmembrane and secreted synaptic proteins. In particular, the NTD and CTD are highly sequence-variable between AMPAR subunits, providing selective protein binding sites (**Figure 5A**). Recent evidence has identified a key role for NTD interactions in synaptic anchoring of AMPARs at hippocampal CA1 synapses (Watson et al., 2017). While the underlying interaction partners are yet to be identified, subunit-specific interactions of both GluA1 and 2 NTDs appear critical for efficient signaling. In particular, the NTD of GluA1 appears to be essential for receptor anchoring in both basal transmission and synaptic potentiation.

The AMPAR ECR protrudes an impressive ~130 Å into the synaptic cleft (Garcia-Nafria et al., 2016; Meyerson et al., 2014) (**Figure 3**). As the peptide linkers connecting the NTD to the LBD are sufficiently flexible to permit large-scale movement and reorganization of the receptor (**Figure 5B**), conformational changes of the ECR would facilitate dynamic interactions with various protein partners in the crowded cleft environment, including auxiliary subunits (Garcia-Nafria et al., 2016).

One example is interaction of the distal NTD with TARPs (Cais et al., 2014). As TARPs protrude very little above membrane surface, substantial bending of the receptor would be required to facilitate this interaction. Such flexibility is evident in both single-particle images of native AMPARs (Nakagawa et al., 2005) and coarse-grained simulations (Dutta et al., 2015; Krieger et al., 2015), and has the potential to influence AMPAR subunit-specific auxiliary interactions or stabilization of particular receptor conformations, but such functional significance has not yet been established.

Emerging importance of trans-synaptic complexes in AMPAR clustering

An emerging feature of iGluRs is an interaction of the ECR with soluble factors secreted from the presynaptic terminal and neighboring glia. These extracellular protein ligands form a molecular bridge, linking the ECR of postsynaptic receptors with presynaptic transmembrane proteins such as neurexins (**Figure 5B**), thereby acting as synaptic organizers by mediating receptor clustering. One striking example is cerebellin (Cbln), a member of the C1q/TNFα superfamily that forms tripartite trans-synaptic complexes, binding postsynaptically to the delta iGluRs (GluDs), and presynaptically to neurexin isoforms. Interestingly, GluD2's metabotropic signaling is impeded when its NTD and LBD are uncoupled by introducing an extended and glycosylated peptide linker, providing an elegant example of crosstalk within the ECR, where downstream signaling requires anchoring of the NTD to the presynaptic membrane (Elegheert et al., 2016; Yuzaki and Aricescu, 2017).

Recent work has extended this premise to KARs at the hippocampal mossy fiber-to-CA3 synapse. Here GluK2 and GluK4 are physically anchored at the postsynaptic membrane by binding of their NTDs to presynaptically secreted C1ql2/3. These, in turn, interact with neurexins, thereby forming a transynaptic complex (Matsuda et al., 2016).

Given the emerging role for the NTD in synaptic anchoring it is likely that such transynaptic anchoring mechanisms also control AMPAR function (Watson et al., 2017). Secreted NTD binders have been reported for AMPARs, yet their role in

clustering and receptor anchoring upon LTP induction is currently unclear. GluA1 has been shown to bind selectively to C1q2/3 in vitro, raising the possibility that AMPARs are clustered by this protein family as described for KARs (Matsuda et al., 2016). There is also compelling evidence that the secreted neuronal pentraxins (NPs), which appear to bind all four GluA subunits (O'Brien et al., 1999; Sia et al., 2007), mediate AMPAR clustering and possibly synapse formation (Lee et al., 2017). GluA4 NTD interactions with NPs are best characterized, and appear essential for maintaining synaptic receptors in fast-spiking hippocampal interneurons through interaction with the presynaptic neuronal pentraxin receptor (O'Brien et al., 1999; Pelkey et al., 2015; Sia et al., 2007) – a mechanism reminiscent of C1ql2/3's effect on KARs. This emerging theme offers substantial potential for controlling AMPAR anchoring and alignment with presynaptic release sites.

Role of TARPs in AMPAR localization, diffusional trapping and recruitment
TARPs and other auxiliary subunits play a major role not only in AMPARs trafficking to the cell surface, but also in their accumulation at postsynaptic sites (Jackson and Nicoll, 2011). This requires the interaction of the C-terminal PDZ-binding motif of the AMPAR-associated TARP, with scaffolding proteins in the postsynaptic density (SAP102 and PSD-95/93 MAGUKs). CKAMP-52 and -44 (called Shisa-6 and -9, respectively) also contain a PSD-95 binding PDZ motif (Karataeva et al., 2014), which facilitate retention of AMPARs at synaptic sites (Klaassen et al., 2016). However, whether other auxiliary subunits play a similar role in AMPAR localisation is currently unclear. For example, type-2 TARPs contain an unconventional PDZ motif (T/S SPC), while GSG1L and CNIHs appear to lack PDZ binding sites.

'Diffusional trapping' has been suggested to be one of the key mechanisms by which synapses recruit AMPARs during plasticity (Opazo et al., 2010). Recovery from postsynaptic depression during high frequency transmission depends in part on fast lateral diffusion of desensitized synaptic AMPARs, and their rapid replacement by nearby non-desensitized receptors (Heine et al., 2008). It has been proposed that glutamate binding and AMPAR desensitization causes a partial loss of the AMPAR-stargazin interaction increasing AMPAR mobility and thereby allowing rapid recovery

from desensitization (Constals et al., 2015). Indeed, it has previously been reported that desensitization triggers functional dissociation of AMPAR from postsynaptically anchored TARPs, which could contribute to short-term synaptic modulation (Morimoto-Tomita et al., 2009). However, such agonist induced physical dissociation of TARPs and AMPARs has been questioned and remains to be elucidated (Semenov et al., 2012).

The C-termini of TARPs include a string of serine resides that can be phosphorylated by CaMKII. Due to electrostatic repulsion from membrane lipid head groups, increased phosphorylation of the C-terminus decreases its membrane association. Extending the C-tail of stargazin facilitates its binding to PSD-95 (Hafner et al., 2015; Opazo et al., 2010; Tomita et al., 2005b). As CaMKII is activated by Ca^{2+} influx through the NMDAR, and is well known to be involved in the induction of synaptic plasticity (Hell, 2014), it is highly likely that this contributes to the increase in AMPAR number and synaptic strength.

Differential TARP expression appears to offer functional heterogeneity of AMPARs not only between circuits and cell types, but between synapses within individual cells (Devi et al., 2016). For example, recent quantitative immunogold electron microscopy has shown that γ-2 and γ-8 play important roles in such differential regulation of AMPARs number at individual hippocampal CA1 synapses (Yamasaki et al., 2016). The synaptic presence of γ-2 effectively increased AMPAR expression, converting synapses from ones with a low to a high number of AMPARs. By contrast, TARP γ-8 appeared essential for basal expression of AMPARs at certain synapses. On the face of it this might seem to imply that γ-8 would play only a modest role in LTP expression in the hippocampus. However, recent experiments have provided additional evidence that TARP γ-8, and not γ-2/3/4, acts as a critical CaMKII substrate in synaptic potentiation (Park et al., 2016). Doubtless there are plenty more surprises waiting in the wings, when it comes to auxiliary subunits.

Conclusion

We have highlighted the complexity of the AMPAR signaling apparatus, its domain architecture, assembly and interplay with auxiliary subunits at multiple levels. This,

together with posttranscriptional regulation of the GluA core and developmental and regional expression of receptor constituents, contributes to the generation of functionally diverse signaling machines. Major challenges ahead include a better understanding of the spatio-temporal expression patterns of the core AMPAR and auxiliary constituents, their precise stoichiometry, and their (potentially regulated) expression at defined synapses and circuitries. Future advances in cryo-EM, cryoelectron tomography and superresolution light microscopy, combined with further functional studies will lead to a better understanding of how auxiliary subunits and synaptic interaction partners modulate AMPAR function to generate the exceptionally diverse signaling system required for information processing in the brain.

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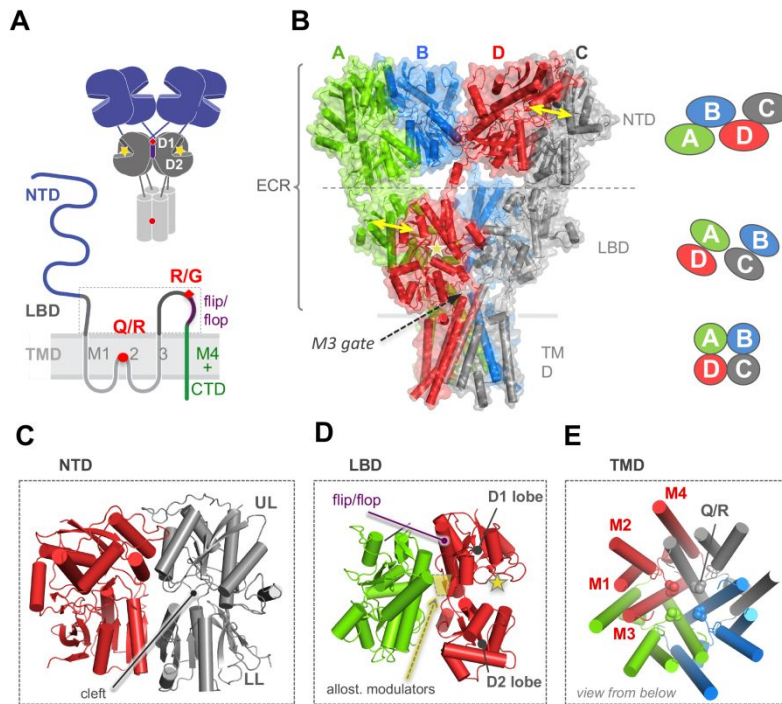
Figure legends

Figure 1. A. Receptor schematic diagram indicating domain contributions of the primary polypeptide chain (bottom panel), mapped onto structural AMPAR schematic (above). The LBD is formed from two regions of primary sequence (boxed with dashed line, lower panel) also called 'S1' (the N-terminal part) and 'S2' (the C-terminal loop). mRNA processing sites (Q/R, R/G and flip/flop) as well as the 4 membrane segments (3 trans-membrane, and a pore loop) plus CTD are indicated. The 3D schematic (top) highlights domain layers and sites of RNA editing in subunit interfaces (coloured as in **A**); agonist (yellow star) docking between LBD lobes (D1/2) is indicated. **B**, Structure of a GluA2 homomer (PDB: 3KG2), coloured by individual polypeptide chains, with agonist in the LBD cleft (yellow star) and the position of the M3 gate (formed by the C-terminal part of the M3 helix) is indicated. Yellow arrows signify dimerization between subunits, occurring differentially within each domain layer, giving rise to the domain swap between the different ECR layers. A top view schematic outlining the domain arrangement in each layer is shown on the right. **C-E**, Insets illustrate details of each domain layer. **C**, NTD dimer (Upper (UL) and lower

(LL) lobes of NTD. **D**, inter-LBD-dimer site of allosteric modulator binding, position of the flip/flop helix and docking position of agonist (yellow symbol). **E**, TMD helices from below, Q/R RNA editing site in pore shown as spheres.
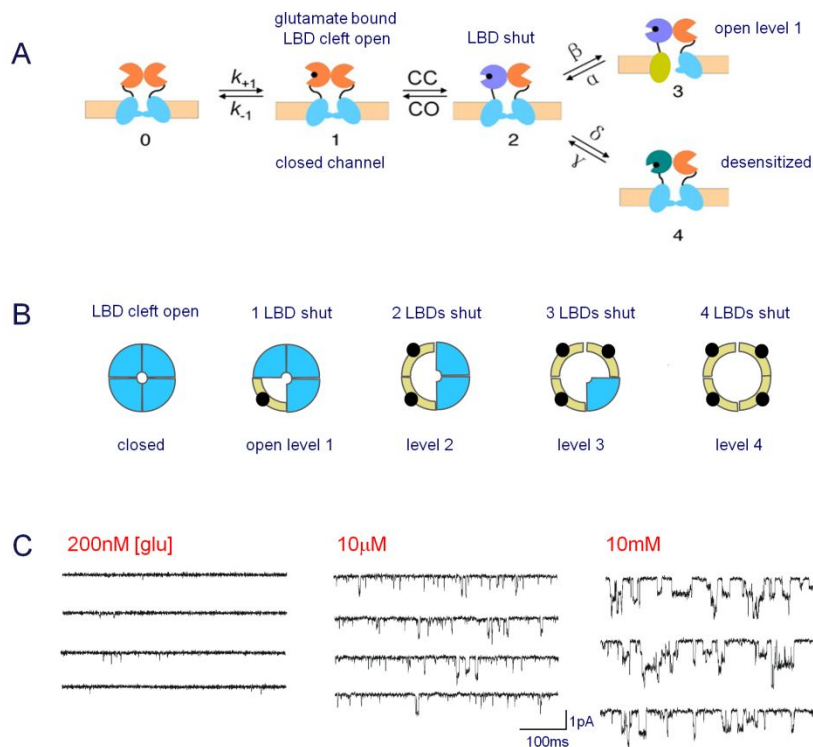

Figure 2. A, Schematic of AMPAR gating in cross section though a single dimer. Transitions between states 0 and 1 represent binding of a single glutamate molecule (black dot) to, and dissociation from, the cleft-open (CO) conformation of the LBD of one subunit. Transitions between states 1 and 2 represent opening and closing of the LBD (CC, 'closed cleft'). In state 2, with the LBD and channel pore closed, the channel can either open (state 3, yellow), or the dimer interface break leading to desensitization (state 4, green) (modified from Zhang et al., 2008). **B,** Hypothetical states of the AMPAR channel if subunits gate independently. Glutamate (black dot) can bind to LBDs on all four subunits. Binding of glutamate to a single subunit may be sufficient to trigger a conformational change in the gate from closed channel (blue) to partially open (yellow), giving rise to open level 1; the binding of additional

bound glutamates then increases the probability of channels opening to higher conductances (levels 2, 3 or 4). With all four LBDs closed, the channel is capable of giving its maximum response (level 4). **C,** single-channel activity recorded in an outside-out patch from a hippocampal CA1 neuron exposed to increasing concentrations of glutamate: 200 nM, 10 μM and 10 mM. Note, that the frequency of events, the proportion of high conductance openings, and open-time all increased with glutamate concentration (from Gebhardt and Cull-Candy, 2006).
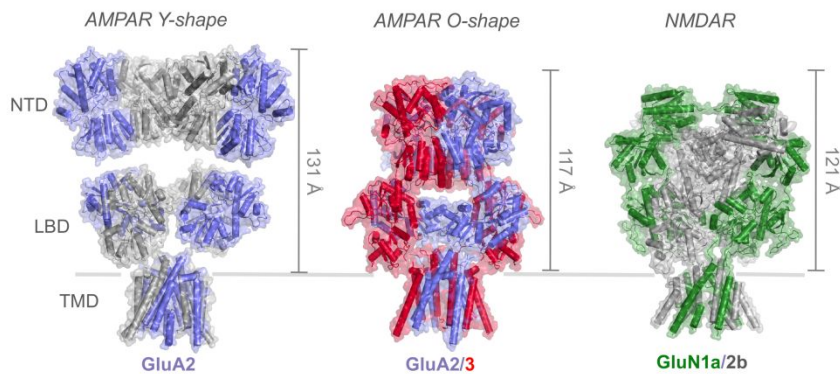

Figure 3. A, Structures of homomeric GluA2 (left, PDB: 4UQJ), heteromeric GluA2/GluA3 (centre, PDB: 5IDE) and the GluN1a/2B NMDAR (right, PDB: 4PE5), highlighting some of the potential rearrangements of the extracellular region (NTD and LDB) of the AMPAR, which can vertically compress, roughly approximating that of a compressed-NMDAR-like structure.
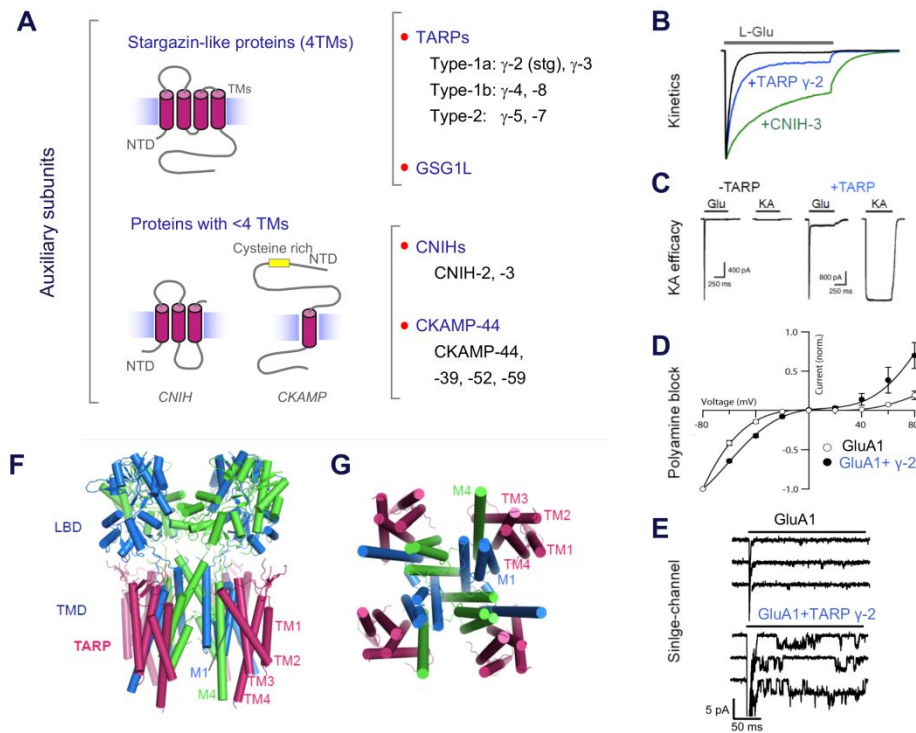
Figure 4. A, Schematic of AMPAR auxiliary proteins, classified into stargazin like (containing 4 transmembrane helices) and other auxiliaries, with generalised protein schematics indicated. **B**, both TARP and cornichon association markedly slow desensitisation kinetics of GluA2(Q) in HEK cells (kindly provided by O. Cais). **C**, TARP association greatly increases the AMPAR response to kainate converting it from a partial to a full agonist (this panel is derived from (Nicoll et al., 2006); **D**, TARP γ-2 partially alleviates polyamine block, allowing enhanced current flow at depolarised potential (seen as a reduction in the rectification of the I-V relationship) (from Soto et al., 2007); **E**, TARP γ-2 increases single-channel conductance (from Coombs & Cull-Candy, 2009). **F**, The architecture of the TARP associated AMPAR (PDB: 5KK2, A/C chains indicated green, B/D chains in blue and four TARP molecules in magenta). The NTD was not incorporated in this model. **G**, cytoplasmic view of the AMPAR-TARP complex (PDB 5KK2).
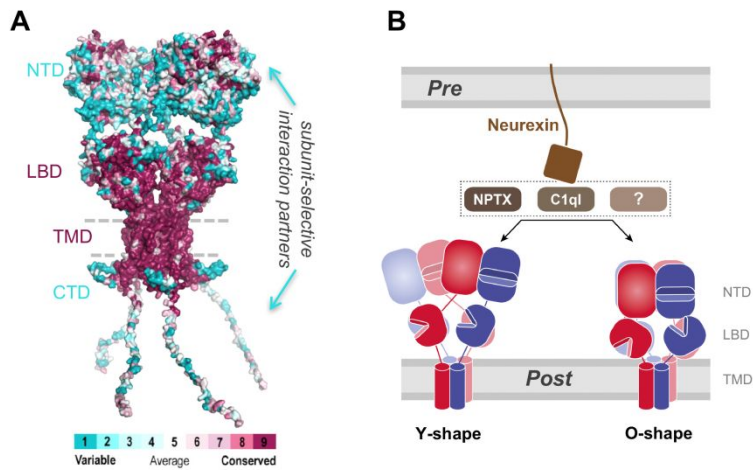
Figure 5. A, AMPAR structure coloured by sequence conservation between receptor paralogues and orthologues, where teal indicates low and magenta high levels of conservation. The receptor N- and C- termini are highly diverse receptor regions, permitting subunit-selective protein interactions (modified from Watson et al. 2017). **B**, schematic representation of AMPAR extracellular region showing the potential rearrangement from Y to O shape conformations, which reduces the receptor's vertical length. Synaptic protein interactors (NPTX= neuronal pentraxin, C1ql = C1q-like; indicated above the double-headed arrow), some of which are anchored to presynaptic neurexin, would perceive drastically different NTD interaction platforms in each case.