Defining the structural relationship between kainate-receptor deactivation and desensitization

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Desensitization is an important mechanism curtailing the activity of ligand-gated ion channels (LGICs). Although the structural basis of desensitization is not fully resolved, it is thought to be governed by physicochemical properties of bound ligands. Here, we show the importance of an allosteric cation-binding pocket in controlling transitions between activated and desensitized states of rat kainate-type (KAR) ionotropic glutamate receptors (iGluRs). Tethering a positive charge to this pocket sustains KAR activation, preventing desensitization, whereas mutations that disrupt cation binding eliminate channel gating. These different outcomes explain the structural distinction between deactivation and desensitization. Deactivation occurs when the ligand unbinds before the cation, whereas desensitization occurs if a ligand is bound without cation pocket occupancy. This sequence of events is absent from AMPA-type iGluRs; thus, cations are identified as gatekeepers of KAR gating, a role unique among even closely related LGICs.

Structural and functional biologists have long sought to understand the mechanisms by which LGICs respond to small chemical ligands and modulators. Seminal work established the general principle that LGICs not only are activated by biologically derived molecules, such as neurotransmitter acetylcholine, but also are inactivated by prolonged exposure to these molecules through a process universally known as desensitization. Since this work, almost all LGICs have been shown to desensitize. For example, desensitization is thought to shape signaling within the vertebrate central nervous system by affecting the fast chemical transmission mediated by iGluRs along with GABA and glycine receptors. From all of this work, it has been shown that most LGICs have distinct for different ion-channel families. For the iGluR family, numerous mechanistic details of activation and desensitization have been identified and extensively commented upon. After the elucidation of the ligand-binding domain (LBD) structure, a mechanism of iGluR desensitization was proposed, involving the separation of subunits that are assembled as dimers at the LBD. This mechanism has been supported by additional crystal structures that captured AMPARs in different functional states. Accordingly, efforts to engineer iGluR receptors that lack desensitization have focused on constraining movement at the LBD dimer interface. From this, coherent cross-linking of the dimer interface has been shown to generate AMPARs and KARs that yield nondecaying currents upon sustained agonist application. Similar experiments on NMDA-type iGluRs have offered a more nuanced explanation of LBD function by uncovering the structural and single-channel effects of dimer cross-linking. Specifically, they propose that conformational changes at the dimer interface primarily affect open-channel probability and not desensitization. This observation suggests that a more in-depth single-channel analysis of the mechanism of AMPAR and KAR desensitization is warranted.

Here, we set out to study the molecular basis of KAR desensitization by evaluating mutants that are proposed to block it. In both cases, the mutations are located in the GluK2 KAR LBD dimer interface, which not only is implicated in receptor desensitization but also contains binding pockets for both sodium and chloride ions. Prior work from our laboratory shows that external ions are an absolute requirement for GluK2 receptor activation, yet their precise role in desensitization is unresolved. Our present data identify that desensitization of KARs proceeds only if a ligand is bound without cation pocket occupancy, whereas deactivation occurs when the ligand unbinds before the cation. This sequence of events identifies external cations as pivotal in directing KARs into active states or long-lived desensitized states.

RESULTS
KARs desensitize with or without prior channel activation
To observe the microscopic behavior of KAR desensitization, we excised outside-out patches from transfected mammalian cells.

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expressing homomeric GluK2 receptors (Online Methods). Using an ultrafast agonist-perfusion system, we recorded single-channel events and then selected, for analysis, recordings in which most responses corresponded to the conductance expected of a single channel\textsuperscript{24}. Although the actual number of active receptors per patch is not known, these single-channel recordings nevertheless reveal the different routes taken by KARs before entering into desensitization. In most cases, rapid application of saturating glutamate (10 mM l-glutamate) activated GluK2 receptors, which open to one of several conductance levels (Fig. 1a–c). Once in the open state, KAR channels typically closed within tens of milliseconds and did not reopen for any measurable duration of time afterwards, thus indicating that the receptor desensitized. Because desensitization is not thought to occur directly from the open state, it presumably proceeded shortly after channel closure. In agreement with this latter point, ensemble averages of single-channel sweeps exhibited decay time constants (6.49 ± 0.41 ms, \( n = 6 \); Fig. 1d,e) that were statistically indistinguishable from decay rates of macroscopic responses (6.28 ± 0.43 ms, \( n = 9 \), \( P = 0.74 \)), thus reaffirming that the onset of KAR desensitization is approximated by the duration of channel activity.

In some cases, 10 mM l-glutamate failed to elicit a measurable response during the entire 250-ms application (Fig. 1a) corresponding to about 31.7 ± 5.5\% of the 525 total sweeps from five patches (Fig. 1e). The apparent failure to respond to the agonist may reflect an intrinsic inability of l-glutamate to reliably convert its energy of binding to activation. If this was the case, however, channel opening would eventually be observed, as the continued presence of l-glutamate would ensure that the energy threshold for activation would be overcome. Consequently, the inability of l-glutamate to activate GluK2 receptors must represent the onset of desensitization without prior passage through the open state(s).

The discrete molecular events that bring about desensitization are currently unresolved. Several studies, however, identify the LBD dimer interface\textsuperscript{15} and the cation-binding site\textsuperscript{19,22} as taking part in the conformational events that initiate KAR macroscopic desensitization. Whether one site or the other has a more direct effect on desensitization has yet to be directly studied. As discussed below, we examined this by studying the single-channel properties of two apparently nondesensitizing GluK2 receptors, namely the mutants D776K and Y521C L783C.

**Figure 1** Kainate-receptor desensitization occurs with or without channel activation. (a) Typical GluK2 receptor unitary current events elicited by 10 mM l-glutamate (l-Glu, 250-ms pulse duration) in an outside-out patch recording (patch no. 12212p1, −60 mV). (b) Overlay of 45 individual current records from the same patch shown in a. A typical opening elicited by l-glutamate is shown in thick line. (c) GluK2 conductance distributions plotted after time-course fitting. (d) Averaged individual current records from the patch in a and b, showing an ensemble response with a decay fit by a single exponential function whose time constant, \( \tau \), is shown. (e) Left, decay time constants of ensemble responses from several patches. Right, fraction of l-glutamate applications that did not elicit a measureable response from receptors. Error bars, s.e.m. from five or six independent patch experiments as indicated.

**The D776K mutation abolishes GluK2 receptor desensitization**

The LBD dimer interface of wild-type GluK2 receptors contains binding sites for two sodium ions and a single chloride ion (Fig. 2a)\textsuperscript{20,21}. Both GluK2 receptor mutations (D776K and Y521C L783C) are also located at the LBD dimer interface (Fig. 2b,c), where they are proposed to eliminate desensitization by constraining subunit movement. The positively charged lysine of D776K establishes new interprotomer contacts by tethering to the cation-binding pocket (Fig. 2b)\textsuperscript{25}, whereas the cysteine residues of Y521C L783C are thought to achieve this through the formation of covalent disulfide bridges between subunits (Fig. 2c)\textsuperscript{15}. Because both mutant receptors are expected to affect the functional properties of KARs similarly, we were surprised to observe that their single-channel behavior was quite different.

Like wild-type receptors, single D776K channels were rapidly activated by 10 mM l-glutamate. However, instead of opening only briefly before desensitization, agonist binding led to sustained activation of the 21–22 pS main open state (i.e., most frequented) (Fig. 2d). In support of this, repetitive applications of 10 mM l-glutamate to patches containing a single D776K receptor elicited activity in every case, thus demonstrating that this mutant GluK2 receptor displays close to the maximum probability of opening. Averaged ensemble responses were nondecaying in nature with rapid off kinetics of ~2–3 ms due to l-glutamate removal (Fig. 2d). These persistent openings were nevertheless interrupted by transient closures too brief to represent long-lived desensitized states and which, consequently, must represent sojourns to lower conductance levels or closed or ligand-free states.

Unlike the D776K receptor, the double-cysteine mutant did not yield persistent channel activity in saturating l-glutamate. Instead, recordings were dominated by submillisecond openings that were separated by longer apparent closures (Fig. 2e)\textsuperscript{26}. Given the infrequent nature of gating, we concluded that responses observed in the excised patches were likely to originate from multiple channels. Despite the transient openings, averaging sweeps from many agonist applications generated a nondecaying ensemble response. The decay kinetics of the ensemble average current of Y521C L783C receptors were nevertheless at least five times slower (14.8 ± 2.9 ms, \( n = 4 \)) than those of D776K receptors (Fig. 2e).

For GluK2 D776K, its consistent gating behavior allowed us to make additional inferences. Time-course fitting of resolvable single-channel events estimated conductance levels of 21, 35 and 40 pS, which were
calculated by a measured reversal potential of 0 mV (Fig. 2f). The open level most frequently visited was 21–22 pS, closely matching the predominant 19-pS conductance level of wild-type receptors, with the two largest conductance levels corresponding to brief sojourns from this state (i.e., 35 and 40 pS). Fitting Gaussian functions to an all-points histogram of D776K data further shows that >90% of the analyzed records corresponded to the main open state (Supplementary Fig. 1). These conductance levels are likely to originate from single channels rather than from several channels opening simultaneously, as lowering the concentration of l-glutamate interrupted openings to the 21- to 22-pS state with clear closures to baseline (Fig. 2g).

In summary, our single-channel data reveal that GluK2 D776K exhibits all the hallmarks expected of a nondesensitizing KAR: sustained activation, high unitary conductance and an absence of long-duration closures. GluK2 Y521C L783C responds quite differently, and therefore we could conclude that the structural basis of its logical behavior of kainate receptors, then activation could depend on the occupancy of the cation pocket, and cation unbinding would promote channel closure and/or desensitization.

GluK2 D776K receptors activate without external cations
If occupancy of the cation-binding pocket is a prerequisite for wild-type KAR activation, removal of all external ions should result in the absence of any detectable current. Although such recordings have already been shown to abolish wild-type KAR activity, this original finding has been disputed by more recent work claiming residual channel activity in ion-free conditions. To re-examine this issue, we repeated experiments comparing GluK2 receptors in the presence and absence of external ions. If Lys776 acts as a tethered cation, as suggested by MD simulations (Fig. 3) and structural data, we reasoned that the GluK2 D776K would gate in the absence of Lys776 substitutes for sodium at the cation-binding pocket

We used MD simulations to explore how electrostatic interactions affect occupancy of the cation-binding pocket, a relationship that cannot be clarified with X-ray crystal structures or electrophysiology. Over the course of each of two 100-ns simulations, the cation pockets of the D776K receptor first released both sodium ions and then formed new contact points with the amino groups of Lys776 (Fig. 3a–d and Supplementary Movie 1). Consequently, the cation-binding pocket was nearly continuously occupied by a positive charge during the entire simulation period, a result consistent with previous structural data. In contrast, simulations of the Y521C L783C receptor predict that these mutations destabilize sodium- and chloride-ion binding, thus facilitating rapid ion release in both simulations performed (Supplementary Fig. 2a, b and Supplementary Movie 2). There was also a tendency for water molecules to more readily occupy the cation pockets of Y521C L783C, and this may explain the instability in sodium- and chloride-ion binding. Measurements of the surface area accessible to solvent indicated a much higher propensity for water molecules to interact with residues lining the cation pocket in the double-cysteine mutant compared to wild-type GluK2 receptors (Supplementary Fig. 2c, d). If these simulations reflect the physiological behavior of kainate receptors, then activation could depend on the occupancy of the cation pocket, and cation unbinding would promote channel closure and/or desensitization.
Figure 3  Lys776 can act as a tethered ion at the GluK2 cation-binding pocket. (a) Coordination distances between sodium ions (bound to chains A and B) and several oxygen atoms found on residues lining the cation-binding pocket (E524, I527 and D528) during a 100-ns MD simulation (version or repeat 1, v1) of the D776K mutant. (b) Coordination distances for the positively charged Nζ of Lys776 (simulation repeat 1, v1). Distances were measured from oxygen atoms normally involved in sodium ion coordination. (c) Sodium ion coordination in the crystal structure of wild-type GluK2 LBD. (d) Snapshot after 100 ns of MD simulation of the D776K mutant. Orange, chain A and its residues; cyan, chain B and its residues; purple, sodium ion; green, chloride ion. Coordination distances are indicated with black lines for the sodium ion (c) and the Lys776 amine (d). Water molecules and nonpolar hydrogen atoms are omitted. Black boxes surround mutated residues.

Interestingly, the Y521C L783C receptor was also able to gate in the absence of external cations (Fig. 4c,f). This finding is in agreement with a prior study21 but is inconsistent with the lack of responsiveness of wild-type GluK2 receptors in ion-free conditions (Fig. 4a,b), thus suggesting the need for an alternative explanation. With this in mind, we considered the possibility that cross-linking of the dimer interface of the GluK2 receptor may eliminate the requirement of external cations for activation. We tested this possibility by identifying mutations in the LBD dimer interface that would disrupt cation binding without forming interprotomer cross-links.

Desaturating cation binding impairs GluK2 activation

We studied disruption of the cation-binding pocket by examining two mutant receptors, namely GluK2 E524G and L783C, which MD simulations suggest destabilize sodium binding to the cation-binding pocket. Importantly, these mutations do not affect receptor surface expression (Supplementary Fig. 3a,b). For E524G, which has a less electronegative cation pocket, two 50-ns simulations of sodium coordination both estimated that sodium is released within 5 ns. In contrast, the wild-type receptor retained sodium for the duration of two 100-ns

Figure 4  GluK2 D776K receptors gate in the absence of external ions. (a,c,e) Membrane currents evoked by L-glutamate acting on wild-type GluK2 (a), D776K (c) and Y521C L783C (e) receptors, in either 150 mM NaCl (top) or in nominal ion-free (bottom) external solution (Vm = −60, −30, 0, 30 and 60 mV). For wild-type GluK2, the same patch was recorded in both ionic conditions (patch no. 121106p2). Mutant responses were taken from different patches (D776K ion, patch no. 11510 p1; ion free, patch no. 12925p5; Y521C L783C ion, patch no. 121002p2; ion free, patch no. 121023p2). (b,d,f) Averaged current (i norm) voltage (V) plots in 0 mM (filled circles) and 150 mM (open circles) NaCl for wild-type GluK2 (b), D776K (d) and Y521C L783C (f) receptors. Currents were normalized to responses at −60 mV in 150 mM NaCl. Error bars, s.e.m. from three independent experiments for each receptor.
Simulations (Fig. 5a–d and Supplementary Movies 3 and 4). In this respect, E524G mimics the Y521C L783C receptor; however, it differs in that 10 mM l-glutamate fails to elicit a measurable response in most excised patches (Supplementary Fig. 3c). We did observe responses in 3 out of the 18 patches tested, but they were small (<10 pA at ~60 mV) in amplitude and thus consistent with the E524G mutation acting to destabilize cation binding.

Interestingly, when only one of the cross-linking residues (i.e., L783C) was mutated, 10 mM l-glutamate failed to elicit a response in all cases, whether we examined whole-cell recordings (B.A.D. and D.B., unpublished data) or excised patches (n = 15) (Supplementary Fig. 3c). MD simulations suggested that the L783C mutant has a less pronounced effect than does E524G on sodium stability, yet the ions managed to dissociate from their binding pockets within 100 ns in one of two simulations (Fig. 5e,f). One potential explanation for the sodium dissociation is that the L783C mutant permits access of additional water molecules into the cation-binding pocket (Supplementary Movie 5), as observed in simulations of Y521C L783C. In comparison to the wild-type GluK2 receptor, the sodium ions in L783C interacted more frequently with water molecules and less frequently with residues of the cation pocket (M.M. and P.C.B., unpublished data). In both mutants, our data point to the lack of responsiveness of E524G and L783C arising from their disruptive effects on the cation-binding pocket, a condition that may be similar to desensitization in a wild-type receptor. Because mutant receptors that disrupt l-glutamate binding are retained within mammalian cells, we do not think that an inability to bind agonists can account for the phenotypes of E524G and L783C. As a result, an explanation is required to account for an additional cysteine (Y521C) restoring channel gating when introduced atop the L783C mutation. We conclude that the cation-independent activation of GluK2 Y521C L783C is due to its covalent cross-linking of the dimer interface circumventing the normal gating requirements of the wild-type receptor (additional information in ref. 26).

**KAR desensitization proceeds after cation unbinding**

MD simulations and single-channel data suggest that GluK2 D776K receptors are nondesensitizing, because Lys776 becomes tethered to the cation-binding pocket. We therefore conclude that cation binding primes KARs for activation by the agonist. We also conclude that cation-unbound states are not primed for activation, and thus agonist binding promotes entry into desensitized states, as observed with the L783C and E524G mutant receptors. These different outcomes are important because they will determine the degree to which desensitization, and by implication cation unbinding, contributes to the wild-type KAR response. For example, during long agonist applications routinely used to measure desensitization rates, most receptors should desensitize because cations will eventually unbind with the agonist still bound. In contrast, with brief applications of 1-glutamate used to measure deactivation rates, fewer GluK2 receptors should desensitize because cations will unbind before the agonist. Importantly, this sequence of events can be tested experimentally. Specifically, we predict that deactivation rates estimated with a brief agonist application should be minimally affected by the presence or absence of desensitization because decay from the peak response corresponds to agonist unbinding from the cation-bound state(s).

To examine the impact of desensitization on deactivation rates, we compared the relaxation kinetics observed after a brief application (i.e., 1 ms) of 10 mM l-glutamate onto wild-type and nondesensitizing D776K KARs (Fig. 6a). For comparison, we also performed a similar analysis of wild-type and a mutant GluA1 AMPA receptor (i.e., L497Y) in which single-channel desensitization is strongly inhibited (Fig. 6b). Wild-type GluK2 receptors exhibited a fast exponential time constant of deactivation of 2.3 ± 0.1 ms (n = 7) (Fig. 6a), which was statistically indistinguishable from the off kinetics of...
D776K receptors regardless of whether 1-ms (2.0 ± 0.2 ms, n = 9; P = 0.63) or 250-ms agonist pulses (2.4 ± 0.2 ms, n = 12; P = 0.82) were applied (Fig. 6a,c). These observations support our assertion that KAR desensitization proceeds after cation unbinding. Accordingly, deactivation and desensitization can therefore be viewed as being structurally distinct and separable processes. In contrast, the decay time constant observed after a 1-ms application of 10 mM l-glutamate to GluA1 AMPARs had a fast exponential time constant of 1.0 ± 0.1 ms (n = 6) (Fig. 6b), which was about 10 times faster than the off kinetics of the nondesensitizing L497Y mutant (12.4 ± 1.6 ms, n = 5; Fig. 6b,c,e). This finding is consistent with the effect of the allosteric modulator cyclothiazide, which also attenuates AMPAR desensitization.

To further test the impact of desensitization on the activation process, we compared the dose-response relationships of GluK2 D776K and wild-type receptors. We reasoned that because the absence of desensitization had little to no effect on GluK2 deactivation kinetics, rates of l-glutamate unbinding should be high relative to rates of cation unbinding, which equate with desensitization. Under such circumstances, receptors would tend to enter desensitized states only during sustained l-glutamate application. As such, the dose-response relationship of the peak response, occurring less than 1 ms after l-glutamate exposure, should exhibit little change in the absence of desensitization.

In agreement with our predictions, the half-maximal effective concentration (EC₅₀) (and Hill coefficient, n_H) estimated from peak dose-response curves to l-glutamate acting on wild-type GluK2 receptors was 652 ± 47 μM (n_H = 0.87, n = 7), which closely matched that of D776K receptors, whose EC₅₀ values were estimated to be 520 ± 91 μM (n_H = 1.6, n = 8) (Fig. 7a,b). These data differ from past work on AMPARs, which has shown that mutations and allosteric modulators that reduce or eliminate desensitization cause progressive leftward shifts in the wild-type dose-response curve. For example, one study noted a leftward shift of an order of magnitude from the wild-type EC₅₀ to that of GluA1 L497Y (Fig. 7b). Our observations comparing wild-type and D776K receptors support the idea that desensitization has little impact on the time GluK2 receptors remain activated. This is, of course, to be expected if desensitization can proceed only after cation unbinding. Indeed, MD simulations reported here suggest that LBD dimer separation, a structural correlate of desensitization, is promoted for wild-type receptors in the absence of bound sodium ions (Supplementary Fig. 4). Our findings also suggest that desensitization affects the time course of AMPAR activation, and this explains the effect of desensitization on both deactivation kinetics and agonist potency.

**DISCUSSION**

The present study advances the understanding of iGluR gating in several ways. First, we show that cation occupancy is the central requirement in keeping agonist-bound KARs in the activated state and out of desensitization. Second, we propose a structural model for the sequence of events that give rise to deactivation and desensitization. Deactivation is observed when the ligand unbinds from cation-bound states, whereas desensitization proceeds when the ligand is bound to cation-unbound states. Third, and finally, closely related AMPARs do not share this reliance on cation-dependent gating; as a result, desensitization appears able to curtail AMPAR channel activation. As discussed below, this unique property of KARs may provide clues as to how subunit composition and/or auxiliary proteins affect native receptors at glutamatergic synapses.

**The KAR dimer interface is a multifaceted structure**

It is remarkable that subunit cross-linking at two neighboring sites (residues 776 and 783) along the GluK2 LBD dimer interface produces...
very different functional consequences. The Y521C-L783C mutation bridges opposing subunits, yet the crystal structure of its LBD suggests a separation of the upper D1 segment of the dimer interface15. Although separation of the dimer interface is thought to underlie both KAR and AMPAR desensitization13, it is not clear how much separation would be tolerable before channel activation could no longer be maintained. Given microscopic recordings showing that Y521C-L783C channels cannot stably access the main open state of wild-type GluK2 (ref. 26), we propose that this mutant is a mostly desensitized receptor typified by an open interface and/or a poorly activating receptor by virtue of its sporadic channel openings.

Targeted slightly higher along the LBD interface, the mutant residue Lys776 occupies the GluK2 cation-binding pocket and has two related consequences on receptor function: it increases open-channel probability to such an extent that no failures are observed, and it sustains activation for the duration of agonist application. The latter effect supports the idea that the molecular events leading to desensitization are triggered at the apex of the interface rather than being coordinated through the interface as a whole. Whether these interactions are further complicated according to an emerging idea that KAR subunits desensitize with a tetrameric symmetry and not as a dimer of dimers30,31 awaits future study.

**The cation-binding pocket and its relation to gating events**

Although structural rearrangements of the LBD accompany iGluR desensitization13, it is presently unknown how such conformational changes are initiated. The matter is further complicated in KARs, in which bound ions have been proposed to stabilize the LBD dimer interface20. Here, we establish a framework to specify when KARs activate and desensitize by identifying the cation-binding pocket as the molecular switch between these processes. In short, cation pocket occupancy maintains KAR activation, and by implication desensitization cannot occur until cations unbind. The link between cation binding and activation is based on several key observations reported above: the sustained single-channel activation in the GluK2 D776K mutation (Fig. 2), in which the cation-binding pocket is thought to be continuously occupied; the inability of GluK2 to activate in the absence of external ions (Fig. 4); and the gating deficiencies among mutants designed to disrupt cation binding (Fig. 5 and Supplementary Fig. 3). Furthermore, the assertion that cation unbinding precedes desensitization can be deduced from other observations we reported. Specifically, we showed that deactivation kinetics of wild-type KARs were unaffected by desensitization, thus confirming our assertion that the decay of the KAR peak response corresponds to agonist unbinding from the cation-bound state(s) (Fig. 6a,c). This conclusion is consistent with previous work showing that GluK2 deactivation kinetics are made faster by lowering of the external cation concentration or replacement of sodium with another cation12. With long agonist applications (i.e., 250 ms), we propose that the decline in KAR activity is due to cation unbinding because besides the presence of the agonist, the only other known requirement of KARs to activate is allosteric ions22. Given this, we concluded that their departure was the most plausible explanation to trigger the onset of desensitization. In accordance with this notion, MD simulations reported here (Supplementary Fig. 4) predict that removal of cations from the LBD dimer interface can induce structural changes associated with the desensitized state(s).

An alternative explanation for the observations above is that KAR desensitization is triggered by intrinsic rearrangements to the LBD structure, which are countered through the occupancy of bound cations. From this perspective, the relation between bound cations and decay kinetics is attributable to a direct modulation of the intrinsic rate of desensitization (by stabilization of LBD dimers), as has been suggested previously21. This interpretation, however, is difficult to reconcile with several observations. To begin with, if desensitization is merely opposed but not blocked by the presence of bound cations, some residual activation should be detected in solutions lacking external ions, but this is not the case. Furthermore, from this perspective, the effect of cation species on deactivation kinetics would have to be explained by desensitization rates overlapping with those of deactivation. Experiments reported here show that deactivation kinetics are unaffected by desensitization (i.e., comparison of D776K to wild-type GluK2 receptors) (Fig. 6), meaning that desensitization must therefore occur on a slower time scale. Thus, the two processes do not overlap, and activation must be directly regulated by cations.

**Ion channels use different strategies to desensitize**

Desensitization of LGICs has been classically thought to arise from agonist molecules converting receptor complexes into nonreactive forms33, in much the same way that even earlier work linked changes in membrane potential to voltage-gated ion-channel inactivation34. Since then, structural explanations have emerged to account for how the processes of inactivation and desensitization occur at the amino acid level. Some of the first insights came from work on voltage-gated sodium and potassium channels, which were shown to possess intracellular inactivation gates35-36, whereas work on cysteine-loop LGICs hinted at a broader rearrangement of quaternary structure37. Pioneering studies also identified coupling between activation and inactivation of voltage-gated channels38, although this coupling has been more difficult to establish at LGICs. Such coupling might be expected to occur at iGluRs because closure in the agonist-binding domain initiated by ligand binding is thought to bring about both activation and subsequent desensitization, as the agonist becomes entrapped in a stable yet inactive conformation12,39. In keeping with this, data presented in this study suggest a tight coupling between these structural events in AMPARs. Interestingly, this is not the case for KARs, which uncouple the process of activation from desensitization through cation-dependent gating. This unique aspect of KAR gating provides an ideal target by which native receptor responses could be modulated at central synapses. For example, alterations in cation affinity through protein-protein interactions could explain how heteromeric subunits40 and/or auxiliary proteins24 regulate the duration of synaptic KAR activity41. Clearly, much still remains to be examined in future studies, including how this allosteric cation-binding pocket might be exploited to regulate KAR signaling within the vertebrate central nervous system.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

G.B.D. designed and performed experiments, analyzed data and wrote the paper; M.M., B.A.D. and M.R.P.A. designed and performed experiments and analyzed

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data; E.D.A. analyzed data; P.C.B. designed experiments; and D.B. designed and performed experiments, analyzed data and wrote the paper.

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ONLINE METHODS

Cell culture and transfection. HEK293T cells were transiently cotransfected with cDNA encoding wild-type or mutant GluK2(Y521C L783C) or GluA1(Y521C L783C) AMPAR subunits and enhanced GFP (eGFP$_{SH}$), as previously described$^{49,50}$, or transfected with iGluK-subunit cDNA on plasmids also encoding eGFP behind an internal ribosomal entry site. The cDNA for the mutant receptors was generated in two steps from wild-type plasmid with QuickChange II XL site-directed mutagenesis (Stratagene). After transfection for 4–8 h with the calcium phosphate precipitation method, cells were washed twice with divalent cation–containing PBS and maintained in fresh medium (MEM containing Glutamax and 10% FBS). Electrophysiological recordings were performed 24–48 h later.

GluK2 receptor surface expression. To test for possible trafficking defects in mutants used in this study, we measured the fluorescence emitted by an elliptic psfGFP genetically fused to the extracellular N-termini of mutant or wild-type GluK2 receptors (Supplementary Fig. 3a,b). Unlike that of eGFP, the fluorescence emission of psfGFP is almost entirely quenched at pH 5.45 (ref. 42), which we used to evaluate the cellular location of the fluorophores$^{43}$. With this approach, a substantial but reversible attenuation in the fluorescence signal emitted by wild-type psfGFP-GluK2 was observed ($n = 17$ cells) after acidification of the external milieu (Supplementary Fig. 3a,b), thus demonstrating that most of the fluorescence signal was emitted by tagged GluK2 receptors on the plasma membrane. In contrast, acidification of the external solution had little effect on the weak fluorescence emitted by psfGFP-GluK2 R523A receptors ($n = 6$ cells) (Supplementary Fig. 3a,b), consistent with previous work showing that this mutant has poor surface expression$^{27}$. Fluorescence emitted by psfGFP-GluK2 ES242G and L783C receptors ($n = 10$ and 6 cells respectively) was robust, much like that of wild-type GluK2, and was reversibly attenuated by acidification (Supplementary Fig. 3a,b), thus suggesting that trafficking to the plasma membrane is not substantially perturbed for either mutant.

Electrophysiological solutions and recordings. External recording solutions typically contained 150 mM NaCl, 5 mM HEPES, 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$ and 2% phenol red. The internal recording solution contained 115 mM NaCl, 10 mM Na$_2$ATP, 5 mM HEPES, 5 mM Na$_2$BAPTA, 0.5 mM CaCl$_2$, 1 mM MgCl$_2$ and 10 mM Na$_2$ATP to chelate endogenous polyamines. The osmotic pressure was adjusted the pH appropriately. In the case of recordings conducted in nominal sodium milieu ($\Omega = 10$ and 6 cells respectively) was robust, much like that of wild-type GluK2, and was reversibly attenuated by acidification (Supplementary Fig. 3a,b), thus suggesting that trafficking to the plasma membrane is not substantially perturbed for either mutant.

Macroscopic response analysis. Data were analyzed with Clampfit 9.0 and tabulated with Microsoft Excel. Curve fittings for determining the off-rate was performed with first- or second-order exponential functions: $y = A_1 \times \exp(-x/w_1)$. Dose-response data to l-Glu were normalized, pooled across patches and fit with the logistic equation of the following form: $I = I_{max}(1 + (EC_{50}/[Glu])^{n_H})$, where $I$ is the normalized maximal current at any agonist concentration, $I_{max}$ is the interpolated maximal response, $EC_{50}$ is the concentration of l-Glu that elicits the half-maximal response, and $n_H$ is the slope or Hill coefficient.

Single-channel analysis. For wild-type GluK2 receptors, analysis was conducted on patches ($n = 5$) from which 50 or more agonist applications were made at 15-s intervals. For GluK2 D776K, which displayed uniform current responses, analysis was limited to 58 agonist applications, which were divided among four patches. Single-channel data were subjected to digital low-pass filtering at 3 kHz (or 1 kHz for presentation in figures), which resulted in r.m.s. baseline noise values that averaged 0.22 ± 0.024 pA ($n = 5$) and 0.22 ± 0.043 pA ($n = 4$) for wild-type and D776K receptors, respectively. These noise values corresponded to <50% of the smallest difference between adjacent conductance levels in the wild-type receptor. The 3-kHz frequency was chosen on account of our data containing many rapid transitions between conductance levels, as described previously for AMPARs$^{44}$. Accordingly, a resolution of two filter rise times ($2 \times 111 \mu s$) was imposed to detect and account for brief events while maintaining resolution of small conductances. Digitally filtered data were exported to Signal 5.0 (Cambridge Electronic Design) for time-course fitting analysis with SCAN45. The idealized records were then used to provide information on response amplitudes, which could be fit with Gaussian functions whose peaks reflect discrete conductance levels: $y = \Sigma_i \frac{A_i}{\sqrt{2\pi}} \times \exp(-2 \times (x-x_i)/\sigma_i^2)$ where $A$ = area, $x_i$ = center of the peak, and $\sigma_i$ = error associated with $x_c$. From this analysis, the distribution and amplitude of single-channel events observed in patches containing a few channels (Fig. 2f) were similar to events measured at equilibrium in multichannel patches (Supplementary Fig. 5).

Molecular dynamics simulations. All crystal structures used in this manuscript were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank. Two protein structures were used for building models for the MD simulations: an l-Glu–bound GluK2 LBD dimer (PDB 3G3F (resolution 1.38 Å (ref. 46)) and an l-Glu–bound GluK2 R523A LBD dimer (PDB 210C (resolution 2.25 Å (ref. 15)), which were used only for simulations concerning the double-cysteine mutant. Together with the crystallographically resolved water molecules, l-Glu ligands and ions were retained in the simulation setup, whereas two bound isopropyl alcohol molecules were deleted. In simulations of GluK2 without bound sodium ions (Supplementary Fig. 4), these were removed before system setup. The protein was solvated in water in a (90 Å) box with the TIP3P water model47, whereafter the system was neutralized and 150 mM NaCl was added. Mutations, except for Y521C L783C, were imposed manually before simulation setup, either by editing or deleting atoms in the PDB file or by using the mutate function of PyMOL (http://www.pymol.org/) and adjusting the side chain rotamer. For the double-cysteine mutant, the GluK2 double-cysteine (Y521C L783C) mutant structure was used. This structure had no ions bound, so the interface–bound ions from the wild-type structure were added, and rotamers for side chains surrounding the ion sites were optimized in PyMOL before solvation, neutralization and ionization as described above.

The MD simulations were performed in Gromacs 4.5 (ref. 48) with the OPLS all-atom force field$^{49,50}$. The systems were first energy minimized until the maximum force on an atom was <100 kJ/mol/nm. After energy minimization, a 200-ns restrained simulation with position restraints on protein heavy atoms and on bound ions with a force constant of 1,000 kJ mol$^{-1}$ nm$^{-2}$ was performed in the NVT ensemble with a temperature of 300 K maintained by a Berendsen thermostat$^{51}$. Periodic boundary conditions were used, and van der Waals interactions were cut off at 10 Å. Long-range electrostatics were accounted for by the Particle-Mesh Ewald method$^{52}$. All bonds were treated as constraints with the LINCS algorithm, allowing a time step of 2 fs. Subsequently, 100 ns of production run were performed (only 30–50 ns for ES24G). The NPT ensemble was used with the temperature maintained at 300 K and the pressure at 1 bar by the Berendsen thermostat and barostat, respectively$^{51}$. Two repeats for each...
mutational variant were produced. Analyses were performed with VMD\textsuperscript{53} and analysis tools of GROMACS\textsuperscript{48}.

**Statistical methods.** Results are expressed as mean ± s.e.m. Statistical analyses of sample means were performed with two-tailed Student’s \( t \) tests. \( P < 0.05 \) was considered to be statistically significant.

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