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TOPICAL REVIEW

Retour aux sources: defining the structural basis of glutamate receptor activation

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Abstract Ionotropic glutamate receptors (iGluRs) are the major excitatory neurotransmitter receptor in the vertebrate CNS and, as a result, their activation properties lie at the heart of much of the neuronal network activity observed in the developing and adult brain. iGluRs have also been implicated in many nervous system disorders associated with postnatal development (e.g. autism, schizophrenia), cerebral insult (e.g. stroke, epilepsy), and disorders of the ageing brain (e.g. Alzheimer's disease, Parkinsonism). In view of this, an emphasis has been placed on understanding how iGluRs activate and desensitize in functional and structural terms. Early structural models of iGluRs suggested that the strength of the agonist response was primarily governed by the degree of closure induced in the ligand-binding domain (LBD). However, recent studies have suggested a more nuanced role for the LBD with current evidence identifying the iGluR LBD interface as a "hotspot" regulating agonist behaviour. Such ideas remain to be consolidated with recently solved structures of full-length iGluRs to account for the global changes that underlie channel activation and desensitization.

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Abbreviations AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; ATD, amino terminal domain; ED, extracellular domain; GlnBP, glutamine binding protein; iGluR, ionotropic glutamate receptor; KA, kainate; KAR, kainate receptor; LBD, ligand binding domain; L-Glu, L-glutamate; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl D-aspartate; NMDAR, NMDA receptor; QA, quisqualate; TARP, transmembrane AMPA receptor regulatory protein; TM, transmembrane domain.

Introduction

Fast excitatory neurotransmission in the vertebrate CNS is mediated by a class of plasma membrane-bound, multimeric proteins called ionotropic glutamate receptors (iGluRs). iGluRs assemble as tetramers from subunits encoded by eighteen genes that are divided into three major subgroups, based on their differential sensitivities to the agonists *N*-methyl D-aspartate (NMDAs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAs) and kainate (KAs) (Nakanishi, 1992; Seeburg, 1993; Hollmann & Heinemann, 1994; Collingridge *et al.*

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2009), while a fourth, orphan group, comprising the δ subunits, does not appear to form functional ion-conducting channels (Yuzaki, 2003).

At central synapses, iGluRs respond to the transient presence of the neurotransmitter L-glutamate (L-Glu) in the synaptic cleft through a series of binding and conformational steps that lead to membrane depolarization by the opening of a cation-permeable transmembrane pore (Dingledine *et al.* 1999; Traynelis *et al.* 2010; Huettner, 2014). Although iGluRs are often found together in the postsynaptic membrane, their global distribution and specific functions differ. The rapid millisecond activation of AMPA receptors (AMPARs) allows them to facilitate postsynaptic depolarization, which is also a necessary first step in relieving tonic Mg^{2+} block of NMDA receptors (NMDARs) (MacDonald & Nowak, 1990). Once unblocked, NMDARs affect neuronal signalling by transporting extracellular Ca^{2+} into the cytoplasm, a process prolonged by the slow intrinsic gating properties of these receptors (Qian & Johnson, 2002; Paoletti *et al.* 2013). In contrast, kainate receptors (KARs) are thought to fulfil more of a modulatory role on synaptic transmission, acting from both pre- and postsynaptic locales via ionotropic and metabotropic signalling mechanisms (Huettner, 2003; Contractor *et al.* 2011; Lerma & Marques, 2013). Curiously, the orphan-class δ iGluRs exhibit a much restricted expression pattern in the CNS with their most notable role in cerebellar Purkinje cells, where they regulate synaptic plasticity and synaptogenesis via a non-ionotropic pathway (Yuzaki, 2012).

Over the past decade, a wealth of structural information on iGluRs has emerged, describing the topology and assembly of domains (Gouaux, 2004; Mayer, 2011; Sobolevsky, 2013). Importantly, insight into their structural properties is expected to have a significant impact in the long term, as we start to unravel how iGluRs are implicated in several prominent CNS disorders (Bowie, 2008) and continue the rational design of therapeutic compounds (Lipton, 2006; Sanacora *et al.* 2008; Santangelo *et al.* 2012; Pirotte *et al.* 2013). Despite this, our understanding of the basic events that lead to iGluR activation is still emerging. In this review, we re-examine this issue and highlight recent studies that place a greater emphasis on the iGluR ligand binding domain (LBD) dimer interface as a 'hotspot' of channel activation. Particular emphasis will be placed on AMPARs and KARs, as more structural information is available for these families than NMDARs. With the recent elucidation of (near) full-length, tetrameric AMPAR structures in several conformations (Dürr *et al.* 2014; Meyerson *et al.* 2014; Yelshanskaya *et al.* 2014), a more complete understanding of iGluRs will require a multidisciplinary approach that brings together functional and structural data with dynamic simulations of protein movement.

iGluR pharmacology and channel activation

By the mid-1980s it was accepted that iGluRs exist as pharmacologically distinct receptor families (for reviews see Watkins & Evans, 1981; McLennan, 1983; Mayer & Westbrook, 1987; Collingridge & Lester, 1989), though their rapid, millisecond time course of activation and inactivation was only fully appreciated with the application of the concentration-clamp technique (reviewed in Akaike, 1995). The use of the concentration-clamp permitted the first detailed kinetic analysis of iGluR channel kinetics in response to rapid agonist application. Typical exchange rates in whole-cell recording conditions were about 10 ms using stepper motors (Vyklicky *et al.* 1990), while sub-millisecond (300–500 μs) exchange was achieved in outside-out patch recordings with the help of a piezo translator (Jonas, 1995). Since exchange rates in whole-cell recordings are markedly slower than AMPAR or KAR gating kinetics, peak agonist responses can only be measured with accuracy in excised patches (see discussion in Bowie *et al.* 2003).

Using concentration-clamp recordings, it was demonstrated that native AMPAR/KARs expressed by rodent hippocampal neurons exhibited two distinct kinetic phenotypes in response to agonists: rapid desensitization with the neurotransmitter L-Glu or quisqualate (QA) and a non-desensitizing or non-decaying profile with kainate (KA) (Kiskin *et al.* 1986). Kinetic models developed to explain this behaviour proposed that KA binds to the desensitized state of AMPAR/KARs with lower affinity than L-Glu, QA or AMPA (Patneau & Mayer, 1991) (Fig. 1*A* and *B*). In keeping with this, the use of the benzothiadiazine diuretic, cyclothiazide, to attenuate receptor desensitization potentiated equilibrium responses elicited by KA much less than L-Glu (Patneau *et al.* 1993). Analysis of a more extensive range of receptor ligands, including sulfur-containing amino acids and willardiine derivatives, further established that neuronal AMPAR/KARs responded in an agonist-dependent manner (Patneau & Mayer, 1990; Patneau *et al.* 1992), presumably due to the varying degrees of desensitization (Fig. 1*C*). To complicate matters further, single-channel recordings demonstrated that KA primarily activated a low conductance open state, whereas L-Glu or QA gated channels with a much larger unitary conductance(s) (Ascher & Nowak, 1988; Cull-Candy & Usowicz, 1989; Tang *et al.* 1989). Although it was debated whether KA may activate distinct receptor families from L-Glu and QA (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987), cross-desensitization experiments on native receptors (Kiskin *et al.* 1986; Patneau & Mayer, 1991) and the cloning of AMPAR receptor subunits (Hollmann *et al.* 1989; Keinanen *et al.* 1990) confirmed that different agonists gate the AMPAR channel pore in quite distinct ways. At NMDARs, gating

behaviour was revealed to be more complicated in that the binding of not one but two neurotransmitters, namely L-Glu as well as the inhibitory transmitter glycine, were required for activation (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). In this regard, KARs were more comparable to AMPARs, though studies of native receptors (Huettner, 1990; Lerma *et al.* 1993) and receptor clones (Egebjerg *et al.* 1991) revealed that KA elicited a strongly desensitizing response. As discussed below, an ongoing challenge has been to develop structural models of iGluR activation that can account for many of these complications. The first steps in this process began with the study of recombinant iGluRs.

Early insights into the topology of the agonist binding site

The cloning of the many genes that encode iGluR subunits (Nakanishi, 1992; Seeburg, 1993; Hollmann & Heinemann, 1994) permitted a detailed characterization not only of their functional properties, but also led the way to obtaining structural information (Madden, 2002; Gouaux, 2004; Mayer & Armstrong, 2004). Sequence analysis of the earliest cloned iGluRs revealed a large (approximately 500 amino acid) extracellular domain (ED), while overall amino acid sequence identity ranged from about 70% between AMPARs (Keinanen *et al.* 1990), down to 40% and 25% when they were compared to KARs and NMDARs, respectively (Bettler *et al.* 1990; Moriyoshi *et al.* 1991). Interestingly, a 100 amino acid segment of the ED just prior to the first predicted transmembrane domain (TM 1) shared 30% sequence identity with the bacterial periplasmic glutamine binding protein (GlnBP) (Nakanishi *et al.* 1990; Moriyoshi *et al.* 1991). The cloning of the first metabotropic glutamate receptors (mGluRs) revealed that these proteins also possess an approximately 500–600 residue ED (Masu *et al.* 1991; Tanabe *et al.* 1992), but retained significant homology within only two shorter, discontinuous segments (Masu *et al.* 1991). Through more refined sequence analysis techniques, it was later appreciated that iGluRs have two distinct EDs. The first, now referred to as the amino terminal domain (ATD), is homologous to the mGluR ED and the bacterial leucine-isoleucine-valine binding protein (LIVBP), while the second, now referred to as the ligand binding domain (LBD), is homologous to the GlnBP (O'Hara *et al.* 1993). At the time, X-ray crystal structural information was available for LIVBP (Adams & Oxender, 1989; Quiocho, 1990).

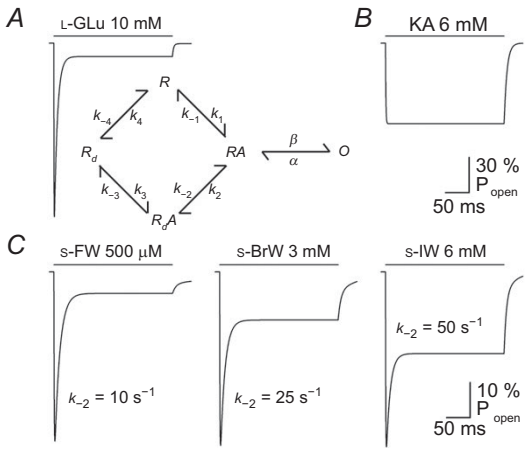

Figure 1. A cyclic gating model recreates AMPAR responses to willardiine series agonists
A, simulated response of AMPARs to L-Glu, generated from a five-state cyclic gating model (Patneau & Mayer, 1991). R, RA, R_d, and O represent resting, agonist-bound, desensitized, and open-channel states of the receptor, respectively. The rate constants for state transitions with L-Glu are adapted from values in another study (Vyklicky *et al.* 1991). *B* and *C*, simulated responses of AMPARs to KA (*B*), as well as to the willardiine series agonists, *S*-5-fluorowillardiine (s-FW), *S*-5-bromowillardiine (s-BrW), and *S*-5-iodowillardiine (s-IW) (*C*). As the halogen substituent increases in size, the equilibrium to peak current ratio recorded from AMPAR/KARs in hippocampal neurons also increases. To recapitulate this effect, the rate constant k_{-2} was increased, while k_{-1} and k_{-3} were adjusted to maintain microscopic reversibility. The rate constants are adapted from published values (Patneau *et al.* 1992), except for the s-BrW simulation, in which values were assigned to reproduce experimental observations.

Drawing upon this structural information led research groups to use distinct, but complementary approaches to conclude that the agonist specificity of iGluRs and mGluRs is governed by two discontinuous segments of amino acid residues located in the extracellular domain (ED). For mGluRs, molecular modelling techniques were used to construct a homology model of mGluR1, which was then validated by assessing the impact of mutating conserved residues on agonist selectivity (O'Hara *et al.* 1993). For iGluRs, the replacement of native glycosylation sites with mutant sites was exploited to determine a topology of three membrane-spanning domains (TM 1, 3 and 4) with a re-entrant loop (TM 2) in between (Hollmann *et al.* 1994). From this framework, chimeric receptors (generated by swapping extracellular regions of GluA3 AMPARs and GluK2 KARs) were used to verify that two separate domains confer agonist selectivity (Stern-Bach *et al.* 1994). These domains were subsequently named S1 for the amino acid residues prior to transmembrane region 1 (TM 1) and S2 for residues between TM 3 and 4. The structural elucidation of an *E. coli* GlnBP (Hsiao *et al.* 1996) further established the unexpected link between the structural motifs of bacterial periplasmic transport proteins and neurotransmitter receptors expressed in the vertebrate CNS (Paas, 1998).

Two final observations led the way in permitting the resolution of the iGluR agonist/ligand binding site at

atomic-level resolution. First, a truncated fusion protein consisting of the S1 and S2 domains of the GluA4 AMPAR, separated by a linker peptide, was shown to exhibit near-identical pharmacological properties to its wild-type counterpart (Kuusinen *et al.* 1995); a strategy that was exploited to obtain large quantities of the GluA2 AMPAR LBD for crystallographic analysis (Chen & Gouaux, 1997). Second, the upper and lower lobes were proposed to act in a 'Venus flytrap' arrangement (Mano *et al.* 1996), which helped further conceptualization of how the iGluR ligand binding cleft might trigger the process of channel activation.

A structural model of agonist efficacy

A new era in the study of iGluRs was heralded when Armstrong and colleagues solved the first high-resolution structure of a LBD; in this case it was the GluA2 AMPAR in complex with KA (Armstrong *et al.* 1998). As anticipated from earlier studies, the GluA2 structure was remarkably similar to that of the *E. coli* GlnBP (Sun *et al.* 1998), with the ligand binding cleft formed between two α-helix- and β-sheet-containing domains that were subsequently named D1 and D2 for the upper and lower lobes, respectively. Appreciating this structural similarity, the authors concluded that the GluA2–KA complex represented a partially closed structure, since the degree of domain closure was intermediate relative to GlnBP's open and closed conformations (Armstrong *et al.* 1998). Given this, they correctly predicted that further domain closure would be possible with different ligands, such as AMPA, with the rearrangement of a few key residues being required to accommodate the ligand. Interestingly, despite the overall low degree of sequence homology amongst iGluR family members, all seven residues that form direct contacts with KA in the structure were shown to be either identical or conservatively substituted, further underlining the importance of keeping these contact points as functionally diverse iGluR subtypes emerged during evolution.

Following this initial study, additional structures of the GluA2 AMPAR LBD in either apo, antagonist-bound (6,7-dinitroquinoxaline-2,3-dione, or DNQX), or agonist-bound (e.g. glutamate, AMPA and kainate) (Fig. 2*A*) conformations were described, which enabled comparisons to be made concerning what rearrangements of the LBD occur for different ligands (Armstrong & Gouaux, 2000; Hogner *et al.* 2002; Jin *et al.* 2002; Armstrong *et al.* 2003). Specifically, what distinguishes an agonist that opens the channel pore from an antagonist that does not? One measure that appeared to correlate well with agonist responsiveness was the angle of LBD closure formed by lobes D1 and D2 around the ligand binding cleft (relative to the apo structure), ranging from about 20 deg for the full agonist L-Glu to 5 deg for the antagonist DNQX (Armstrong & Gouaux, 2000). In support of the cleft closure hypothesis, a relationship between LBD closure, efficacy, and agonist response (in the presence of cyclothiazide, but see below for more details) held for a series of willardiine agonists, each with a different halogen-substituted group at the same position (Jin *et al.* 2003) (Fig. 2*B* and *C*). As predicted, the larger the halogen atom, the less closure around the agonist could be observed in structures, accounting for the lower weighted channel conductance and reduced efficacy seen in electrophysiological experiments (Jin *et al.* 2003). Taken together, all of these studies helped establish the idea that the degree of agonist-induced domain closure determines the extent of receptor activation and thus may represent the structural basis of agonist efficacy.

The need for a revised model of agonist efficacy

In subsequent years, high resolution structures of the LBD of KARs, NMDARs and orphan-class iGluRs were also described, in almost all cases, using the same approach as successfully applied to GluA2 AMPARs (Furukawa & Gouaux, 2003; Mayer, 2005; Naur *et al.* 2007) (though see Nanao *et al.* 2005). However, additional structural analyses of the three functional iGluR families has shown that full cleft closure can in fact be induced by partial agonists, and even antagonists. Structures of a GluA2 mutant for which AMPA becomes a partial agonist captured the AMPA-bound LBD cleft in intermediate, as well as fully closed conformations (Armstrong *et al.* 2003). Concurrently, the first structures of the GluN1 NMDAR subunit (Furukawa & Gouaux, 2003; Inanobe *et al.* 2005) indicated similar cleft closure for full and partial agonists, contrary to the model of agonist behaviour proposed for AMPARs. Moreover, although the initial structural analysis of GluK1 KARs suggested a close correlation between the degree of LBD closure and agonist behaviour (Mayer, 2005), subsequent work suggested that a more complicated relationship was at play (Fay *et al.* 2009; Frydenvang *et al.* 2009). This finding was not entirely surprising since earlier work had already established that external Na^+ and Cl^- ions profoundly affected the KAR agonist response (Bowie, 2002; Bowie & Lange, 2002; Wong *et al.* 2006) by binding to discrete sites at the interface between subunits (Plested & Mayer, 2007; Plested *et al.* 2008; Bowie, 2010) (see below). Taken together, these studies suggested that the structural basis of agonist behaviour was different between iGluR family members and/or that the initial structural model of agonist action at AMPARs needed further refinement.

On that note, Robert and colleagues addressed this issue through a series of structural and functional experiments that examined the impact of mutating a single amino-acid

residue (i.e. T686A) that was expected to destabilize the closed conformation of the GluA2 AMPAR ligand binding cleft (Robert *et al.* 2005). Importantly, the authors were later able to more directly examine agonist efficacy by studying AMPARs at the single-channel level, taking into account sub-conductance levels. These studies suggested a model whereby more efficacious agonists can better stabilize the closed conformation of a dynamic ligand binding cleft (Zhang *et al.* 2008), an idea that has also been used to explain agonist behaviour at KARs (Maclean *et al.* 2011). In agreement, spectroscopic measurements of the willardiine-bound GluA2 S1S2 constructs pinpointed specific residues in the cleft that undergo dynamic motions correlated with agonist potency (Fenwick & Oswald, 2008). Thus, variations in cleft stability could potentially explain why crystal structures might capture this domain in multiple – and sometimes unexpected – conformations given the nature of the agonist or antagonist being examined. Such structures capture low energy states and poorly reflect stochastic processes that underlie channel gating (Lau & Roux, 2007). An alternative to the cleft closure paradigm proposed in recent studies is that other movements influence efficacy. In particular, the need to evaluate ligand-induced conformational changes in three dimensions has been emphasized (Birdsey-Benson *et al.* 2010) and at multiple locations, including the LBD dimer interface (Nayeem *et al.* 2011). As explained below, there has been a renewed focus on the dimer interface in determining agonist efficacy, particularly in KARs, where a novel role for the cation binding pocket has been identified.

Agonist efficacy, desensitization, and the LBD dimer interface

Although much emphasis was placed on relating conformations of iGluR LBDs to activation, it was also appreciated that subunits assemble back-to-back as a dimer of dimers (Armstrong & Gouaux, 2000; Tichelaar *et al.* 2004). Interactions between subunits along the LBD dimer interface are thought to remodel upon activation

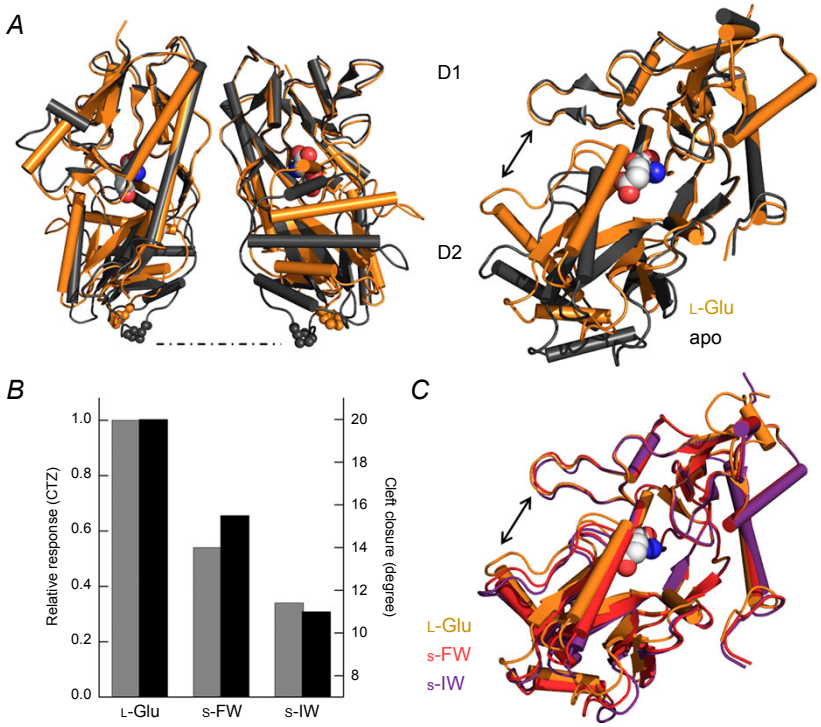

Figure 2. Agonist efficacy correlates with closure of the GluA2 ligand binding cleft
A, side view of the GluA2 LBD with apo (protein data bank (PDB) 1FTO) and L-Glu-bound (PDB 1FTJ) structures overlaid (left). The residue P632, found at the base of the D2 domain, is emphasized to illustrate how this region lifts up and separates upon agonist binding. Visualization of a single subunit highlights how the cleft between D1 and D2 is narrowed in the L-Glu-bound structure (right). *B* and *C*, in the presence of the allosteric modulator cyclothiazide (CTZ) to attenuate desensitization, agonist responsiveness correlates to the degree of closure between D1 and D2 at the ligand binding cleft. For example, willardiine series agonists with smaller halogen substituents were more efficacious and produced a greater degree of cleft closure. (Jin *et al.* 2003).

and desensitization (Fig. 3). Activation is proposed to lift apart the lower D2 lobes of the interface, while desensitization is proposed to reflect the rupture of cross-interface interactions as the upper D1 lobes separate (Sun *et al.* 2002) (Fig. 3). This structural arrangement is also significant from a functional perspective, since it helps explain the onset and recovery from AMPAR desensitization, which occurs in two sequential, kinetic steps (Robert *et al.* 2001; Bowie & Lange, 2002). This model emerged in part because the binding site for the cyclothiazide, and location of the leucine to tyrosine (L–Y) mutation, both of which strongly attenuate AMPAR desensitization (Patneau *et al.* 1993; Stern-Bach *et al.* 1998), are situated where subunits are predicted to come together. Accordingly, studies of the LBD dimer interface have often examined how individual residues affect the kinetics of desensitization (Horning & Mayer, 2004), with less attention paid to their role in agonist efficacy. However, other studies show that mutations along the KAR interface affect the relative efficacies of L-Glu and KA (Fleck *et al.* 2003; Zhang *et al.* 2006; Maclean *et al.* 2011), in addition to the time course of desensitization. Comparisons of agonist behaviour at GluA2 AMPARs in conditions with desensitization present or attenuated (in the presence of cyclothiazide or atop the L–Y mutation) even suggested that desensitization generally inverts the rank order of efficacy (Jin *et al.* 2003; Holm *et al.* 2005).

In light of evidence linking the LBD dimer interface to desensitization, experiments were designed to directly test if it was possible to trap AMPARs or KARs into specific conformational states (i.e. active or desensitized) by introducing disulphide bonds via cysteine residues on opposing sides of the dimer interface to restrict protein movement (Armstrong *et al.* 2006; Priel *et al.* 2006; Weston *et al.* 2006) (Fig. 4*A*). As anticipated, the rapidly decaying response elicited by L-Glu was converted into a non-decaying phenotype via crosslinking of the D1 interface, suggesting that restricting dimer movement in this region locks the AMPAR or KAR into the activated state (Priel *et al.* 2006; Weston *et al.* 2006) (Fig. 4*B*). Despite this, it was surprising that responses elicited by the crosslinked GluA2 AMPAR were potentiated by cyclothiazide to a greater extent than the wild-type receptor (Weston *et al.* 2006). In principle, if desensitization is blocked, the agonist response would be expected to be unaffected by cyclothiazide unless a more complicated mechanism of cyclothiazide action is at play (see Mitchell & Fleck, 2007). Similarly, GluK2 KARs crosslinked to restrict dimer movement (Priel *et al.* 2006; Weston *et al.* 2006) elicited responses that were more consistent with the equilibrium

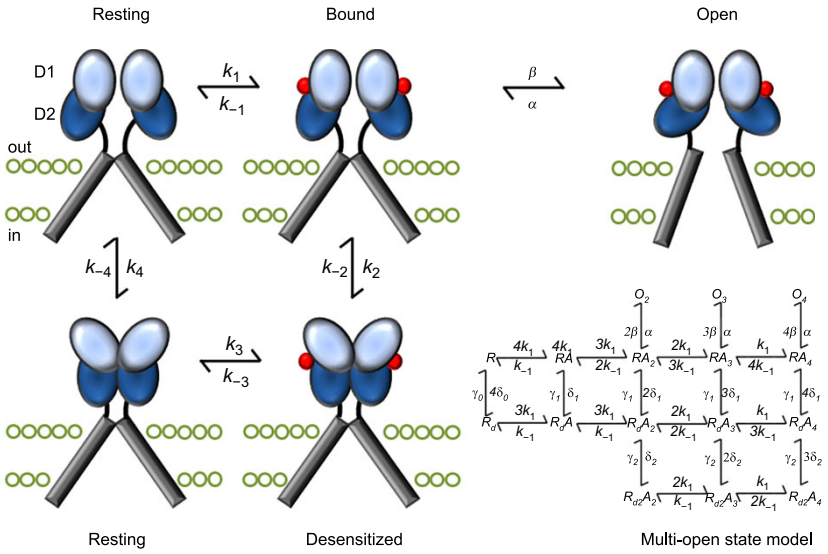

Figure 3. Early structural model of iGluR activation and desensitization
The binding of agonist molecules (red) to the LBD dimer permits subsequent rearrangement to one of two conformations: activated or desensitized. The activated state occurs when the two D2 lobes (dark blue) are lifted apart, generating forces on the transmembrane domains (grey bars) to open the channel pore. Alternatively, the desensitized state stems from the separation of the two D1 domains (light blue) at the dimer interface, relaxing the LBD such that the energy provided by ligand binding cannot open the pore. The structural states are arranged according to the cyclic gating model described in Fig. 1, although to better account for the complex functional behaviour of iGluRs, more advanced kinetic models (Robert & Howe, 2003) have been developed, into which the four agonist binding sites are incorporated (bottom right). Moreover, is not clear to what extent movements of LBD dimers occur in the absence of bound ligands. The ATD has also been excluded for simplicity.

response of wild-type receptors, where the occurrence of desensitization accounts for the slow decay kinetics and the higher apparent agonist affinity (Bowie *et al.* 2003). A clue to the unexpected behaviour of these cysteine mutants could be found in the GluK2 Y521C/L783C crystal structure (Weston *et al.* 2006), which showed the dimer interface to be in a relaxed conformation, in between an activated arrangement and the pseudo-desensitized arrangement of GluA2 S729C (Armstrong *et al.* 2006) (Fig. 5*A*). An important caveat in these studies is that the authors relied on macroscopic responses to infer the absence or presence of desensitization. However, given that desensitization represents long-lived inactive states of the channel (Sakmann *et al.* 1980), its occurrence can only be truly confirmed by single-channel analysis. As explained below, unitary measurements of crosslinked iGluRs revealed an unappreciated importance of the LBD dimer interface in governing the degree of activation.

Single-channel measurements of the GluN1/GluN2A NMDAR crosslinked at equivalent positions to those in AMPARs and KARs demonstrated that restraining dimer movement had a profound effect on activation (i.e. P_{open}), whereas desensitization was still present (Borschel *et al.* 2011). The authors' findings suggested two possibilities: (i) the structural basis of NMDAR desensitization is distinct from that of AMPARs and KARs and/or (ii) earlier studies implicating the dimer interface with AMPAR and KAR desensitization had overlooked the impact of cross-linking on the activation process. In keeping with the latter possibility, single-channel recordings comparing wild-type GluK2 receptor responses to the crosslinked receptor (i.e. GluK2 Y521C/L783C) showed that the mutant receptor activated poorly (Daniels *et al.* 2013) (Fig. 5*B*). Specifically, the crosslinked GluK2 receptor is effectively locked out of the main open-state, rather than having a preference for it, which would be expected if desensitization was abolished (Fig. 5*C*). Preliminary single-channel data on crosslinked GluA2 receptors (B. A. Daniels & D. Bowie, unpublished observations) reveals that channel activation is similarly disrupted. Taken together, these data suggest that the architecture of the LBD dimer interface of all iGluR subtypes is a key location for channel activation.

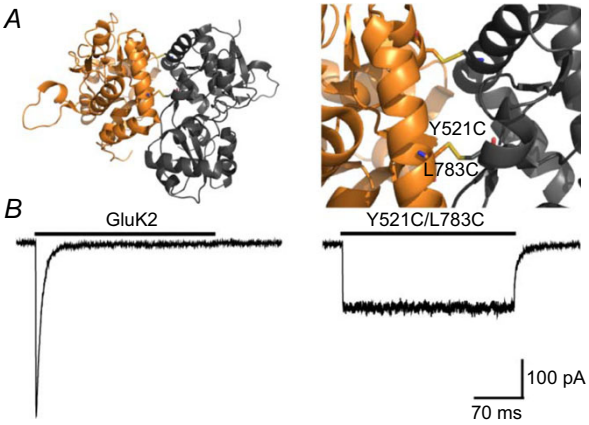

Figure 4. Crosslinking of the GluK2 LBD dimer interface yields non-decaying current responses
A, side views of the LBD dimer interface of GluK2 Y521C/L783C (PDB 2I0C), in its entirety (left) and close-up (right), detailing the inter-protomer disulphide bond (yellow). *B*, activation profiles of wild-type GluK2 and Y521C/L783C receptors in response to 10 mM L-Glu (holding potential –60 mV). Adapted from Daniels *et al.* (2013) with permission.

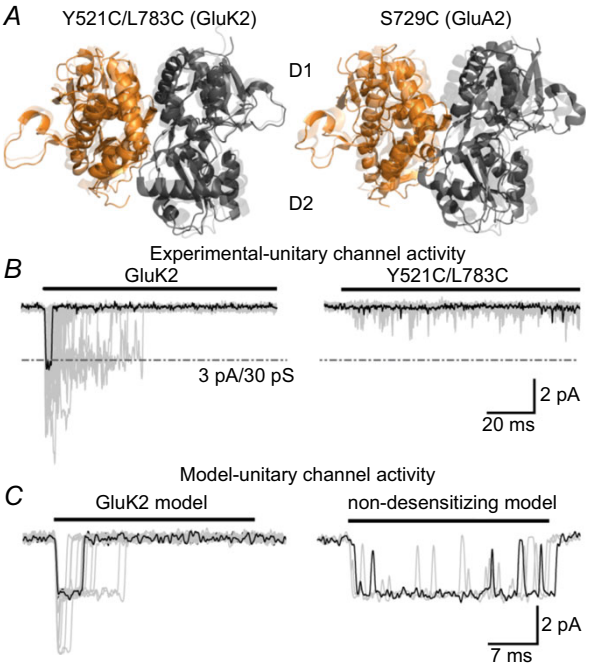

Figure 5. Cysteine crosslinking of the GluK2 LBD dimer interface disrupts activation
A, side views of the LBD dimer interface of GluK2 Y521C/L783C (left, PDB 2I0C) and GluA2 S729C (right, PDB 2I3W) in front of the corresponding L-Glu-bound wild-type receptors (transparent, PDB 3G3F and 1FTJ). For both mutants the distance across the interface between the D1 domains is increased. *B*, activation profiles of wild-type GluK2 and Y521C/L783C receptors at the single-channel level with a typical unitary response highlighted (black) for both receptors. *C*, simulations of GluK2 unitary channel activity in the presence (left) or absence (right) of desensitization, generated from a cyclic gating model (Bowie *et al.* 1998). When a single channel is simulated with desensitized states removed, the open channel probability is much greater than observed experimentally for the double cysteine mutant. Responses were recorded or simulated using 10 mM L-Glu and a holding potential of −100 mV, and experimental data were filtered at 1 kHz. Adapted from Daniels *et al.* (2013) with permission.

The cation binding pocket of KARs acts as an on/off switch

Although the strategy of covalent crosslinking did not lock KARs into the main activated state as expected, another GluK2 receptor mutant, namely GluK2 D776K, had also been proposed to eliminate macroscopic desensitization (Nayeem *et al.* 2009), but had yet to be studied at the single-channel level. Encouragingly, structural data showed that the positively charged Lys776 established a new inter-protomer contact across the dimer interface by tethering to the cation binding pocket (Nayeem *et al.* 2011) and thus affected KARs through a different mechanism from the Cys521/Cys783 disulphide bridge (Fig. 6*A*). This observation was also intriguing given the fact that earlier functional data had shown that occupancy of the cation binding pocket by external cations, such as Na^+, was an absolute requirement for KAR activation (Wong *et al.* 2006; Bowie, 2010). Consequently, it was possible that Lys776 mimicked the effect of external cations and, by near-permanent occupancy of the cation binding pocket, was able to sustain activation and thus eliminate desensitization. In keeping with this, single channel recordings of individual GluK2 D776K receptors activated to the main conductance state of approximately 20 pS and remained there in the continued presence of the agonist (Dawe *et al.* 2013) (Fig. 6*B* and *C*). Using a combination of molecular dynamics simulations and electrophysiological recordings, it was shown that the cation binding pocket acts like an on/off switch with cation binding priming the KAR for activation, whereas desensitization proceeds when the cation site is unoccupied (Dawe *et al.* 2013). From this perspective, GluK2 D776K maximizes agonist efficacy and sustains the agonist response by keeping the KAR in the activated state rather than affecting the process of desensitization directly. Curiously, GluA1 AMPARs contain many of the residues that make up the cation binding pocket in KARs, but remain insensitive to modulation by external cations (Bowie, 2002). Previous studies have explained cation insensitivity of AMPARs by speculating that the Lys residue that lies in the pocket acts as a surrogate cation (Wong *et al.* 2006, 2007). This idea has yet to be tested experimentally; however, given that the Lys residue is conserved amongst some NMDARs, the KAR 'cation binding pocket' may prove to be a hotspot for activation of all iGluR families.

New insights from full-length iGluR structures

Despite the identification of several discrete sites that regulate iGluR activation, the structural domains (ATD and LBD) to which they belong have been studied largely in isolation, making it difficult to ascribe a role to them during any global protein rearrangements that may accompany channel gating. Part of the problem has been the difficulty of obtaining full-length structures at atomic resolution (Mayer, 2011; Sobolevsky, 2013), though lower resolution, single particle electron microscopy (EM) images have actually been available for some time (Nakagawa, 2010). The first 'image' of a tetrameric iGluR was obtained following large-scale expression of recombinant GluA2 (Safferling *et al.* 2001), followed by a more refined three-dimensional reconstruction of the receptor at 20 Å resolution (Tichelaar *et al.* 2004). Native AMPAR complexes, with and without associated transmembrane AMPA receptor regulatory proteins (TARPs), were later imaged in multiple conformations at 30–40 Å

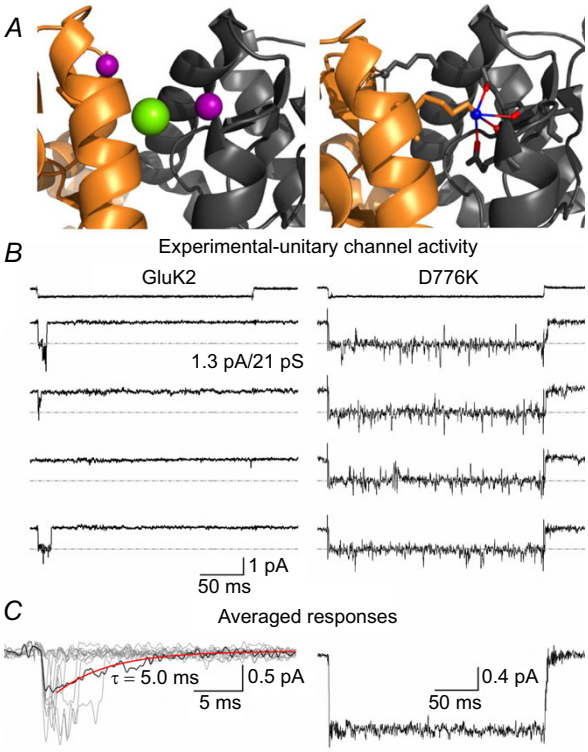

Figure 6. Occupancy of the GluK2 cation binding pocket sustains activation
A, side views of the LBD dimer interface of wild-type GluK2 (left, PDB 3G3F) and the D776K mutant (right, PDB 2XXX). The former includes two allosteric sodium ions (purple) and a chloride ion (green) bound at the apex of the interface, while the latter possesses a charged amino group (blue) on residue 776 tethering into the electronegative pocket (red) normally occupied by sodium. *B*, representative single-channel responses of GluK2 (left) and D776K (right) to 10 mM L-Glu (holding potential −60 mV, filtered at 1 kHz). *C*, averaged responses of individual sweeps taken from the same patch recordings as shown in *B*, which mimic the phenotype exhibited by a large population of receptors. For wild-type GluK2 several individual responses (grey) are overlaid behind the average response, while a fit of the current decay (red) suggests the unitary events are representative of those that contribute to macroscopic decay kinetics, which occur over a similar time course. (Dawe *et al.* 2013).

using cryo-EM techniques (Nakagawa *et al.* 2005, 2006). The AMPARs were sorted into two major classes: a resting and/or activated class possessing a more compact extracellular region, and a desensitized class notable for separation of lobes at the ATD level (Nakagawa *et al.* 2005). This arrangement contrasts with an unliganded GluA2 receptor comprising a compact ATD and separate LBD lobes reported elsewhere (Midgett & Madden, 2008; Midgett *et al.* 2012), and suggests that protein purification conditions may heavily influence the resulting structure. Full-length tetrameric crystal structures have since supported the notion of a Y-shaped structure with a twofold axis of symmetry for apo and antagonist-bound GluA2 AMPARs (Sobolevsky *et al.* 2009; Dürr *et al.* 2014) (Fig. 7*A*). Meanwhile, crystallization in the presence of agonists and allosteric modulators or toxins that increase channel open probability suggest an activated state not too structurally dissimilar from the resting state: amongst many interesting movements, these structures reveal greater closure of the ligand binding cleft, opening between pairs of LBD dimers around the axis of symmetry, and an increase in inter-subunit LBD–TM linker distances (Fig. 7*B*), indicative of a pulling force that precedes channel opening (Chen *et al.* 2014; Dürr *et al.* 2014). These results were largely corroborated by cryo-EM data published simultaneously in which GluA2 is characterized in resting, activated and desensitized states, and contrasted with GluK2 in a desensitized conformation (Meyerson *et al.* 2014). Through improved image-processing techniques, resolution approaching 7 Å was achieved, in contrast to earlier images of apo and desensitized GluK2 receptors of around 20 Å (Schauder *et al.* 2013). Perhaps the most intriguing finding from the later EM study was an asymmetric rearrangement of the LBD during desensitization (Fig. 7*C*), in which the two subunits of each dimer rotate 125 deg and 13 deg in a horizontal plane (Meyerson *et al.* 2014), a movement captured to some extent in an fluorowillardiine-bound crystal structure (Dürr *et al.* 2014), but possibly constrained due to the extensive mutagenesis required for crystallization. GluA2 was also crystallized with nitrowillardiine in another study, but the LBD did not exhibit any drastic

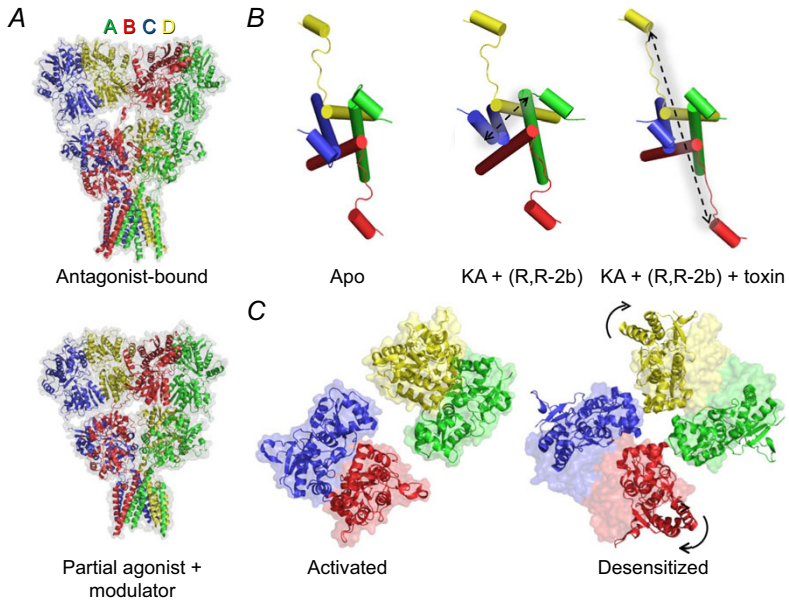

Figure 7. Structural rearrangements of full-length iGluRs during activation and desensitization
A, full-length GluA2 receptor bound by the competitive antagonist ZK200775 (top, PDB 3KG2) or KA and the allosteric modulator R,R-2b (bottom, PDB 4U1W), which potentiates GluA2 current responses in equilibrium conditions (Kaae *et al.* 2007). Both structures retain a twofold axis of symmetry, with the A/C and B/D subunits having distinct arrangements. The latter structure is thought to represent a 'pre-open' state of the receptor during the activation process. *B*, in contrast to the unliganded, apo state of GluA2 (left, PDB 4U2P), the binding of an agonist with positive modulator (centre, PDB 4U1W) causes separation between A/C subunits at the level of the LBD–TM 3 linker, generating forces that could open the pore. Addition of the con-ikot-ikot snail toxin further increases the B/D distance (right, PDB 4U5D). *C*, the tetrameric LBD of GluA2 bound by L-Glu and the allosteric modulator LY451646 (left, PDB 4UQK), a potentiator of AMPAR equilibrium currents (Gates *et al.* 2001), believed to be in an activated state. In contrast, the LBD of GluK2 bound by 2*S*,4*R*-4-methylglutamate (right, PDB 4UQQ) is believed to be in a desensitized state, characterized by large horizontal rotation of the B/D subunits.

rearrangements relative to an antagonist-bound structure of the same, modified receptor (Yelshanskaya *et al.* 2014). Differences in agonist-bound structures are difficult to interpret, since it is unknown whether such structures represent closed or desensitized states, and to what extent mutagenesis of the GluA2 crystal construct or crystal lattice contacts affect the conformations adopted by these proteins (Yelshanskaya *et al.* 2014). Ultimately, the ideas presented in these structural studies remain to be consolidated, yet they will certainly generate many testable hypotheses concerning the mechanisms of AMPAR/KAR gating.

Conclusion

Although we have witnessed great advances in our understanding of iGluR activation in the last three decades, the way forward faces two interrelated obstacles. First, we still do not have a detailed kinetic model of iGluRs that explains the relationship between different activated (i.e. channel conductance(s)) and desensitized states. This obstacle could be addressed with computational approaches that take into account the hierarchical structure of the iGluR (Ollivier *et al.* 2010). However, to provide an accurate starting template for the rule-based modelling of iGluRs, we require a clearer understanding of the stoichiometry of their activation and desensitization. The use of photo-switchable ligands tethered to the iGluR ligand binding cleft (Volgraf *et al.* 2006) could help address this problem, particularly if certain subunits are selectively stimulated (Reiner & Isacoff, 2014), although receptor modification and difficulty replicating response kinetics observed with rapid solution exchange will complicate interpretations made from this technique.

The second major roadblock to understanding iGluR activation is the scarcity of structural correlates for functional states. Despite the recent determination of the full-length structures of AMPARs in a range of agonist and antagonist-induced states (Dürr *et al.* 2014; Meyerson *et al.* 2014), it remains to be seen if these few 'snapshots' can account for the diversity of functional behaviour exhibited by iGluRs. It is also worth noting that no crystal structure has yet shown the pore in an open configuration. Consequently, there is still much to study regarding the signal transduction pathway that allows agonist binding to promote conformational changes that gate the channel pore. In NMDARs, where full-length structures are now available (Karakas & Furukawa, 2014; Lee *et al.* 2014), recent work has shown that the LBD–TM linker provides a mechanical force that increases the likelihood of channel opening (Kazi *et al.* 2014). In AMPARs and KARs, which have a low open probability in equilibrium conditions (e.g. Zhang *et al.* 2008), carrying out such investigations remains difficult. Molecular dynamics simulations of the GluA2 receptor have suggested that small increases in pore diameter can be achieved through rearrangement in the LBD (Dong & Zhou, 2011), but whether this mechanism can permit ion permeation is unclear. In the short term, full-length iGluR structures will provide more insight into which micro-domains may be critically linked to channel activation. But as an alternative approach, recent work on Cys-loop receptors suggests that insight into the allosteric nature of the ligand-gated ion channels may be achieved by mapping out the energy contributions of different structural domains during gating (Purohit *et al.* 2013). In either case, the identification of regions which contribute to the activation and desensitization of intact iGluRs opens up several new avenues of investigation. For instance, it is presently uncertain how dynamic the interactions are between auxiliary proteins (known to affect receptor trafficking and gating kinetics) and iGluRs at the synapse (Jackson & Nicoll, 2011), but having a three-dimensional model of AMPAR activation could shed light on state-dependent modulatory effects. In addition, such models might reveal previously unappreciated sites as new targets for therapeutic compounds. While it is clear that many secrets of glutamate receptor physiology remain to be uncovered, we have learned a great deal from 30 years of research on this neurotransmitter receptor.

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Additional information

Competing interests

None declared.

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