A Domain Linking the AMPA Receptor Agonist Binding Site to the Ion Pore Controls Gating and Causes *lurcher* Properties when Mutated

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Ionotropic, AMPA-type glutamate receptors (GluRs) critically shape excitatory synaptic signals in the CNS. Ligand binding induces conformational changes in the glutamate-binding domain of the receptors that are converted into opening of the channel pore via three short linker sequences, a process referred to as gating. Although crystallization of the glutamate-binding domain and structural models of the ion pore advanced our understanding of ligand-binding dynamics and pore movements, the allosteric coupling of both events by the short linkers has not been described in detail. To study the role of the linkers in gating GluR1, we transplanted them between different GluRs and examined the electrophysiological properties of the resulting chimeric receptors in *Xenopus laevis* oocytes and HEK293 cells. We found that all three linkers decisively affect receptor functionality, agonist potency, and desensitization. One linker chimera was nondesensitizing and exhibited strongly increased agonist potencies, while fluxing ions even in the absence of agonist, similar to properties reported for the GluR1 *lurcher* mutation. Combining this new *lurcher*-like linker chimera with the original *lurcher* mutation allowed us to reassess the effect of *lurcher* on GluR1 gating properties. The observed differential but interdependent influence of linker and *lurcher* mutations on receptor properties suggests that the linkers are part of a fine-tuned structural element that normally stabilizes the closed ion pore. We propose that *lurcher*-like mutations act by disrupting this element such that ligand-induced conformational changes are not necessarily required to gate the channel.

Key words: glutamate; AMPA receptor; gating; *lurcher*; linker; desensitization

Introduction

Ionotropic glutamate receptors (GluRs) are responsible for the majority of excitatory neurotransmission in the mammalian brain (Hollmann and Heinemann, 1994). Each GluR subunit harbors an extracellular N terminus, an intracellular C terminus, three transmembrane domains (TMDs; A–C), and a hairpin loop between TMDs A and B (see Fig. 1B). TMD B and the hairpin loop are the major channel-lining domains (Wollmuth and Sobolevsky, 2004) and together with TMD A are inserted between two discontinuous stretches of sequence, S1 and S2, which make up the ligand-binding domain (LBD).

Gating in GluRs encompasses the conformational change induced by ligand binding and its subsequent translation into pore opening. This movement is propagated via three short linkers of 9–17 aa that connect the LBD with the TMDs, presumably forming the outer vestibule of the ion channel (Beck et al., 1999). Inclusion of these linkers in the crystal structure of the LBD was precluded by their flexibility (Armstrong et al., 1998), and struc-

tural determinants of gating that reside within them still remain completely unknown. So far it has only been reported that mutations in the linker preceding TMD A (linker A) (Stern-Bach et al., 1998), as well as a point mutation in the linker following TMD B (linker B), attenuate AMPA receptor (AMPA) desensitization (Yelshansky et al., 2004).

We transplanted all three linkers of the nonfunctional delta2 subunit separately and in all possible combinations into the AMPAR GluR1 and analyzed chimeric receptors in heterologous expression systems. Interestingly, the linker A chimera showed strongly increased agonist potencies and was constitutively open in the absence of agonist, similar to properties reported for the GluR1 *lurcher* mutation (Taverna et al., 2000). Originally, the *lurcher* mutation was discovered as an alanine-to-threonine point mutation in the most conserved region of GluRs, the SYTANLAAF motif downstream of TMD B. This mutation occurs spontaneously in the delta2 subunit of *lurcher* mice where it opens the subunit constitutively (Zuo et al., 1997). All other described *lurcher*-like mutations are located exclusively in the SYTANLAAF motif (Kohda et al., 2000; Taverna et al., 2000; Williams et al., 2003; Hu and Zheng, 2005; Yuan et al., 2005). We find that combining the original GluR1 *lurcher* mutation and the *lurcher*-mimetic linker A mutation has an additive effect on the constitutive current but does not increase agonist potencies, arguing in favor of true spontaneous activation, an interpretation that has come under dispute recently (Meier Klein and Howe,

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2004). We interpret original *lurcher* and *lurcher*-mimetic properties as consequences of two effects: increased coupling efficiency explaining ligand-mediated receptor properties and occurrence of a shunting trigger signal downstream of the LBD that uncouples channel opening from LBD conformation, explaining ligand-insensitive constitutive currents. In addition, we find receptor properties strongly dependent on the introduced combination of linkers A and C, suggesting that both linkers control stability of the closed state. Because current gating models of the TM region focus on TMD B and the pore loop structure only (Wollmuth and Sobolevsky, 2004), the observed impact on gating by linkers A and C provides an interesting new piece to the gating puzzle.

Materials and Methods

Molecular biology. Wild-type cDNAs of rat delta2 (kind gift from J. Boulter, University of California, Los Angeles, CA) and rat GluR1 were used for the construction of the linker chimeras between GluR1 and delta2. For expression in oocytes, all chimeras and GluR1 point mutants were inserted into the vector pSGEM, a modified version of pGEMHE (Villmann et al., 1999). For subsequent experiments in HEK293 cells, mutants were subcloned into pcDNA3 (Invitrogen, San Diego, CA). All chimeras and point mutants were generated via PCR-directed mutagenesis. Primers containing the required mutations (18–54 bp in length; Biomers, Ulm, Germany) were used in overlap extension PCRs to engineer the mutants. We defined the respective native linker sequences as follows (see Fig. 1A): linker A connects the S1 domain with TMD A (17 aa in GluR1, K505–I521; 17 aa in delta2, E536–L552), linker B connects TMD B with the S2 domain (9 aa in GluR1, F619–S627; 9 aa in delta2, F638–S646), and linker C connects the S2 domain with TMD C (17 aa in GluR1, S771–N787; 18 aa in delta2, L796–S813). Numbering of amino acids always starts with the first codon of the mature protein. To name the resulting chimeras, we used the following nomenclature: acceptor-(exchanged part)donor [e.g., GluR1 provided with the linker A of delta2 was named GluR1-(linkerA)delta2]. All linker chimeras, the GluR1 *lurcher* mutation [GluR1-(A618T)] and the nondesensitizing mutant GluR1-(L479Y) (Stern-Bach et al., 1998) were flop splice variants. Where indicated, wild-type cDNA of the transmembrane AMPAR regulatory protein (TARP) $\gamma 2$ from rat in pSGEM vector was used to boost the expression of receptors in oocytes.

cRNA synthesis. cDNA was transcribed and capped for each construct using a modified *in vitro* transcription protocol that uses 400 μ M GpppG (GE Healthcare, Little Chalfont, UK) for capping and an extended reaction time of 3 h with T7 polymerase (Schmidt et al., 2006). Trace labeling was performed with [α - 32 P] UTP to allow calculation of yields and evaluation of transcript quality by agarose gel electrophoresis.

Heterologous expression in *Xenopus* oocytes. Stage V or VI oocytes were surgically removed from the ovaries of anesthetized *Xenopus laevis* and prepared as described previously (Schmidt et al., 2006). For homomeric expression of mutants, oocytes were injected with 10 ng (50 nl) of cRNA within 24 h after surgery. For heteromeric expression with the TARP $\gamma 2$, oocytes were injected with a mixture of 10 ng (50 nl) of GluR cRNA and 1 ng (5 nl) of TARP cRNA. Two-electrode voltage-clamp recordings were performed at room temperature 5–6 d after injection with a TurboTec 10CX amplifier (NPI, Tamm, Germany) controlled by Pulse software (HEKA Elektronik, Lambrecht, Germany). Electrodes were filled with 3 M KCl and had resistances of $\sim$ 1–3 M Ω . Oocytes were clamped at -70 mV and continuously superfused with calcium-free Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 10 mM HEPES-NaOH, pH 7.2) to minimize the activation of endogenous Ca²⁺-gated chloride channels. Agonists and antagonists were applied for 20 s unless stated otherwise. Glutamate, kainate, CNQX, DNQX, and 1-naphthylacetyl spermine (NASP) were obtained from Sigma (Taufkirchen, Germany), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) was purchased from Tocris (Bristol, UK). Leak currents were recorded after they had reached a stable baseline and before starting the first agonist application. The total leak current during measurements of *lurcher*-like mutants

originates from two different sources: one is caused by recording procedures such as impaling the oocyte with sharp electrodes, and one is mediated by open GluR channels in the nominal absence of agonist (termed constitutive current). To distinguish between both components, we applied the open-channel blocker NASP (10 μ M) in the nominal absence of agonist, assuming that the recorded block reflects only the GluR-mediated component (Blaschke et al., 1993; Kohda et al., 2000).

To determine the EC₅₀ values for kainate and glutamate, 8–10 different agonist concentrations were applied to the same oocyte, and steady-state values of the evoked currents were measured. Data from each oocyte were normalized to the maximal current response at saturating concentrations of agonist and fitted separately, and the calculated EC₅₀ values were averaged for 3–10 oocytes. Data were analyzed using Pulse software (HEKA Elektronik) and Prism 4.0c (Graph Pad, San Diego, CA).

Transient expression in HEK293 cells. HEK293 cells were grown in 35 mm dishes and transfected with 2.5 μ g of recombinant plasmid DNA using a modified calcium precipitation technique (Chen and Okayama, 1987). Whole-cell recordings were performed 24–48 h after transfection using an EPC-9 amplifier (HEKA Elektronik) controlled by Pulse 8.7 (HEKA Elektronik). Currents were digitized with a sampling rate of 10 kHz and filtered with 2 kHz. Pipettes were pulled from borosilicate glass and had resistances of 2–10 M Ω . The external buffer consisted of (in mM) 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES-NaOH, pH 7.3; the internal buffer included (in mM) 130 CsF, 33 KOH, 4 NaCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, and 10 HEPES-KOH, pH 7.3. Ligands were applied using a two-channel theta glass capillary mounted on a piezoelectric translator (Colquhoun et al., 1992). Current responses were measured at room temperature at a holding potential of -60 mV.

Glutamate concentration assay. The Amplex Red Glutamic Acid/Glutamate Oxidase Assay kit (Invitrogen) was used to assess the background glutamate level in the oocyte recording chamber. Experiments were performed according to the manufacturer's manual. To maximize the sensitivity of the kit, all reactions were started in the dark, and blackened microplates were used.

Labeling of cell-surface protein and Western blotting. Oocytes were used for plasma membrane-resident protein analysis 5 d after cRNA injection, following a previously described protocol (Hollmann et al., 1994). Isolation exclusively of cell-surface proteins was performed through biotinylation labeling, which spares all intracellular proteins, followed by streptavidin–agarose-mediated precipitation of glycosylated surface proteins. Proteins were separated on SDS-polyacrylamide gels and blotted onto Hybond enhanced chemiluminescence nitrocellulose membranes (GE Healthcare) as described previously (Villmann et al., 1999). The detection of proteins was performed using a primary antibody (dilution 1:1000) directed against the C terminus of GluR1 (kind gift from R. Haganir, Johns Hopkins University School of Medicine, Baltimore, MD). Peroxidase-labeled mouse anti-rabbit IgG (Sigma, Munich, Germany) was used as a secondary antibody (dilution 1:20,000), and immunoreactive bands were visualized by the chemiluminescence method (Pierce, Rockford, IL).

Results

Three short linkers critically influence GluR1 gating properties

Our study focused on the linkers A, B, and C connecting the LBD with the TMDs (Fig. 1). All linkers of the delta2 subunit were transplanted separately as well as in each possible combination into the AMPAR GluR1. In an attempt to maximize the effect of the exchange on receptor properties, we deliberately chose the distantly related delta2 subunit.

The resulting GluR1 mutants were GluR1-(linkerA)delta2, GluR1-(linkerB)delta2, and GluR1-(linkerC)delta2 plus their respective combinations. All seven mutants were expressed in *Xenopus* oocytes for functional analysis (Fig. 2A). Surprisingly, two mutants, the ones containing either linker A or linker A+B, showed much larger current responses to kainate application than wild-type GluR1 ($1578 \pm 109\%$ of wild-type GluR1 for

Figure 1. Alignment of linker regions. **A**, Sequence alignment of the linkers A, B, and C, shown for all mammalian GluR subunits. All identical amino acids within the seven receptor subgroups are marked in pink. Differences are highlighted in blue. The conserved SYTANLAAF motif is included in the alignment because it is directly adjacent N-terminally to the linker B and contains the *lurcher* site (*). The first five amino acids of the linker C in AMPARs are part of the differentially spliced flip/flop region. Although linker B is predictive only for either non-NMDA or NMDA receptors, linkers A and C are highly indicative of their specific subgroups, with the single exception of the δ subunits. **B**, Schematic topology of GluRs including the crystal structure of the AMPAR GluR2 LBD (Armstrong and Gouaux, 2000). Linkers A, B, and C are indicated in yellow.

linker A and $1163 \pm 84\%$ for linker A+B) (Table 1). Two mutants, the ones containing either linker B or linker A+C, were also functional but showed only very small current responses ($3.7 \pm 0.4\%$ for linker B and $1.6 \pm 0.4\%$ for linker A+C). The other three mutants (containing linker C, linker B+C, and linker A+B+C of delta2) did not display any detectable current responses, neither to kainate nor to glutamate application.

AMPA current responses can be potentiated by coexpressing the TARP $\gamma 2$ (Chen et al., 2003). We therefore tested whether coexpression with $\gamma 2$ could endow function to the nonfunctional linker C, linker B+C, and linker A+B+C mutants. Current responses of all mutants already functional in the absence of $\gamma 2$ were potentiated when coexpressed with $\gamma 2$, as expected (Fig. 2B). Despite the coexpression with $\gamma 2$, mutants containing linker C or linker B+C did not show any current responses after agonist application. However, the triple linker combination produced current responses in coexpression with $\gamma 2$. Western blot analysis revealed that all seven linker mutants were expressed in the plasma membrane, including the nonfunctional linker C and linker B+C constructs, and that their expression was slightly increased by coexpression with $\gamma 2$ (Fig. 2E).

To exclude fast receptor desensitization as the reason for the nonfunctionality of the linker C and linker B+C mutants in oocytes, we expressed both mutants in HEK293 cells and recorded from them using the patch-clamp technique and fast agonist application. No current response to glutamate application could be detected for either mutant ($n = 10$; data not shown). Because very fast desensitizing currents can escape detection even in the HEK cell expression system (Horning and Mayer, 2004), we examined both linker mutants in the background of the nondesensitizing GluR1-L479Y mutation (Stern-Bach et al., 1998) in the

oocyte system. However, neither the introduction of the L479Y mutation ($n = 10$) nor coexpressing the resulting mutants with $\gamma 2$ could endow any function to GluR1 receptors containing linker C or linker B+C of delta2 ($n = 5$; data not shown). Therefore, the transplantation from delta2 into GluR1 of linker C alone renders the channels nonfunctional, and linker B is not capable of rescuing function.

The transplantation of linker A had the most profound effect on GluR1 ion channel properties. Apart from much larger current responses, the expression of either linker A or linker A+B mutants in oocytes raised the leak current well above levels of wild-type GluR1 (Fig. 2C). AMPAR open-channel blockers such as NASP could block this large inward current, suggesting that the mutant channels are open in the nominal absence of agonist. This kind of aberrant gating behavior has been reported before for a number of so-called *lurcher* point mutations, all of which were restricted to the conserved SYTANLAAF motif located directly adjacent to linker B (Fig. 1) (Kohda et al., 2000; Taverna et al., 2000; Williams et al., 2003; Hu and Zheng, 2005; Yuan et al., 2005). We next recorded dose-response curves for our linker mutants, because one hallmark characteristic of *lurcher* mutants are strongly increased agonist potencies. Indeed, we found a 100-fold reduction in EC_{50} values for the mutants containing either linker A or linker A+B of delta2 (Fig. 2D, Table 1). Because of small current responses of the mutants containing linker B, linker A+C, or linker A+B+C, agonist potencies had to be determined in coexpression with $\gamma 2$. Wild-type receptors show slightly reduced EC_{50} values (4.5-fold) when coexpressed with $\gamma 2$. Although we did not observe this influence of $\gamma 2$ on agonist potencies of the *lurcher*-like mutants containing either linker A or linker A+B (Table 1), EC_{50} values for the linker combinations B,

Figure 2. delta2 linker transplantation into GluR1. **A**, Representative current responses elicited by 150 μ M KA recorded from *Xenopus* oocytes expressing wild-type GluR1 or various linker chimeras. The duration of agonist application is indicated by horizontal black bars. **B**, Representative kainate-induced current responses for all constructs shown in **A** when coexpressed with γ 2. **C**, Mean leak currents of oocytes expressing wild-type GluR1 or linker chimeras. Each bar represents the mean ($\pm$ SEM) of 7–42 oocytes originating from at least two independent experiments. **D**, Dose–response curves for the indicated mutants. For a complete list of EC₅₀ values, see Table 1. **E**, Western blot analysis of whole-cell (W) and surface (S) protein fractions (15–20 oocytes) was performed to check for expression of the nonfunctional linker chimeras (left multicolumn panel), in the absence (left column) or presence (middle column) of γ 2. For comparison, wild-type GluR1 was analyzed in the same set of experiments. +, Coexpression with γ 2; –, expression in the absence of γ 2. Note that all chimeras are expressed in the plasma membrane.

A+C, or A+B+C, which could only be recorded in the presence of γ 2, might have been slightly reduced by γ 2, like the wild type. Interestingly, we did not find the *lurcher*-like high agonist potency of the linker A mutant when linker C was cotransplanted. Hence, the EC₅₀ value of the linker A+C mutant for kainate did not show a reduction but was increased 27-fold compared with wild type. Transplantation of either linker B alone or all three linkers together decreased the kainate potency threefold to fourfold.

Because the original *lurcher* effect is caused by a point mutation in linker B, we split the linker A in three parts, in an attempt to delineate more closely which part of the linker is responsible

for the observed *lurcher* properties. None of the three mutants constructed, however, reproduced the full effect of the complete linker A, suggesting a cumulative effect of several of the 14 introduced mutations in linker A (data not shown).

In summary, the GluR1 linker A chimera showed the same aberrant gating behavior as reported previously for *lurcher* mutations. Cotransplantation of linker C, however, removed these *lurcher* features, even decreasing kainate potency compared with wild type. This antagonistic behavior of linkers A and C is intriguing and suggests that the amino acid sequences of these two linkers are fine-tuned in native GluRs to guarantee normal gating. Mutations in these regions can apparently either increase agonist potencies and facilitate gating (even to an extent in which ligand binding is not required any more, as in our linker A mutant) or decrease agonist potencies and obstruct gating (even to an extent in which channels do not gate at all, as in our linker C mutant). Between these two extremes, normal gating constitutes “intermediate” behavior, similar to what we see in our linker A+C mutant.

Introduction of linker A disrupts GluR1 desensitization and increases kainate efficacy

Glutamate application induced similarly large current responses at linker A mutant channels as kainate application. In *Xenopus* oocytes expressing GluR1 mutants, high current responses to glutamate application are indicative of nondesensitizing mutants. Therefore, we directly analyzed desensitization properties of the linker A mutant in HEK293 cells. For *lurcher*, previous studies had revealed that desensitization is reduced as well (Kohda et al., 2000; Meier Klein and Howe, 2004), representing another parallel to the linker A mutant. One well described mechanism of how GluR desensitization can be fine-tuned by mutations is modulation of the stability of the dimer interface of two LBDs (Stern-Bach et al., 1998; Sun et al., 2002; Fleck et al., 2003; Horning and Mayer, 2004; Priel et al., 2006; Weston et

al., 2006). The linkers are not part of any solved LBD crystal structure, and *lurcher* and linker A mutations are positioned outside the proposed dimer interface. Therefore, the mechanism by which they reduce desensitization ought to be different. To more directly assess any inhibitory impact on desensitization of the linker A mutant, we examined it along with the *lurcher* mutation and the nondesensitizing GluR1-(L479Y) mutant, which is known to stabilize the dimer interface (Sun et al., 2002). In addition, we tested all possible combinations of these three mutations to detect any mutual influence on channel properties.

Whole-cell recordings of transiently transfected HEK293 cells confirmed complete desensitization of glutamate-evoked cur-

Table 1. Currents, leak currents, and agonist potencies for KA with and without $\gamma 2$ at GluR1 linker transplantation mutants

Construct GluR1-(linkerX) $\gamma 2$	$I_{\max}$ KA ($-\gamma 2$) (nA) (n)	$I_{\max}$ KA ($+\gamma 2$) (nA) (n)	Average leak (nA) (n)	EC ₅₀ KA ($-\gamma 2$) (μ M) (n)	EC ₅₀ KA ($+\gamma 2$) (μ M) (n)
GluR1(Q)flop	241 $\pm$ 63 (5)	6448 $\pm$ 375 (11)	221 $\pm$ 38 (24)	27.82 $\pm$ 1.32 (10)	6.00 $\pm$ 1.01 (3)
A	3805 $\pm$ 263 (10)	6246 $\pm$ 821 (8)	2216 $\pm$ 221 (42)	0.28 $\pm$ 0.02 (3)	0.37 $\pm$ 0.04 (3)
B	9 $\pm$ 1 (12)	3413 $\pm$ 566 (13)	94 $\pm$ 12 (15)	nr	22.59 $\pm$ 2.91 (3)
C	0 $\pm$ 0 (5)	0 $\pm$ 0 (5)	113 $\pm$ 50 (7)	nf	nf
A+B	2804 $\pm$ 204 (7)	3566 $\pm$ 271 (7)	2545 $\pm$ 191 (20)	0.31 $\pm$ 0.04 (4)	0.24 $\pm$ 0.03 (2)
A+C	4 $\pm$ 1 (12)	1396 $\pm$ 146 (17)	266 $\pm$ 43 (11)	nr	164.92 $\pm$ 20.60 (4)
B+C	0 $\pm$ 0 (5)	0 $\pm$ 0 (5)	272 $\pm$ 69 (10)	nf	nf
A+B+C	0 $\pm$ 0 (8)	129 $\pm$ 18 (21)	127 $\pm$ 37 (8)	nf	17.36 $\pm$ 0.41 (4)

Mean current responses and agonist potencies ($\pm$ SEM). nr, Nonrecordable; nf, nonfunctional.

rents for wild-type GluR1, whereas for both the linker A and *lurcher* mutants we found a markedly reduced extent of desensitization (Fig. 3, Table 2). Responses to glutamate of all four possible combinations of linker A, *lurcher*, and L479Y mutations displayed virtually no desensitization. Hence, determination of desensitization time constants was impossible. A similar picture emerged for kainate-evoked currents (Fig. 3A).

Figure 4A shows typical current responses of all mutants to saturating concentrations of glutamate (300 μ M) and kainate (150 μ M), recorded in oocytes (see Table 3 for EC₅₀ data). Glutamate-evoked current amplitudes of the linker A mutant and GluR1-(L479Y) were in the same range. In contrast, *lurcher* only showed 48 $\pm$ 6% of the L479Y-mediated current, and the combination linker A+*lurcher* reduced the current amplitudes even further to 18 $\pm$ 1% of GluR1-(L479Y) (for recording conditions, see Table 2). Unexpectedly, adding the *lurcher* and linker A mutants to L479Y decreased the current amplitudes: current responses to glutamate were reduced to 46 $\pm$ 5% for the linker A+L479Y construct, to 25 $\pm$ 2% for the L479Y+*lurcher* construct, and to 9.3 $\pm$ 0.6% for the combination of all three mutations compared with GluR1-(L479Y) (Fig. 4A,B). For agonists that induce strong desensitization such as glutamate, current amplitudes in the oocyte system normally increase when desensitization is blocked. Yet here, three mutations that individually block desensitization and thus individually increase current responses decreased glutamate-induced current amplitudes when combined. In primary neuronal cultures, reduced desensitization has been shown to hamper trafficking of mutant receptors to the plasma membrane (Greger et al., 2006, 2007). Thus, the observed reduction in current amplitudes might be interpreted as resulting from reduced surface expression in the oocytes. However, we measured the current amplitude reduction for all combinatorial mutants relative to the already nondesensitizing and thus trafficking-minimized L479Y mutant. Any additional reduction in current therefore must be attributable to mechanisms other than decreased trafficking because of reduced desensitization. In addition, reduced agonist-induced current amplitudes coincide with a larger fraction of constitutive currents, offering an alternative explanation for the reduced agonist-induced currents (see next section).

Based on the recorded current amplitudes, we then calculated glutamate (Glu)-to-kainate (KA)-induced current ratios ($I_{\text{Glu}}/I_{\text{KA}}$). Two effects influence this ratio in oocytes: desensitization and the efficacy of the respective agonist. For the essentially nondesensitizing GluR1-(L479Y) mutant, the ratio simply reflects the agonist efficacies, and hence kainate (as a partial agonist) elicited only 29 $\pm$ 5% of the glutamate-induced current, resulting in a ratio of 4.4 $\pm$ 0.7. For the wild type, the ratio is 0.16 $\pm$ 0.02, reflecting approximately fivefold higher currents induced by kainate compared with glutamate. For all mutants that exhibit a

strongly reduced desensitization, one would expect an $I_{\text{Glu}}/I_{\text{KA}}$ close to the value for GluR1-(L479Y). Yet here, for the linker A and *lurcher* mutants as well as all their possible combinations with the L479Y mutation, kainate-evoked currents were increased to 50–100% of glutamate-induced currents (Fig. 4C, Table 2), indicating that the introduction of linker A or *lurcher* mutations increases kainate efficacy.

Together, the linker A mutation has two effects besides generating spontaneously active channels: a reduced desensitization and an increased kainate efficacy, both parallels to *lurcher*.

Combination of linker A and *lurcher* mutations locks more GluR1 channels in an open conformation

All mutants carrying one of the three mutations (linker A, *lurcher*, or L479Y) did show elevated GluR-mediated constitutive currents when expressed in oocytes (Table 2). We quantified these constitutive currents by applying NASP (10 μ M), assuming that it exclusively blocks current mediated by constitutively open channels in the nominal absence of agonist (see Materials and Methods). Figure 5B shows the mean NASP block for all examined linker A, *lurcher*, and L479Y mutations (Table 2). Except for wild-type GluR1, where NASP application did not have any effect, a minimum of 80% of the recorded basal currents was attributable to GluR-mediated constitutive currents.

The quantification of GluR-mediated constitutive current amplitudes allows the calculation of the relationship between mean glutamate-evoked current responses on the one hand and the mean constitutive current on the other hand (Fig. 5A). Clearly, for wild-type GluR1, 100% of GluR-mediated currents are induced by glutamate application. Quite surprisingly, however, this does not hold true for the L479Y mutant. Here, 14.2 $\pm$ 2.0% of the total GluR-mediated current were constitutive current, suggesting that the L479Y mutation also generates some open channels in the absence of agonist (Fig. 5A, Table 2), a fact that previously had been overlooked. Introduction of either linker A or the *lurcher* mutation shifts the balance in favor of higher constitutive currents. Only 64.1 $\pm$ 3.0% of the total current mediated by the linker A mutant were found to be evoked by glutamate application. The rest of the current response was mediated by GluR gating in the nominal absence of agonist. The *lurcher* mutant showed a virtually identical balance: 64.8 $\pm$ 3.0% of GluR-mediated current were agonist evoked. The most striking shift, though, was produced by the combination linker A+*lurcher*. Here, only 12.8 $\pm$ 1.0% of the total GluR-mediated current could be induced by glutamate application, whereas the remaining 87.2 $\pm$ 1.0% were present in the absence of agonist. Interestingly, adding the L479Y mutation to either linker A or *lurcher* mutants decreased the agonist-inducible current, whereas the constitutive current increased. Only approximately one-third of the linker A+L479Y and *lurcher*+L479Y mutant channels

Figure 3. Analysis of desensitization in HEK293 cells. **A**, Representative responses of whole-cell patches to application of Glu (3 mM) or KA (600 μ M) recorded from HEK293 cells expressing the specified mutants. Black bars indicate the duration of agonist application. **B**, Extent of desensitization in percentage $\pm$ SEM of glutamate-activated peak currents recorded from four to nine HEK293 cells.

were closed in the absence of agonist. The triple mutant linker A + *lurcher* + L479Y showed the same drastic shift as the linker A + *lurcher* mutant. Agonists could only activate about one-tenth of these channels.

Apparently, the mechanisms by which the linker A and *lurcher* mutants evoke high constitutive currents are additive, indicating two independent mechanisms. The surprisingly small agonist-evoked current responses of mutants containing combinations of *lurcher*, linker A, or L479Y described in the previous section can thus be explained by a larger fraction of channels that are already in an open state before agonist application, and can thus not be activated any further.

Constitutive activation or residual glutamate acting at super-potent channels?

Currently, two possible mechanisms are discussed to explain the elevated constitutive current amplitudes of *lurcher* channels. One theory attributes the inward current to active channels that gate in the absence of agonist (Kohda et al., 2000; Taverna et al., 2000), whereas the other assumes that the constitutive current is caused by residual glutamate in external solutions during measurements (Meier Klein and Howe, 2004). The latter explanation is based on the observation that the competitive antagonist NBQX can block a fraction of the GluR-mediated constitutive current. According to Meier Klein and Howe (2004), NBQX competes with glutamate for the binding sites, and any effect NBQX has on the constitutive current should be attributable to replaced glutamate.

We therefore tested the response of all mutants to application of NBQX (10 μ M). In all cases except wild type, NBQX blocked a fraction of the constitutive current (Fig. 5B). The largest block was observed for the L479Y mutant ($68.7 \pm 4.2\%$), whereas linker A and *lurcher* mutants as well as the combinations linker A + L479Y and L479Y + *lurcher* responded to NBQX to a lesser extent (29–48%). Both mutants containing the combination of linker A and *lurcher* mutations displayed the most drastic reduction in NBQX block, because only 5–8% of the leak current could be blocked by NBQX application. For all examined mutants [except wild type and GluR1-(L479Y) alone], a substantial amount of leak current could not be blocked by NBQX, indicating that residual glutamate cannot be the sole source for the GluR-mediated fraction of leak current.

Previous characterizations of the GluR1 *lurcher* mutant had revealed that the quinoxalines CNQX and DNQX, competitive antagonists at AMPARs structurally related to NBQX but carrying smaller side groups, can activate *lurcher* channels (Taverna et al., 2000; Meier Klein and Howe, 2004). Therefore, we tested the effects of both quinoxalines on all analyzed mutants. Although the application of these antagonists in the absence of ligand had no effect on GluR1 wild type, their application reduced leak current levels of oocytes expressing the L479Y mutant (Table 3). Quite the opposite happened when we applied CNQX and DNQX to mutants containing either linker A or *lurcher* mutations. At these mutants, both compounds induced considerable current responses, revealing further similarity between those two mutants (Fig. 5C, Table 3).

All quinoxalines were applied at a concentration of 10 μ M, which, according to reported EC_{50} (CNQX, DNQX) and IC_{50} (NBQX) values for GluR1 *lurcher*, should be well in the saturating range (Taverna et al., 2000; Meier Klein and Howe, 2004). To make sure the used concentration was saturating, we tested for an increase in the block of the constitutive currents of selected mutants (L479Y, *lurcher*, linker A, and *lurcher* + linker A + L479Y)

Table 2. Glu- and KA-induced currents, current ratios, average leak current, and extent of desensitization at selected GluR1 mutants

Construct	$I_{\max}$ Glu (nA) (n)	$I_{\max}$ KA (nA) (n)	$I_{\text{Glu}}/I_{\text{KA}}$ ratio (n)	Average leak (nA) (n)	$I_{\max}$ Glu as a fraction of total GluR-mediated current ^a (%) (n)	Desensitization (%) (n)
GluR1(Q)flop	35 ± 5 (8)	241 ± 63 (5)	0.16 ± 0.02 (5)	173 ± 34 (13)	100.0 ± 0.0 (10)	100.0 ± 0.0 (8)
L479Y	4220 ± 356 (10)	1222 ± 226 (6)	4.44 ± 0.67 (10)	746 ± 171 (15)	85.8 ± 2.4 (5)	1.9 ± 0.5 (5)
Linker A	3940 ± 255 (16)	3805 ± 263 (10)	1.13 ± 0.04 (14)	2549 ± 242 (20)	64.1 ± 2.8 (13)	8.1 ± 0.5 (5)
<i>lurcher</i>	2035 ± 266 (15)	1600 ± 210 (19)	1.41 ± 0.08 (9)	2105 ± 255 (25)	64.8 ± 4.7 (5)	13.0 ± 0.8 (3)
Linker A + <i>lurcher</i>	767 ± 47 (14)	364 ± 41 (12)	2.13 ± 0.13 (13)	4908 ± 264 (23)	12.8 ± 0.8 (14)	1.6 ± 0.9 (5)
Linker A + L479Y	1966 ± 233 (19)	1711 ± 189 (19)	1.18 ± 0.04 (15)	4052 ± 106 (21)	34.9 ± 3.7 (9)	1.9 ± 0.5 (5)
L479Y + <i>lurcher</i>	1062 ± 110 (19)	582 ± 86 (18)	2.44 ± 0.24 (11)	2064 ± 160 (31)	37.8 ± 5.1 (7)	1.0 ± 0.4 (4)
Linker A + L479Y + <i>lurcher</i>	394 ± 26 (19)	207 ± 19 (11)	2.06 ± 0.08 (14)	2875 ± 146 (25)	10.0 ± 0.7 (9)	3.5 ± 1.4 (5)

The ratios of glutamate-to-kainate-induced currents ($I_{\text{Glu}}/I_{\text{KA}}$ ratios) were calculated for each oocyte and averaged ($\pm$ SEM). Concentrations of the applied agonists were 150 μ M for kainate and 300 μ M for glutamate. The extent of desensitization was determined using the patch-clamp recording technique in HEK293 cells; all other values were determined by two-electrode voltage-clamp recording from *X. laevis* oocytes.

^aSee Figure 5 for explanation.

when applying 10 times more NBQX. Constitutive currents were blocked marginally better (0–7%; $n = 5$ –6; data not shown) by 100 μ M NBQX than by 10 μ M NBQX, proving that the observed differences in NBQX block of constitutive current were not simply attributable to reduced potency of the quinoxaline.

The drastically increased agonist potencies of *lurcher*, which allow glutamate concentrations in the nanomolar range to evoke substantial current responses, were another major argument of Meier Klein and Howe (2004) to attribute the elevated leak currents to contaminating glutamate.

To address this, we first determined the background glutamate level using an enzymatic cycling assay, to test for contaminating glutamate in the oocyte recording system. We found residual glutamate levels in the recording chamber to be below the detection level of 15 nM ($n = 3$), as long as after each glutamate application sufficient time (6 min) was allowed for washing out the agonist. We then determined agonist potencies for all six mutants (Fig. 6, Table 3). If the constitutive currents were attributable to residual glutamate, one would expect the highest agonist potencies to correspond with the highest constitutive currents.

Glutamate was ~ 10 -fold more potent at linker A and ~ 20 -fold more potent at *lurcher* mutants ($EC_{50} = 426 \pm 46$ nM; $EC_{50} = 208 \pm 45$ nM) than at wild-type GluR1 ($EC_{50} = 4.4 \pm 0.2$ μ M). The L479Y mutation increased the potency for glutamate approximately twofold ($EC_{50} = 2.4 \pm 0.1$ μ M). Surprisingly, the combination linker A + L479Y increased glutamate potency by a factor of 100 compared with wild type, resulting in an EC_{50} value of only 45 ± 8 nM. Combining the *lurcher* with the L479Y mutation increased potency as well, but only approximately fivefold ($EC_{50} = 743 \pm 83$ nM). Thus, after their combination with the L479Y mutation, the increased agonist potencies of linker A and *lurcher* were shifted in opposite directions. The triple combination linker A + *lurcher* + L479Y again showed a drastically increased glutamate potency ($EC_{50} = 79 \pm 10$ nM), suggesting that the com-

Figure 4. Comparison of linker A, *lurcher*, and L479Y mutations. **A**, Modulation of maximal current amplitudes by linker A, *lurcher*, and L479Y mutations and all their possible combinations. Shown are representative current responses induced by 300 μ M Glu or 150 μ M KA recorded from oocytes. **B**, Mean glutamate- and kainate-evoked current responses. Data shown are means ($\pm$ SEM) of 8–19 oocytes originating from at least two independent experiments. Results from independent experiments were only included in the analysis if the wild-type controls did not differ significantly. **C**, Resulting glutamate-to-kainate-induced current ratios for all mutants.

bination of linker A and L479Y is the critical determinant for glutamate potencies in the nanomolar range. The extremely low EC_{50} values of both linker A + L479Y and the triple combination linker A + *lurcher* + L479Y explain well the very slow return to baseline in the whole-cell patch-clamp measurements with glutamate (Fig. 3A), which we only saw for those two mutants containing a combination of linker A and L479Y mutations. When comparing the potencies with the corresponding constitutive current levels, we could not find a positive correlation for every mutant. Although combining the linker A with the L479Y mutation resulted in the highest glutamate potencies, constitutive current levels were not the

Table 3. Agonist potencies for glutamate (Glu) and efficiencies of blockers at selected GluR1 mutants

Construct	Block of leak current after application of drugs at 10 μ M				EC ₅₀ Glu (nM) (n)
	NASP (%) (n)	NBQX (%) (n)	CNQX (%) (n)	DNQX (%) (n)	
GluR1(Q)flop	0.5 $\pm$ 0.4 (7)	1.3 $\pm$ 1.2 (5)	2.0 $\pm$ 1.8 (4)	1.9 $\pm$ 1.4 (5)	4445 $\pm$ 264 (3)
L479Y	90.2 $\pm$ 2.6 (4)	68.7 $\pm$ 4.2 (3)	39.7 $\pm$ 8.4 (3)	36.9 $\pm$ 9.3 (3)	2424 $\pm$ 112 (3)
Linker A	83.8 $\pm$ 2.4 (10)	32.5 $\pm$ 3.2 (6)	−52.7 $\pm$ 6.7 (6)	−36.6 $\pm$ 4.5 (6)	426 $\pm$ 46 (3)
<i>lurcher</i>	81.8 $\pm$ 3.0 (10)	29.2 $\pm$ 2.4 (6)	−59.3 $\pm$ 10.1 (7)	−47.8 $\pm$ 7.9 (7)	208 $\pm$ 45 (3)
Linker A + <i>lurcher</i>	90.7 $\pm$ 1.3 (12)	5.2 $\pm$ 1.4 (6)	−3.7 $\pm$ 0.5 (6)	−3.3 $\pm$ 0.3 (6)	260 $\pm$ 4 (3)
Linker A + L497Y	80.8 $\pm$ 5.1 (5)	30.8 $\pm$ 2.1 (8)	−15.5 $\pm$ 2.1 (5)	−14.0 $\pm$ 1.4 (5)	45 $\pm$ 8 (5)
L497Y + <i>lurcher</i>	94.3 $\pm$ 1.2 (5)	48.1 $\pm$ 2.8 (5)	−17.0 $\pm$ 3.3 (5)	−13.3 $\pm$ 2.9 (5)	743 $\pm$ 83 (5)
Linker A + L497Y + <i>lurcher</i>	95.7 $\pm$ 0.3 (3)	7.9 $\pm$ 0.4 (7)	−3.8 $\pm$ 0.6 (3)	−3.8 $\pm$ 0.6 (3)	79 $\pm$ 10 (3)

Mean block of leak current after application of specified drugs at 10 μ M was calculated for each oocyte and averaged ($\pm$ SEM). Negative values indicate no block but additional current responses that were induced after application of the antagonist on top of the elevated leak current.

Figure 5. Analysis of leak currents. **A**, Relationship between mean Glu-induced currents (gray columns, b) and mean NASP-blockable constitutive currents (white columns, a), which together represent the total GluR-dependent current (c). This total GluR-dependent current was set to 100% for each mutant to facilitate comparison between different mutants. Each column represents the mean percentage ($\pm$ SEM) of 5–14 oocytes (left). To illustrate the calculation, examples of current responses to NASP and Glu are shown (right). **B**, Mean block of leak currents by NASP (gray columns) and the competitive antagonist NBQX (black columns), each at 10 μ M. Data are given as means ($\pm$ SEM) of 8–14 oocytes. **C**, Typical responses of GluR1, GluR1-(L479Y), linker A, and *lurcher* mutants to application of 10 μ M CNQX or 10 μ M DNQX recorded from *Xenopus* oocytes.

highest. In addition, the EC₅₀ value for glutamate for linker A + *lurcher* (260 $\pm$ 4 nM) was almost unaltered compared with the value for *lurcher* alone (208 $\pm$ 45 nM), although constitutive current levels for both mutants differed substantially. The described constitutive current for linker A + *lurcher* is elevated to such an extent that it cannot be caused by residual glutamate alone, even assuming a glutamate contamination in the range of our detection level (15 nM). Thus, although background glutamate would certainly influence the EC₅₀ values, especially for the very low values measured for linker A + L479Y, it cannot account for the extensive differences in EC₅₀ values alone.

The interpretation of the constitutive current in *lurcher* channels is an important question to solve with regard to GluR and, in particular, δ receptor function. If the constitutive current in GluR1 *lurcher* was solely caused by residual glutamate,

the same mechanism would have to be expected for delta2 *lurcher*. A recent study reported the binding of glycine and D-serine to the LBD of delta2 (Naur et al. 2007). Surprisingly, though, application of D-serine or glycine to delta2 *lurcher* leads to a concentration-dependent block of the constitutive current with unexpectedly low potency (IC₅₀ value of 182 μ M for D-serine and of 507 μ M for glycine). These findings clearly exclude that residual glycine or D-serine are responsible for the constitutive current observed for delta2 *lurcher*. In this and both our findings, the low responsiveness of linker A + *lurcher* mutants to NBQX and the discrepancies in correlation between high agonist potencies and high constitutive current levels are at odds with residual glutamate being the principal cause for the elevated leak currents. Instead, the data strongly imply that unliganded openings have to be considered in *lurcher* gating models (see Discussion).

Discussion

To define the role in channel gating of the linkers connecting the LBD with the TMDs, we mutated AMPAR GluR1 linkers to the corresponding delta2 linkers and showed that all three linkers are critically involved in gating. Although delta2 lacks agonist-induced responses in heterologous expression systems (Araki et al., 1993; Lomeli et al., 1993; Naur et al., 2007), the simultaneous transplantation of its three linkers into GluR1 resulted in functional receptors. Thus, the lack of ion channel function of the delta2 subunit cannot be attributed to inherently nongating linker domains.

Linkers are important determinants of gating properties

Transplantation of linker A resulted in a *lurcher*-like channel showing strongly increased current responses. In contrast, the mutation of linker C generated nonfunctional AMPARs. Combining linker A and linker C mutants resulted in a functional construct lacking the linker A-induced *lurcher* characteristics. Thus, linkers A and C both play a decisive role in gating. Given the close positioning of both linkers as suggested by their mutual interaction with allosteric modulators (Balannik et al., 2005), a direct linker–linker interaction would be an intriguing explanation for our findings. However, to unambiguously prove this, mutant cycle analysis would be required, which, unfortunately, is impossible because the linker C mutant is nonfunctional. Apparently, the linkers comprise a fine-tuned system that regulates GluR gating properties and requires the linkers to be compatible with each other.

A new motif in GluRs mimics *lurcher* characteristics

Surprisingly, GluR1 subunits carrying the linker A of delta2 showed large inward currents in the absence of agonist, almost no desensitization, and a substantial leftward shift in agonist potencies. Furthermore, kainate efficacy was increased, and the competitive antagonists CNQX and DNQX were converted to agonists. Virtually identical characteristics had been reported for GluR1 *lurcher* channels (Taverna et al., 2000; Kohda et al., 2000; Williams et al., 2003; Meier Klein and Howe, 2004). This similarity is intriguing because *lurcher* and linker A mutations are located in structurally different receptor domains. *Lurcher* is part of the SYTANLAAF motif in TMD B, and the amino acid side chain at this position likely is never water-accessible, regardless of the conformational state of the channel (Sobolevsky et al., 2003). In contrast, linker A resides outside the membrane region of GluRs, immediately preceding TMD A. Positioned such, linker A amino acids are probably always water-accessible, as has been demonstrated for linker A in NR1 subunits (Beck et al., 1999). This suggests structurally different mechanisms for the *lurcher* and linker A impact on gating that nevertheless results in similar channel properties.

Coupling efficiency and constitutive activation

None of the previously suggested explanations for the *lurcher* properties (Kohda et al., 2000; Taverna et al., 2000; Meier Klein and Howe, 2004) offered a consistent explanation for increased kainate efficacy, conversion of competitive antagonists into agonists, or NBQX-insensitive constitutive currents.

Figure 6. Dose–response curves for all constructs analyzed. Dose–response curves for glutamate of the various mutants (3–6 oocytes) are shown. All mutants, except L479Y, show a pronounced reduction in EC_{50} values (see Table 3).

Both *lurcher* and linker A mutations are perfectly located to alter GluR coupling efficiency. Coupling efficiency describes the probability with which each subunit undergoes ligand-induced conformational changes from closed to open state (Jin et al., 2003) and is directly proportional to the degree of cleft closure at the LBD. At *lurcher* channels, even very little domain closure is sufficient to gate channels: DNQX binding already triggers gating despite inducing only 3° of domain closure (Armstrong and Gouaux, 2000). Furthermore, only 12° of kainate-induced domain closure suffice to activate the channel almost as completely as glutamate does at 20°. The overall structure and binding mechanism of the LBD is most likely not altered by the mutations because both reside well outside the LBD. We therefore propose that in *lurcher* and linker A channels a smaller degree of cleft closure is sufficient to reach maximal coupling efficiency of full agonists.

How could channels gate in the absence of ligand? In principle, two different mechanisms for unliganded open channels are conceivable (Fig. 7C). An open channel could occur in conjunction with either an unliganded closed-cleft LBD or an unliganded open-cleft LBD. The former would assume obligatory allosteric coupling between the LBD and the channel, with gating events occurring exclusively in concert with domain closure. The latter would require allosteric uncoupling of LBD and channel, allowing channel gating to occur completely independent of the LBD conformational state. Assuming obligatory allosteric coupling, thermal fluctuations between partially closed and open LBD conformations could explain unliganded gating events in *lurcher*-like channels. As soon as LBD intrinsic movements lead to at least 3° of domain closure, *lurcher* and linker A channels would open, thereby producing constitutive current. In wild-type channels, this intrinsic fluctuation rarely leads to gating because a larger degree of domain closure is required. The reduction in constitutive current after application of NBQX could be interpreted such that NBQX binding arrests the LBDs of *lurcher*-like mutants at a degree of domain closure insufficient to gate the channel, thus blocking thermal fluctuations and reducing constitutive current. In addition, an increased coupling efficiency of the mutants can explain the observed low EC_{50} values. If binding of glutamate inevitably leads to channel gating, the EC_{50} value should more closely reflect the K_D of glutamate binding. Indeed, the low nanomolar EC_{50} values found for the combination of linker A and L479Y mutations are comparable

Figure 7. Model of AMPAR gating. **A**, Schematic representation of the open, resting, and desensitized states of a dimer. The diagram features an interface located at the TMD/linker region of two subunits. Numbers in blue are the distances as seen in the crystal structures of the LBD in the respective states of the receptor (Armstrong et al., 2006). **B**, A simplified state diagram and hypothetical reaction coordinate for transitions between open, resting, and desensitized states, modified after Sun et al. (2002). A weakening of the TMD/linker interface in *lurcher* and linker A mutants (red line) would lower the energy barrier between resting and open states, leaving more channels in the open conformation. For comparison, the well described strengthening of the dimer interface by the L479Y mutation (green line) as well as the wild type (black line) are indicated. **C**, Schematic representation of the resting (middle) and the two unliganded open (left and right) states suggested to be responsible for the observed constitutive currents. In the unliganded open state I, thermal fluctuations may partially close the LBD and lead to channel opening, producing constitutive currents that are NBQX-sensitive, because the antagonist prevents thermal fluctuations by arresting the LBD in an open conformation. In the unliganded open state II, uncoupled gating events open the channel regardless of the LBD conformation, explaining ligand-insensitive constitutive currents. **D**, Scheme detailing the different contributions to the total current observed at *lurcher*-like channels. According to the proposed model, the constitutive current can be blocked by open-channel blockers (e.g., NASP) and represents channels that are either in unliganded open state I or II. The NBQX-sensitive fraction of the constitutive current reflects unliganded open state I and is caused by thermal fluctuations. The ligand-independent fraction of the constitutive current corresponds to unliganded open state II.

to previously reported AMPAR K_D values [e.g., 12 nM for AMPA (Keinänen et al., 1990)].

However, a strictly allosteric interpretation of the results can neither account for the substantial constitutive current not blocked by NBQX application, nor for the observed discrepancy between high agonist potencies and high constitutive current levels. We therefore propose that the fraction of constitutive current not blocked by NBQX reflects channel opening uncoupled from the LBD conformational state (Fig. 7D). Combination of linker A and *lurcher* has a clearly additive effect on these uncoupled gating events but not on EC_{50} values or NBQX impact. Both linker A and *lurcher* mutations increase coupling efficiency, which can account for all observed ligand-induced effects. The fraction of constitutive current not susceptible to manipulation by ligands, however, is interpreted here as the result of gating events entirely independent of ligand-induced conformational changes (Fig. 7D). Accordingly, high agonist potencies do not always correlate with high constitutive current levels, because agonist potency is a

ligand-dependent property, but the NBQX-insensitive fraction of the constitutive current is not.

A modified model of AMPAR gating

Recent work suggested that rearrangement of the TMDs is required for gating (Sobolevsky et al., 2004). This rearrangement is presumably impeded by hydrophobic TMD–TMD interactions. The *lurcher* site amino acid should be involved in these hydrophobic TMD–TMD interactions, because the side chain is water-inaccessible and likely directed toward TMD A (Sobolevsky et al., 2003). The introduction of a polar threonine at this site likely weakens hydrophobic interactions. The suggested uncoupled gating and increased coupling efficiency could directly result from weakened TMD–TMD interactions (Fig. 7B). Interpreting these interactions as an energy barrier to be overcome during gating, weakening these interactions would allow gating to occur with much less expense of energy (i.e., much less or even no domain closure). After closure of the two LBD lobes, strain is exerted on both the LBD dimer

interface and the TMD–TMD interactions (Fig. 7A). Channels then can either desensitize (rearrange the LBD dimer interface) or gate (break the TMD–TMD interaction). Following this model, the function-incapacitating linker C mutation can be interpreted as achieving the opposite of the linker A mutation: stabilization of the closed state to keep even a full agonist from providing sufficient energy for channel gating. Intriguingly, the combination of linkers A and C results in intermediate channel properties, consisting of a functional receptor but decreased agonist potency.

Our model provides two fundamentally different ways to inhibit desensitization: stabilization of the LBD dimer interface (Sun et al., 2002), or destabilization of the TMD–TMD interaction. Recently, a structural framework for the desensitized state of the LBD has been proposed (Armstrong et al., 2006). Paradoxically, the desensitized state, being the most stable state in GluRs, was the most difficult to crystallize, prompting the suggestion that the main stabilization of desensitized receptors occurs outside the LBD, likely by linker–TMD interactions (Armstrong et al., 2006). In our view, exactly these interactions are disturbed by *lurcher*-like mutations. Altering them could influence the stability of both closed and desensitized states and, in case of structural differences at the ion channel level, have different effects. Mutations in the TMD area that solely influence desensitization without producing constitutive currents (Krupp et al., 1998; Villarroel et al., 1998; Ren et al., 2003; Yelshansky et al., 2004) might simply destabilize the desensitized state, whereas *lurcher*-like mutations destabilize the closed state or both.

We show that mutation of structurally different sites in the GluR1 linker region can produce constitutively open channels, highlighting the importance of including all linkers in structural gating models. Furthermore, the antagonistic influences of linkers A and C on channel properties suggest the existence of a structural element that determines the stability of the closed pore, analogous to the LBD interface determining desensitization properties. Future studies will have to define this element more closely.

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