Interaction of the M4 Segment with Other Transmembrane Segments Is Required for Surface Expression of Mammalian α-Ámino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors*

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Background: In contrast to prokaryotic ionotropic glutamate receptors (GluRs), eukaryotic GluRs have an additional transmembrane segment, M4, that has unknown functional significance.

Results: Interaction of a specific face of the M4 segment with other transmembrane segments is necessary for AMPAR surface expression.

Conclusion: The M4 segment is required for AMPAR surface expression.

Significance: This work suggests a mechanism regulating AMPAR biogenesis.

Ionotropic glutamate receptors (GluRs) are ligand-gated ion channels with a modular structure. The ion channel itself shares structural similarity, albeit an inverted membrane topology, with P-loop channels. Like P-loop channels, prokaryotic GluR subunits (e.g. GluR0) have two transmembrane segments. In contrast, eukaryotic GluRs have an additional transmembrane segment (M4), located C-terminal to the ion channel core. However, the structural/functional significance of this additional transmembrane segment is poorly defined. Although topologically similar to GluR0, mammalian AMPA receptor (GluA1) subunits lacking the M4 segment do not display surface expression. This lack of expression is not due to the M4 segment serving as an anchor to the ligand-binding domain because insertion of an artificial polyleucine transmembrane segment does not rescue surface expression. Specific interactions between M4 and the ligand-binding domain are also unlikely because insertion of polypeptides into the linker connecting them has no deleterious effects on function or surface expression. However, tryptophan and cysteine scanning mutagenesis of the M4 segment, as well as recovery of function in the polyleucine background, define a unique face of the M4 helix that is required for GluR surface expression. In the AMPA receptor structure, this face forms intersubunit contacts with the transmembrane helices of the ion channel core (M1 and M3) from another subunit within the homotetramer. Thus, our experiments show that a highly specific interaction of the M4 segment with an adjacent subunit is required for surface expression of AMPA receptors. This interaction may represent a mechanism for regulating AMPA receptor biogenesis.

Fast excitatory neurotransmission in the central nervous system is primarily mediated by ionotropic glutamate receptors (GluRs), specifically NMDA, AMPA, and kainate receptor subtypes (1). GluRs are tetramers formed by four identical (homo-) or similar (hetero-) subunits. Each GluR subunit is modular (2, 3) containing four discrete domains as follows: an N-terminal (NTD), a ligand-binding (LBD), a transmembrane (hydrophobic segments M1–M4), and a C-terminal (CTD) domain (Fig. IA, lower panel). Functionally, GluRs also display modularity. The NTD plays a critical role in GluR biogenesis along with modulation of receptor gating (4–8). However, its removal has only minor effects on expression and receptor function (9). Similarly, the CTD is critical for regulation of the expression, distribution, and function of GluRs (10) but can also largely be removed while leaving basic properties intact (11). The LBD (S1S2 construct) also binds glutamate independent of other domains (12). Finally, the NTD (13–15) and the LBD (16–19) have been crystallized as soluble independent entities.

The core of the ion channel (transmembrane helix M1, the re-entrant M2 loop, and transmembrane helix M3) shares a similar membrane topology to pore loop channels such as K+ channels, albeit being inverted 180° in the plane of the membrane (20–22). The two-transmembrane prokaryotic GluR subunit, GluR0, is functional and supports an evolutionary link between the K+ and GluR ion channels (23). In contrast, despite retaining the other modular components, truncated NMDA receptor subunits lacking the M4 segment lose functionality (24, 25). Nevertheless, the specific role of the M4 transmembrane segment remains poorly understood.

In this study, we find that AMPA GluA1 subunits lacking the M4 segment do not express on the membrane surface, an effect

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§1 The abbreviations used are: GluR, ionotropic glutamate receptor; NTD, N-terminal domain; CTD, C-terminal domain; LBD, ligand-binding domain; MTS, methanethiosulfonate; MTSET, 2-(trimethylammonium)ethyl MTS; CTZ, cyclothiazide; AMPAR, AMPA receptor; DEPC, diethyl pyrocarbonate.

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not due to the absence of the CTD. Replacement of M4 in GluA1 with an artificial polyleucine transmembrane helix, as well as polyglycine-mediated decoupling of M4 from the LBD, suggests that the lack of surface expression is not due to the interaction of M4 with the LBD. Rather, tryptophan and cysteine mutagenesis scans identified residues, lining a single face of the M4 segment, that interact specifically with the M1 and M3 transmembrane segments of an adjacent subunit (22). We conclude that the interaction of the M4 segment with the other transmembrane segments (rather than with the LBD) is required for receptor biogenesis in mammalian GluRs.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—Truncated AMPA receptor subunits, polyleucine substitutions, site-directed mutations, and polyglycine insertions were made in and around the M4 segment of the rat GluA1 (old GluR1) (accession number P19490) subunit in the “flip” form (26). For expression in oocytes, we used a construct where a leucine in the ligand-binding domain was substituted with a tyrosine (GluA1(L479Y) or GluA1’) as a reference and as a background for all subsequent mutageneses. For wild-type channels, this construct is essentially nondenaturing (27, 28). For HEK 293 cells, wild-type GluA1 was used as a background for all mutagenesis. Point mutations and insertions were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (see Ref. 29 for additional details). Numbering of amino acids is for the mature protein (signal peptide, 18 amino acids).

The M4 segment was initially defined by hydrophobicity encompassing Val-788 to Ile-808 (30, 31). However, the recent crystal structure of a GluA2 homotetramer in a closed state (bound to a competitive antagonist) identified that the M4 segment is extended by about one turn of an $\alpha$-helix on each end (for GluA1, Leu-785 to Asn-813) (22). Nevertheless, our conclusions based on the core of the M4 segment (Val-788 to Ile-808) are valid for the entire segment.

We generated deletions of the M4 segment (and the CTD) by replacing Val-788 with a stop codon (TGA) (GluA1(V788Stop)) referred to as GluA1-ΔM4 (or GluA1’-ΔM4 when in the nondenaturing background). The S2-M4 linker, from the C-terminal end of S2 to the N-terminal end of M4, is present in the ΔM4 constructs. The CTD deletion (GluA1-ΔCTD) was made by introducing a stop codon (TAA) at Ser-814 (GluA1(S814Stop)).

Wild-type and mutant AMPA receptor subunits were expressed in *Xenopus laevis* oocytes and/or human embryonic kidney 293 (HEK 293) cells (32, 33). Oocytes were treated as described previously (32) and were maintained in a nutrient OR-3 medium containing 50% L-15, 50 mg/ml penicillin/streptomycin, 5 mM glutamine, and 15 mM NaHEPES (all Invitrogen) (pH 7.2, NaOH) as well as in 6-cyano-7-nitroquinoxaline-2,3-dione (25 mM). HEK 293 cells were transfected with GluR subunits (4 μg total) using FuGENE 6 (Roche Applied Science). A vector for enhanced green fluorescent protein (pEGFP-C1, Clontech) was typically co-transfected at a ratio of 1:9 (pEGFP-C1/GluR subunits). Recordings were typically made 1–3 days after transfection. Immunocytochemistry and biotinylation experiments were performed 2 days after transfection.

Current Recordings in *X. laevis*—Membrane currents in *Xenopus laevis* oocytes were recorded at room temperature (20–23 °C) using a two-electrode voltage clamp (DAGAN TEV-200A, DAGAN Corp., Minneapolis, MN) with Cell Works software (npi electronic, Tamm, Germany). Microelectrodes were filled with 3 M KCl and had resistances of 1–4 megohms. The external solution consisted of (mM) the following: 115 NaCl, 2.5 KCl, 0.18 CaCl$_2$, and 5 HEPES (pH 7.2, NaOH). All reagents, including glutamate (typically 1 mM), were bath-applied.

Concentration-response curves were measured in *Xenopus* oocytes using GluA1’ as a reference. Solutions containing various concentrations of glutamate (1.3–3 mM) were applied to cells held at −60 mV. Current amplitudes normalized to the maximal response were fitted as a function of concentration and fitted with the Hill equation, $I/I_{max} = 1/(1 + (EC_{50}/(34)^n))$, where $EC_{50}$ is the concentration to achieve half-maximal response, and $n$ is the Hill coefficient.

Substituted Cysteine Accessibility Method—AMPAR cysteine-substituted mutant channels were probed from the extracellular side of the membrane with the positively charged methanethiosulfonate (MTS) reagent 2-(trimethylammonium)ethyl MTS (MTSET). MTSET was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada), and was prepared, stored, and applied as described.

Steady-state reactions were quantified at −60 mV. Base-line agonist-activated current amplitudes ($I_{pre}$) were established by three to five consecutive 15–20 s applications of glutamate separated by 60–120 s washes in glutamate-free solution. Subsequent to the last wash, MTSET (2 mM) was applied for 60 s in the presence of agonists. After the MTSET exposure, current amplitudes ($I_{post}$) were determined again using three to five agonist applications. The change in the agonist-activated current amplitude, expressed as a percentage (% change), was calculated as $\Delta I = (I_{post} - I_{pre})/I_{pre} \times 100$.

Current Recordings in HEK 293—Currents in the whole-cell mode or outside-out patches, isolated from HEK 293 cells, were recorded at room temperature (20–23 °C) using an EPC-9 amplifier with Patchmaster software (HEKA Elektronik, Lambrecht, Germany), digitized at 10 kHz, and low pass filtered at 2.9 kHz (−3 dB) using an 8 pole low pass Bessel filter. Pipettes had resistances of 2–5 megohms when filled with the pipette solution and measured in the standard NaCl external solution. We did not use series resistance compensation nor did we correct for junction potentials.

For HEK 293 cell recordings, external solutions were applied using a piezo-driven double barrel application system. One barrel contained the external solution, and the other barrel contained the same solution with added glutamate (5 mM). To optimize solution exchange for fast agonist application, we briefly
treated the tips of the theta glass with hydrofluoric acid to reduce the thickness of the septum.

Our standard internal (pipette) solution consisted of (mm) the following: 105 potassium gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 Mg-ATP, and 0.3 GTP, pH 7.3 (KOH). The standard external solution consisted of (mm) the following: 140 NaCl, 10 HEPES, 1.8 CaCl₂, and 1 MgCl₂, pH 7.2 (NaOH). When measuring current amplitudes, cycloheximide (CTZ, 15–30 μM, stock solution in 100 mM NaOH) was added to the external solutions to minimize AMPA receptor desensitization. Unless otherwise noted, all chemicals were obtained from Sigma or J. T. Baker.

To determine the rate and extent of desensitization, we rapidly (<300 μs) applied glutamate for 100 ms at −60 mV to outside-out patches excised from HEK 293 cells. Time constants of desensitization (τdes) were determined by fitting the current decay with a single exponential function. The extent of desensitization was based on the steady-state (I∞) and peak (Ip) current amplitudes and calculated as the percent desensitization (%des = 100 × (1 − Ip/I∞)).

**Immunocytochemistry**—HEK 293 cells were washed twice with PBS 48 h after transfection. Nonpermeabilized cells were incubated at 37 °C with anti-(NTD)GluA1 (Calbiochem) for 30 min, rinsed with PBS, and fixed in 4% paraformaldehyde. Cells were then rinsed with PBS, blocked in 1% goat serum for 1 h, and incubated with an Alexa Fluor® 633 goat anti-rabbit secondary antibody (Invitrogen). Permeabilized cells were fixed in 4% paraformaldehyde, rinsed with PBS, and permeabilized with 0.25% Triton X-100 for 5 min at room temperature. Permeabilized cells were then blocked and incubated with primary antibody each for 1 h at room temperature and 37 °C, respectively. Cells were rinsed with PBS and incubated with Alexa Fluor® 633 goat anti-rabbit secondary antibody (Invitrogen). HA-tagged constructs were visualized by the same procedures outlined above for both nonpermeabilized and permeabilized conditions using an anti-HA antibody (Y-11) (Santa Cruz Biotechnology). Cells were examined using an upright microscope Axio Imager Z1 with an AxioCam MR3 (Carl Zeiss, Jena, Germany).

**Purification of Membrane Proteins**—Ten healthy oocytes were injected with 0.1 μg/μl mRNA. Two to 3 days after injection, oocytes were washed in PBS containing 0.58 mM N-ethylmaleimide (Pierce), homogenized in lysis buffer (20 mM Tris, 0.58 mM N-ethylmaleimide), and centrifuged (Eppendorf Centrifuge 5417R) at 3000 rpm to separate out the yolk. The supernatant was recovered and centrifuged at 40,000 rpm (Beckman TLA 120.2 rotor) for 10 min at 4 °C. The pellet was rinsed in PBS and recentrifuged at 40,000 rpm. The resulting pellet was resuspended and first sonicated in solubilization buffer without detergent (20 mM Tris, 50 mM NaCl, 1:1000 protease inhibitor mixture (Sigma), 0.58 mM N-ethylmaleimide) and then incubated with detergent (0.03% sodium deoxycholate, 1% Triton X-100) for 1 h at 4 °C. Solubilized proteins were centrifuged at 40,000 rpm for 20 min at 4 °C, and membrane proteins contained in the supernatant were separated by SDS-PAGE under nonreducing conditions.

**Biotinylation**—Forty eight hours after transfection, HEK 293 cells were cooled on ice and washed three times with ice-cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS/CM). Cells were treated with either PBS/CM (control, no biotin) or 2–5 mg/ml Sulfo-NHS-SS-Biotin (Thermo) for 45 min on ice. Cells were then rinsed with ice-cold PBS/CM, quenched in 0.1 M glycine PBS/CM, and pelleted in PBS/CM. Pellets were resuspended in lysis buffer (40 mM DDM dissolved in PBS containing protease inhibitors) and incubated on ice for 30 min. The cell lysate was then centrifuged at 10,000 × g for 2 min, and the supernatant was incubated with Neutravidin-Plus beads (Thermo) end-over-end for 2 h at room temperature. Beads were rinsed three times with PBS/CM, and bound proteins were eluted by incubation with 50 mM DTT in 2× loading buffer for 1 h at room temperature. Eluted proteins, whole-cell lysates, and unbound proteins (flow-through) were separated by SDS-PAGE under reducing conditions.

**Immunoblotting**—Proteins were transferred from the gel to 0.45-mm nitrocellulose membranes by semi-dry transfer (Bio-Rad) using Bjerrum-Schaffer-Nielsen Buffer (35). Blots were blocked with milk and incubated with primary antibodies overnight. The antibodies used were anti-(NTD)GluA1 (GluR1(E6), which is directed against the NTD; sc-13152, Santa Cruz Biotechnology) and anti-β-tubulin (Axyll, H8481, Accurate Chemical & Scientific Corp., Westbury, NY). Blots were washed prior to incubation with HRP-conjugated goat anti-mouse (sc-2302) or HRP-conjugated goat anti-rabbit antibody (sc-2048) and were developed using luminol reagent (sc-2048, all reagents Santa Cruz Biotechnology) before exposure to chemiluminescence Biomax Film (Kodak, Cedex, France). For biotinylation experiments, blots were probed with anti-HA (Covance) and GAPDH (Calbiochem) and visualized using the Odyssey infrared imaging system (LI-COR) with anti-mouse 800CW and 680LT secondary antibodies, respectively.

**Quantification of Immunoblots**—Immunoblot films were scanned using an EPSON flat scanner (Epson Perfection V700 Photo) in an 8-bit gray scale mode, 1200 dpi, reflective and quantified using NIH Image J (1.38×) on a Windows XP platform. GluR protein levels were expressed relative to a loading control (β-tubulin). Immunoblots of biotinylated proteins were analyzed using the Odyssey software (LI-COR).

**Statistical Analysis**—For statistical analysis, we used Igor Pro (WaveMetrics, Inc., Lake Oswego, OR) and Microsoft Excel (Redmond, WA). Results are reported and shown graphically as means ± S.E. An analysis of variance or a Student’s t test was used to define statistical differences. The Tukey or Dunnet’s test was used for multiple comparisons. Significance was assumed if p < 0.05.

**RESULTS**

To investigate the structural and functional significance of the M4 segment in mammalian GluRs, we used the AMPA receptor subunit GluA1 in the flip form due to its robust expression as a homomultimer in heterologous expression systems. For most functional assays, we used Xenopus oocytes because there is no uncertainty whether the oocyte has been injected in contrast to HEK 293 cells where transfection is uncertain even with co-transfected GFP.
AMPAs require the M4 transmembrane segment for surface expression. The upper panels of Fig. 1, A and B, show representative membrane current recordings from Xenopus oocytes injected with mRNA for GluA1 and GluA1-ΔM4. The predicted membrane topology of GluA1 and GluA1-ΔM4 is shown in the lower panels of Fig. 1, A and B. Oocytes injected with GluA1 mRNA showed robust glutamate-activated current amplitudes, typically on the order of several microamperes (Table 1). Similarly, HEK 293 cells transfected with wild-type GluA1 and recorded in CTZ displayed large glutamate-activated currents (Table 1). In contrast, GluA1 subunits lacking the M4 transmembrane segment, as well as the C-terminal domain (CTD), did not show any detectable glutamate-activated currents in Xenopus oocytes (nondesensitizing background) (Fig. 1B) or in HEK 293 cells (wild-type background) (Table 1). Stargazin (γ2), the canonical member of transmembrane AMPAR-related proteins, increases surface expression of AMPA receptors (e.g., 36), but co-expression of the GluA1-ΔM4 construct with Stargazin in HEK 293 cells did not rescue glutamate-activated currents (Table 1).

Immunoblots of whole-cell lysates from oocytes expressing GluA1 or GluA1-ΔM4 showed protein expression at the expected relative sizes (GluA1, 110 kDa; GluA1-ΔM4, 90 kDa) (Fig. 1C). Quantification of protein expression (see "Experimental Procedures") in Xenopus oocytes showed that both...
TABLE 1

Current amplitudes of wild-type and truncated forms of the AMPA receptor GluA1 subunit expressed in Xenopus oocytes (GluA1) or HEK 293 cells (GluA1)

| Construct                  | Xenopus oocytes | HEK 293 cells |
|----------------------------|-----------------|---------------|
|                            | l               | n             | l               | n             |
| GluA1 or GluA1             | −1650 ± 75      | 15            | −3010 ± 170     | 11            |
| A1−ΔM4 or A1−ΔM4           | ND              | >20           | ND              | 11            |
| GluA1 + Stargazin          | NT              | −6850 ± 1500  | 5               |
| A1−ΔM4 + Stargazin         | NT              | ND            | 5               |
| GluA1 or GluA1             | −2190 ± 90      | 11            | −71 ± 7         | 4             |
| A1−ΔCTD or A1−ΔCTD         | −2090 ± 150     | 10            | −15 ± 2         | 8             |

GluA1 and GluA1-ΔM4 are expressed at similar levels that were significantly greater than background (DEPC-injected oocytes) (Fig. 1D). The absence of functional agonist-induced currents for the ΔM4 construct is not a result of lack of translation or due to ubiquitous protein degradation.

The lack of detectable membrane currents but the presence of protein expression by immunoblots suggest that the ΔM4 constructs are synthesized but are not trafficked to the membrane. Although mutants in the nondesensitizing background (L479Y) show functional currents, previous evidence has shown that the L479Y mutation attenuates GluA2 tetramer formation (37). We therefore tested for surface expression of wild-type GluA1 constructs in HEK 293 cells using biotinylation (Fig. 1, E and F) and immunocytochemistry (Fig. 1, G and H). Biotinylation of cell surface proteins showed that wild-type GluA1, but not GluA1-ΔM4 (Fig. 1, E, lower panel, and F), was expressed at the cell surface.Quantification of the biotinylated signal of GluA1-ΔM4 was not statistically different from that of controls (Fig. 1P) suggesting that the GluA1-ΔM4 is not trafficked to the membrane. Furthermore, immunocytochemistry of wild-type GluA1 showed prominent surface expression (nonpermeabilized images, Fig. 1G), whereas the GluA1-ΔM4 construct showed no detectable surface expression (nonpermeabilized images, Fig. 1H), although the protein was expressed robustly (permeabilized images, Fig. 1H). Identical results were found using HA-tagged GluA1 and GluA1-ΔM4 constructs (data not shown).

The ΔM4 construct lacks not only the M4 segment but also the CTD (Fig. 1B, lower panel), an element that is critical for regulating GluR expression (10). Partial deletion of the CTD in GluA1 (removal of last 52 amino acids of 77 total), leaving 25 amino acids after M4, including a PKA-binding site, has little effect on its expression or function (11). To verify further that the absence of the CTD in the ΔM4 construct does not underlie the lack of surface expression, we generated a construct in which even more of the CTD was deleted, placing a stop codon at position 814 (GluA1-ΔCTD or GluA1-ΔCTD) (removal of 77 amino acids of CTD) corresponding to the polar clamp of the M4 transmembrane helix (22). The amplitude of glutamate-activated currents for GluA1-ΔCTD (Fig. 2, A and B) as well as general gating properties such as concentration-response curves (Fig. 2, C and D; Table 1) and rates of entry into desensitization (Table 1) were indistinguishable from wild-type. Thus, although the CTD is critical for modulating GluR trafficking (10, 38), its absence does not underlie the lack of surface expression for the ΔM4 constructs (Fig. 1).

Interaction of the M4 Segment with LBD Does Not Appear Critical to Its Function—Previous experiments have indicated that the ability of the LBD to bind glutamate is a key component of GluR biogenesis (39–42). The M4 segment is coupled to helix K in domain 1 of the LBD via the S2-M4 linker. Helix J, just N-terminal to helix K, is a critical component of the LBD dimer interaction whose stability is key to receptor gating (17, 43). Therefore, one potential role of the M4 segment in GluRs may be to act simply as a membrane spanning element that serves as an additional anchor (along with M1 and M3) to the LBD permitting agonist binding. To test this idea, we replaced the membrane spanning portion of the M4 segment in GluA1 with polyolecines (21 residues). Such polyolecine stretches are frequently used as model transmembrane helices (44, 45) because they form spontaneous, stable α-helices and insert naturally into membranes. We did not use a polyanaline transmembrane segment to avoid any potential helix-helix interactions.

When the M4 segment was replaced with polyolecines (GluA1-‘M4PLeu’) (Fig. 3A), we did not observe any detectable glutamate-activated currents in either oocytes (right panel, Fig. 3B) or HEK 293 cells (data not shown). Like the ΔM4 construct, whole-cell lysates of the polyolecine construct showed robust protein expression (Fig. 3, C and D) but no detectable surface expression as visualized by immunocytochemistry (Fig. 3E). Thus, a polyolecine transmembrane helix that presumably mimics the membrane spanning property of M4, although lacking any helical interaction motifs, does not yield functional receptors suggesting that specific residues within the M4 segment are essential to its function.

As an additional test of whether the connection between M4 and the LBD is critical to GluR function, we inserted up to eight glycine residues in the S2-M4 linker coupling the M4 segment to the LBD (Fig. 4A). Glycine, a small amino acid that lacks a side chain, is a strong helix breaker for soluble proteins. Thus, stretches of polyglycines will introduce flexibility and also lengthen the linker, effectively decoupling the LBD from the M4 segment while retaining the native membrane spanning segment. Hence, if specific interactions between M4 and the LBD are important to AMPA receptor function, we anticipate that the addition of these glycines to the S2-M4 linker should produce a phenotype comparable with ΔM4 constructs (either poor or no surface expression and subsequent deficits in agonist-induced current amplitudes).

As shown in Fig. 4, B and C, receptors containing introduced glycines still showed significant glutamate-activated current amplitudes, considerably different from the nonexpressing ΔM4 construct. The additional glycines also had no notable effect on glutamate sensitivity (Fig. 4D) nor desensitization or activation gating within the time constraints of our exchange system (Fig. 4, E and F). These results further support the idea that the interaction of the M4 transmembrane segment with
Tryptophan Mutagenesis Scan Reveals a Putative Interacting Face of the M4 Segment — The results presented so far argue that the interaction of M4 with the LBD is not required for surface expression. The recent crystal structure of a homomeric AMPA receptor (22) indicates a strong interaction of M4 with the LBD, consistent with this disulfide bond being intact. In contrast, for GluA1-S2-M4<sup>W616</sup>, DTT had no effect on current amplitudes (0 ± 2%, n = 3) (data not shown), suggesting that the disulfide bond is no longer intact. These results suggest that because the disulfide bond is not intact, the polyglycines are not simply “looped out” of the LBD, but rather they perturb the linker joining the LBD to the M4 segment and decouple the LBD from the M4 segment.

One potential complication of these experiments is that introduced polyglycines were inserted N-terminal to a conserved cysteine residue (Cys-769) known to form a disulfide bond that stabilizes the LBD (16). Hence, if this endogenous disulfide bond is intact in the polyglycine-substituted receptors, then it may prevent the introduced glycines from having their desired effect, namely decoupling the LBD from the M4 segment through lengthening the distance of S2 from the M4 segment. DTT, a reducing agent, significantly potentiates current amplitudes in wild-type GluA1 (40 ± 1%, n = 3) in the presence of CTZ (data not shown) expressed in Xenopus oocytes, consistent with this disulfide bond being intact. In contrast, for GluA1-S2-M4<sup>W616</sup>, DTT had no effect on current amplitudes (0 ± 2%, n = 3) (data not shown), suggesting that the disulfide bond is no longer intact. These results suggest that because the disulfide bond is not intact, the polyglycines are not simply “looped out” of the LBD, but rather they perturb the linker joining the LBD to the M4 segment and decouple the LBD from the M4 segment.
Tryptophan-substituted Receptors That Do Not Show Glutamate-Activated Currents Are Not Expressed at the Membrane

In summary, the tryptophan scan of the M4 segment identified a specific face of the M4 segment that strongly disrupts receptor expression. As will be discussed under “Discussion,” this face (especially those positions that showed no glutamate-activated current highlighted in red in Fig. 5E) interacts specifically with the core of the ion channel (M1 and M3) of adjacent subunits.

Tryptophan-substituted Receptors That Do Not Show Glutamate-activated Currents Are Not Expressed at the Membrane—To verify that the absence of membrane currents in tryptophan-substituted receptors coincided with a lack of surface glutamate-activated currents were positioned on one side of the M4 segment.

Because the L479Y mutation attenuates GluA2 tetramer formation (37), we made parallel experiments in HEK 293 cells in which the current amplitudes of tryptophan-substituted receptors in the wild-type GluA1 background were recorded using CTZ. The recordings in oocytes (nondesensitizing background) and HEK 293 cells (wild-type background) were nearly identical (Table 2; summarized in Fig. 5E, right panel) with the exception being that in HEK 293 cells, A789W showed robust currents, and more positions showed no detectable current. This may be an effect of limited sampling or may represent a more restrictive nature of GluR biogenesis in HEK 293 cells because all of the new positions in HEK 293 cells without detectable currents (Val-788, Gly-790, Ile-794, and Val-805) had extremely small currents and/or were inconsistently detected in oocytes. Nevertheless, these results strongly support the idea that this interacting face is common in all expression systems and is not dependent on the nondesensitizing background.
expression, we used immunocytochemistry (Fig. 6) focusing mainly on those tryptophan-substituted receptors that did not show glutamate-activated currents in *Xenopus* oocytes (V791W, L795W, G798W, A802W, and E809W) as well as G790W that showed no detectable glutamate-activated current in HEK 293 cells (Table 2). Immunocytochemical analysis showed that these nonfunctional receptors, generated in the wild-type background, could not be detected at the cell surface (Fig. 6, left panel, Non-permeabilized) but were present intracellularly (right panel, Permeabilized). Furthermore, trypto-
TABLE 2

Functional properties of wild-type and tryptophan-substituted GluA1 receptors expressed in Xenopus oocytes (GluA1) or HEK 293 cells (GluA1)

Values shown and displayed as in Table 1. ND indicates no glutamate-activated currents were detected. NT indicates not tested. For some mutations, injected oocytes showed either no detectable glutamate-activated currents or very small current amplitudes. In these instances, the EC_{50} values in parentheses indicate the total number of oocytes recorded, whereas the other number indicates that showed a detectable glutamate-activated current. EC_{50} is the concentration at half-maximal activation and Hill the Hill coefficient (see “Experimental Procedures”). The average current amplitude is only of those recordings that showed detectable currents. Positions underlined showed significant reductions in glutamate-activated current amplitudes in both Xenopus oocytes and HEK 293 cells. When measuring current amplitudes in HEK 293 cells, where the wild-type background (GluA1) was used, we included cyclothiazide in the recording solution.

| Construct  | Xenopus oocytes | HEK 293 cells |
|------------|----------------|---------------|
|            | I (nA)         | EC_{50} (μM)  | Hill | n (n) |
| GluA1      | −2120 ± 40     | 42 ± 0.1      | 1.6 ± 0.1 | 14 | −1330 ± 50 | 16 |
| N787W      | −14 ± 2        | 7 (13) NT     | −41 ± 11 | 4 |
| V788W      | −66 ± 2        | 3 (16) NT     | ND | 3 |
| A789W      | −15 ± 1        | 14 (18) 14.8 ± 1.0 | 1.8 ± 0.1 | 6 |
| G790W      | −14 ± 2        | 11 (21) NT    | ND | 4 |
| V791W      | ND             | 7 NT          | ND | 3 |
| F792W      | −4 ± 1         | 2 (9) NT      | −19 ± 7 | 3 |
| T793W      | −2430 ± 100    | 11 5.6 ± 0.2  | 1.5 ± 0.1 | 6 |
| F794W      | −7 ± 1         | 7 (16) NT     | ND | 3 |
| L795W      | ND             | 10 NT         | ND | 3 |
| L796W      | −3440 ± 160    | 11 3.0 ± 0.3  | 1.2 ± 0.1 | 5 |
| G797W      | −2940 ± 160    | 10 3.7 ± 0.2  | 1.4 ± 0.1 | 5 |
| G798W      | ND             | 7 NT          | ND | 3 |
| L799W      | −310 ± 20      | 13 7.8 ± 0.2  | 1.5 ± 0.1 | 8 |
| G800W      | −2970 ± 80     | 13 3.9 ± 0.1  | 1.3 ± 0.1 | 6 |
| L801W      | −140 ± 10      | 11 7.2 ± 0.3  | 1.6 ± 0.1 | 7 |
| A802W      | ND             | 7 NT          | ND | 3 |
| L803W      | −1100 ± 60     | 10 8.0 ± 0.5  | 1.4 ± 0.1 | 6 |
| L804W      | −3350 ± 110    | 12 3.0 ± 0.1  | 1.3 ± 0.1 | 6 |
| V805W      | −36 ± 2        | 12 11.0 ± 0.3 | 1.4 ± 0.1 | 6 |
| A806W      | −2070 ± 70     | 10 3.0 ± 0.1  | 1.5 ± 0.1 | 5 |
| L807W      | −5670 ± 120    | 13 3.0 ± 0.1  | 1.5 ± 0.1 | 6 |
| I808W      | −2990 ± 80     | 12 4.1 ± 0.1  | 1.3 ± 0.1 | 6 |
| E809W      | ND             | 9 NT          | ND | 3 |

phosphorylated mutants that showed similar current amplitudes as wild type (e.g. G797W) also showed strong surface expression (Fig. 6, left panel). Thus, these results further support the hypothesis that specific interactions along the interface of the M4 segment with that of the other transmembrane domains are critical for AMPA receptor surface expression.

Cysteine Mutagenesis Scan Highlights the Interacting Face—The tryptophan scan identified a face of the M4 segment that when substituted with tryptophan showed greatly reduced surface expression. To further verify this point, we carried out a cysteine scan of the GluA1 M4 segment. Because cysteine is a smaller residue than tryptophan, it should have less dramatic effects on receptor surface expression.

Fig. 7A (left panel) summarizes normalized current amplitudes for wild-type and cysteine-substituted GluA1 subunits. Many of the cysteine-substituted receptors showed current amplitudes comparable with control, but a subset of positions showed either no detectable glutamate-activated current (X) or greatly reduced current amplitude (<0.3 of control) (Fig. 7B). These positions that showed significant decreases in current amplitude, with the exception of Tyr-793 that was unaffected in the tryptophan scan, were a subset of those positions affected in the tryptophan scan. These results are therefore consistent with the idea that there is a specific interacting face required for surface expression in AMPA receptors.

We also took advantage of the substituted cysteines to look at the water accessibility of the different positions using MTS reagents (Fig. 7A, right panel). Only one position tested, Arg-787, showed a significant effect of the positively charged MTSET. Although we cannot rule out the possibility of silent reactions (the MTS reagent reacts with a position but has no functional effect), these results argue that there are no water-filled cavities around the M4 segment consistent with the idea that it interacts strongly with other transmembrane segments.

Restoration of Function in the Polyleucine Background—Given the observation that certain residues are necessary for receptor surface expression (Figs. 5–7), we wanted to consider the opposite scenario (the minimum number of these key residues required to restore surface expression in a nonexpressing background). Hence, starting with the nonexpressing polyleucine construct as a background (Fig. 3), we systematically re-introduced key residues to identify the minimum number needed to restore surface expression as assayed by glutamate-activated currents (Fig. 8).

Fig. 8A aligns the core of the GluA1 M4 segment with sequence alignments of conserved residues within non-NMDA (upper panel) and NMDA (lower panel) receptor subunits. Across all subtypes, 16 of 23 residues were similar in identity, and three positions (Gly-790, Phe-792, and Glu-809) were fully conserved (Fig. 8). Of these 16 positions, 14 of them (out of 14 total) when substituted with tryptophan yielded poor or no surface expression (Fig. 5). When the most conserved positions in the M4 segments, Gly-790 and Phe-792 in GluA1 (demarcated by dark gray boxes in Fig. 8A) were reintroduced into the M4 polyleucine background (GluA1-M4pLeu(GF)), they did not restore glutamate-activated currents (raw data not shown) (note that Glu-809 was present in GluA1-M4pLeu). Reintroducing a number of other conserved residues (e.g. Gly-798 and Ala-802) in a stepwise fashion also did not yield functional receptors. Function, albeit small in amplitude, was restored only when a cluster of residues was converted back to their native conformation (L790G/L791V/L792F/L798G/L802A/
FIGURE 6. Tryptophan-substituted receptors that do not show glutamate-activated currents are not expressed at the membrane. Immunocytochemistry of HEK 293 cells co-transfected with GFP and either wild-type GluA1 (upper row) or tryptophan-substituted GluA1 subunits (wild-type background) under nonpermeabilized (left panel) or permeabilized (right panel) conditions. Tryptophan-substituted receptors V791W, L795W, G7898W, A802W, and E809W (underlined) showed no detectable glutamate-activated current either in oocytes or HEK cells (Fig. 5B; Table 2), and no surface expression could be detected (Non-permeabilized conditions). Current amplitudes for G790W (underlined) were extremely small (oocytes) or not detected (HEK 293 cells). As a control, G797W showed wild-type-like current amplitudes and robust surface expression. All transfected constructs showed robust intracellular protein expression (Permeabilized conditions). Scale bars represent 10 microns.
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FIGURE 7. Cysteine mutagenesis scan of residues in the M4 segment. A, left panel, mean current amplitudes (± S.E.) at −60 mV and in 1 mM glutamate normalized to those obtained for GluA1 injected and recorded during the same cycle for cysteine substitutions (n > 7) (all recordings were made in oocytes). GluA1 current amplitudes were −1100 ± 41 (n = 32). Positions that did not show detectable glutamate-activated currents are demarcated by x. Positions that showed current amplitudes <0.3 of wild-type are colored blue. A, right panel, mean percent change (± S.E.) in glutamate-activated current amplitudes measured before and after exposure of wild-type or cysteine-substituted GluA1 subunits to MTSET in the continuous presence of glutamate (MTSET + ). Left and right pointing bars indicate inhibition and potentiation, respectively. Filled bars indicate that the value of % change is statistically different from that of wild-type GluA1 (p < 0.05). B, helical net representation of current amplitudes for positions substituted with cysteine in the M4 segment recorded in Xenopus oocytes (nondesensitizing background). Only positions tested are shown. Receptors containing cysteine substitutions that did not show glutamate-activated current are highlighted in red. Those that showed greatly reduced current amplitudes (<0.3 of wild-type) are highlighted in blue. Outside of Tyr-793, where a tryptophan substitution had no significant effect on current amplitudes, the positions that showed significant effects were a subset of those identified in the cysteine scan (Fig. 5E).

Fig. 8D highlights the distribution of those positions necessary to recover function in the M4 polyleucine background on the M4 segment from the GluA2 structure. These results are consistent with the idea that an interacting face in the M4 transmembrane segment is critical to GluR surface expression. In addition, they suggest that it is this interacting face, rather than a single side chain, that underlies this functional action.

DISCUSSION

Mammalian ionotropic GluRs like their prokaryotic relative (GluR0) share a common ion channel core structure (M1–M3) with that of two-transmembrane K⁺ channels, albeit inverted 180° in the plane of the membrane. Yet what distinguishes mammalian GluRs and perhaps all eukaryotic GluRs (48) from GluR0 is the presence of an additional transmembrane segment, the M4 segment, that connects the highly regulated CTD to the core of the ion channel. GluR0 forms fully functional tetrameric receptors without the presence of the additional M4 segment (23). In this study, we investigated the role of the M4 transmembrane segment in AMPA receptor function. Using a truncated GluA1 construct, we show that the M4 transmembrane segment is necessary for surface expression. Through the use of single amino acid tryptophan and cysteine substitutions, we find that a specific face along the M4 α-helix is critical for GluR function. In the AMPA receptor structure, the M4 transmembrane segments are associated with the core of the ion channel (M1–M3) of an adjacent subunit (22), and our functionally defined interacting face aligns extremely well with the adjacent subunit (Fig. 9). We conclude that in addition to linking the highly regulated CTD to the core of the ion channel, the M4 segment itself has functional significance being involved in some form in receptor biogenesis. Our experiments, however, do not address to what specific biogenic process the M4 segment is required.

Interaction of M4 with Other Transmembrane Segments Is Required for Efficient Surface Expression—Our results demonstrate the significance of the M4 segment to the function of mammalian GluRs and delineate, at least at an initial level, the basis for this. Specifically, we find that an interaction among the M4 transmembrane segment and the other transmembrane elements, either M1 and/or M3, is required for GluR function. Various lines of evidence support this idea. First, both deletion (Fig. 1) and substitution of the M4 segment with an inert polyleucine membrane-spanning segment (Fig. 3) results in the loss of surface GluR expression, an effect not dependent on the CTD (Fig. 2). A tryptophan mutagenesis scan revealed a distinct pattern of positions (highlighted in Fig. 5, E and F) that disrupts L805V; note a conserved leucine, Leu-795 (red position, Fig. 5E), is already present in the polyleucine background (Fig. 8, B and C). A slightly different combination starting with the addition of Val-788 (VGFGAV) yielded a comparable outcome (Fig. 8C).

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FIGURE 8. Recovery of function in nonsurface-expressed polyleucine background. A, sequence alignment of residues in and around the M4 segments in mammalian non-NMDA (upper panel) and NMDA (lower panel) receptor subunits. Outside of GluA1 and GluN1, consensus sequences are shown for AMPA (GluA1–GluA4), low affinity kainate (low KA) (GluK1–GluK3), high affinity kainate (high KA) (GluK4–GluK6), GluN2 (GluN2A–GluN2D), or GluN3 (GluN3A and GluN3B). Positions highlighted in dark gray are conserved across all subunits, light gray indicates identical for more than 80% of subunits, and boxed are similar in nature. Positions not highlighted are not conserved. Numbering is given only for the GluA1 subunit. GluA1 positions where function was affected in the tryptophan scan in Xenopus oocytes are highlighted in dark gray ovals (Fig. 5; Table 2). B, representative whole-cell currents recorded from a Xenopus oocyte injected with GluA1-M4pLeu(GVFGAV). In this construct, the GluA1 segment was in the polyleucine background (see Fig. 3) but certain positions had been mutated back to their wild-type form, L790G/L791V/L792F/L798G/L802A/L805V (GVFGAV). Currents are recorded and displayed as in Fig. 1A. C, mean glutamate-activated current amplitudes (± S.E.) at −60 mV and in 1 mM glutamate normalized to those obtained in wild-type (GluA1). D, necessary positions (GVFGAV) required to regenerate function in the nonsurface-expressed polyleucine background are shown space-filled and in dark gray on the M4 segment from the GluA2 structure. Note that one of the leucines (L795) was also demarcated red (see Fig. 5E).

function. Here, red positions (positions that show no glutamate-activated current) line one face of the M4 transmembrane helix. Strategic reintroduction of a series of residues along this face in the nonfunctioning polyleucine background results in functional/surface expressed channels (Fig. 8). Finally, our data indicating an interacting face of the M4 segment is required for GluR function is strengthened by the GluA2 crystal structure (22). Specifically, the functionally defined interacting face is strongly aligned with portions of the M1 and M3 transmembrane segments of an adjacent subunit with positions showing no effect typically located on the portion of the helix in contact with the lipid interface (Fig. 9; Table 3). However, further experiments are required to identify the specific residues within the M1/M3 transmembrane helices that interact along the face of the M4 segment and are critical to receptor function.

One alternative explanation for the role of the M4 segment is that it is required for the proper functioning of the LBD. We believe that this alternative is unlikely because decoupling of the LBD from M4 has no notable effect on receptor function (Fig. 4). Similarly, an artificial polyleucine transmembrane domain, which replicates the membrane spanning aspect of M4 (Fig. 3), but not specific interactions, does not restore channel function. Presumably, this polyleucine transmembrane domain should function like M4 and anchor the LBD. Coupled together, these data suggest that it is specific residues within the M4 segment that are important for GluR function, rather than serving as a physical link between M4 and the LBD.

All available evidence indicates that an interaction of the M4 segment with the other transmembrane segments is essential for surface expression. Nevertheless, the M4 segment may also contribute in some form to receptor gating (49, 50) and/or interactions with transmembrane AMPAR-related proteins (51). Indeed, for those tryptophan-substituted receptors that did show reduced albeit measurable currents, we often saw a
TABLE 3
Residues in the vicinity of the M4 transmembrane segment in the closed state

| Residue | M4 segment, subunit B | M1 segment | M3 segment |
|---------|-----------------------|------------|------------|
| Val-788 | Ser-512 (B) | Ile-610 (A) | Gly-531 (A) |
| Ala-789 | – | Ile-609 (A) | Val-597 (A) |
| Gly-790 | Val-510 (B) | Phe-604 (A) | Leu-592 (A) |
| X Val-791 X | Phe-513 (B) | Ile-607 (A) | Ser-593 (A) |
| Phe-792 | Ile-521 (A) | Phe-604 (A) | – |
| X Val-791 X | Ile-525 (A) | Il-608 (A) | Ala-528 (A) |
| Tyr-793 | – | – | Ser-593 (A) |
| Ile-794 | – | Val-600 (A) | Ile-596 (A) |
| X Leu-795 X | – | Val-600 (A) | Val-597 (A) |
| Val-796 | Phe-604 (A) | – | Val-597 (A) |
| Gly-797 | – | Trp-601 (A) | Val-600 (A) |
| X G798 X | – | Ile-596 (A) | Gly-531 (A) |
| Leu-799 | Gly-531 (A) | Val-600 (A) | Val-532 (A) |
| Gly-800 | – | Val-597 (A) | Val-535 (A) |
| Leu-801 | – | Ile-596 (A) | Gly-798 (A) |
| X Ala-802 X | – | – | Ser-593 (A) |
| Ala-806 | – | Ser-593 (A) | Ser-593 (A) |
| Leu-807 | – | Leu-592 (A) | – |
| Ile-808 | – | Ser-593 (A) | Ser-593 (A) |
| X E809 X | – | Ser-593 (A) | Ile-796 |

small shift in the glutamate EC_{50} (Table 2). Presumably, this may reflect an alteration in the positioning and dynamics of M4 with other transmembrane segments such as the M3 transmembrane helix (the major pore-lining gating domain). In fact, the M3 transmembrane segment interacts most strongly with M4 (Table 3). Thus, mutations in M4 may not directly influence the LBD by the S2-M4 linker, but rather indirectly alter the LBD dynamics and gating via the associated M3 segment and hence the M3-S2 linker.

Helical Interactions—Specific transmembrane packing interactions are critical determinants of the folding, stability, and function of membrane proteins, including ion channels (47). These interacting faces often display specific amino acid side chains or even unique sequence motifs that facilitate such interactions. At the core of this interacting face of the M4 segment is a GXGG-like sequence (798GXXA802). GXGG motifs, in which two amino acids with small side chains, typically glycine but also alanine or serine, separated by three amino acids, are well known to be located at points of transmembrane helix-helix interactions (47, 52, 53). Substitution of either Gly-798 or Ala-802 with tryptophan and/or cysteine resulted in loss of membrane expression (Figs. 5B and 7A).

Deletion of the M4 Segment Does Not Interrupt Early Stages of GluR Biogenesis—Biogenesis of GluRs (the biosynthesis, folding, oligomerization, and trafficking of receptors to the membrane) requires many synchronized interactions to yield surface expressed/functional ion channels (38, 54, 55). Our results suggest that the interaction of the M4 segment with other transmembrane segments affects one or more of these biogenesis steps. Lack of protein biosynthesis is unlikely because immunoblots of whole-membrane lysates demonstrate protein production at the expected size (Figs. 1, C and D, and 5, C and D). Furthermore, protein expression was detected in permeabilized conditions for ΔM4 (Fig. 1H) and for nonfunctional single amino acid substitutions (Fig. 6). However, these mutations could disrupt protein folding. Moreover, we believe this alternative is unlikely for a variety of reasons. Multidomain proteins, including ion channels, are highly modular (56). GluR subunits are also largely modular (see Introduction), and truncation of the M4 segment (ΔM4 construct) yields a subunit with a topology like the functional GluR0. Finally, single tryptophan or cysteine substitutions yield a phenotype like ΔM4, and these substitutions largely line a single side of a helix, one that interacts specifically with other transmembrane segments (Fig. 9). Although substitution with a large amino acid like tryptophan might disrupt folding for some of these positions, it seems highly unusual that it would occur for a single side of a transmembrane segment and that it would occur also for the smaller cysteine side chain (Fig. 7B). Nevertheless, specific experiments will be needed to fully address the question of whether membrane folding is disrupted.

One intriguing possibility is that the M4 segment is involved in the oligomerization of GluR subunits to form tetramers. In the AMPA receptor crystal structure, the M4 of one subunit is associated with the core of the ion channel (M1–M3) of an adjacent subunit (22), and the identified interacting face is strongly aligned with M1 and M3 of an adjacent subunit (Fig. 9). Thus, it is plausible that the M4 segment might be involved in the assembly and/or oligomerization of GluRs via either the stabilization of dimers and/or tetramerization of functional receptors. Alternatively, the M4 may function to mask an unidentified retention signal (25), which would provide support for a role in the trafficking of receptors. Further studies must be performed to discern the precise mechanistic role of the M4 segment in GluR biogenesis.

Perturbations of M4 as Pathway for Modulation of Receptor Trafficking—Modulation of GluR function is critical in regulating synaptic activity, including plasticity (10), and can occur by a variety of means such as phosphorylation, membrane phospholipid composition, auxiliary subunits, and post-synaptic density proteins, many of which interact with the CTD. At present, the molecular mechanism by which these agents act remains largely undefined. Although speculative, perturbations
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in the orientation of M4 relative to other transmembrane segments in the plane of the membrane possibly driven by modification of the CTD or interactions with transmembrane AMPAR-related proteins (51) could represent one such mechanism. Nevertheless, specific experiments will be needed to address any potential role for the positioning of M4 as a regulatory mechanism of GluR biogenesis.

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REFERENCES

1. Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) Pharmacol. Rev. 51, 7–61
2. Mayer, M. L. (2006) Nature 440, 456–462
3. Oswald, R. E., Ahmed, A., Fenwick, M. K., and Loh, A. P. (2007) Curr. Drug Targets 8, 573–582
4. Ayala, G. N., and Simon-Bach, Y. (2001) Neuron 31, 103–113
5. Giebel, M., Siegler, R., and Mody, L. (1999) Nature 400, 594–598
6. Giebel, M., Siegler, R., and Mody, L. (2000) Nature 403, 686–690
7. Yuan, H., Hansen, K. B., Vance, K. M., Ogden, K. K., and Traynelis, S. F. (2009) J. Biol. Chem. 284, 185–192
8. Horak, M., Chang, K., and Wentholt, R. J. (2008) J. Neurosci. 28, 3500–3509
9. Collingridge, G. L., Olsen, R. W., Peters, J., and Spedding, M. (2009) Neuropharmacology 56, 2–5
10. Stern-Bach, Y., Russo, S., Neuman, M., and Rosenmund, C. (1998) Neuron 21, 907–918
11. Schmid, M., Körber, C., Herrmann, S., Werner, M., and Hollmann, M. (2007) J. Neurosci. 27, 12230–12241
12. Talukder, I., Borker, P., and Wollmuth, L. P. (2010) J. Neurosci. 30, 11792–11804
13. Boulter, J., Hollmann, M., O’Shea-Greenfield, A. E., Maron, C., and Heinemann, S. (1997) Science 277, 1033–1037
14. Keinänen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T. A., Sakmann, B., and Seeburg, P. H. (1999) Science 283, 556–560
15. Sobolevsky, V. I., Beck, C., and Wollmuth, L. P. (2002) Neuron 35, 75–85
16. Yelshansky, M., Sobolevsky, A. I., Iatze, C., and Wollmuth, L. P. (2004) J. Neurosci. 24, 4728–4736
17. Ma, B., Tsai, C. J., Hallol, T., and Nussinov, R. (2011) Structure 19, 907–917
18. Bjerrum, O. J., and Schra-Feinberg N. (1986) Analytical Electrochemistry, Weinheim, Germany
19. Tomita, S., Adesnik, H., Sekiguchi, M., Zhang, W. D., Wada, K., Howe, J. R., Nicoll, R. A., and Breidt, D. S. (2005) Nature 435, 1082–1085
20. Shanks, N., Maruo, T., Farina, A. N., Ellisman, H. M., and Nakagawa, T. (2005) J. Neurosci. 30, 2728–2740
21. Traynelis, S. F., Wollmuth, L. P., Mc Bain, C. J., Menini, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J., and Dingledine, R. (2010) Pharmacol. Rev. 62, 405–496
22. Mah, S. I., Cornell, E., Mitchell, N. A., and Fleck, M. W. (2005) J. Neurosci. 25, 2215–2225
23. Valluru, L., Xu, Z., Zhu, Y., Yan, S., Contractor, A., and Swanson, G. T. (2005) J. Biol. Chem. 280, 6085–6093
24. Gill, M. B., Vithanaporn, P., and Swanson, G. T. (2009) J. Biol. Chem. 284, 14503–14512
25. Penn, A. C., Williams, S. R., and Greger, I. H. (2008) EMBO J. 27, 3056–3068
26. Horning, M. S., and Mayer, M. L. (2004) Neuron 43, 379–388
27. Zhou, F. X., Merianos, H. J., Brunger, A. T., and Engelman, D. M. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 2250–2255
28. Caputo, G. A., and London, E. (2004) Nature 430, 789–804
29. Tomita, S., and Noji, H. (2005) J. Biol. Chem. 280, 8250–8257