Polyamine-mediated channel block of ionotropic glutamate receptors and its regulation by auxiliary proteins

Published, Papers in Press, October 17, 2018, DOI 10.1074/jbc.TM118.003794

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Edited by Roger J. Colbran

Most excitatory neurotransmission in the mammalian brain is mediated by a family of plasma membrane–bound signaling proteins called ionotropic glutamate receptors (iGluRs). iGluRs assemble at central synapses as tetramers, forming a central ion-channel pore whose primary function is to rapidly transport Na^+ and Ca^{2+} in response to binding the neurotransmitter L-glutamic acid. The pore of iGluRs is also accessible to bulkier cytoplasmic cations, such as the polyamines spermine, spermidine, and putrescine, which are drawn into the permeation pathway, but get stuck and block the movement of other ions. The degree of this polyamine-mediated channel block is highly regulated by processes that control the free cytoplasmic polyamine concentration, the membrane potential, or the iGluR subunit composition. Recently, an additional regulation by auxiliary proteins, most notably transmembrane AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor regulatory proteins (TARPs), cornichons, and neuropilin and tolloid-like proteins (NETOs), has been identified. Here, I review what we have learned of polyamine block of iGluRs and its regulation by auxiliary subunits. TARPs, cornichons, and NETOs attenuate the channel block by enabling polyamines to exit the pore. As a result, polyamine permeation occurs at more negative and physiologically relevant membrane potentials. The structural basis for enhanced polyamine transport remains unresolved, although alterations in both channel architecture and charge-screening mechanisms have been proposed. That auxiliary subunits can attenuate the polyamine block reveals an unappreciated impact of polyamine permeation in shaping the signaling properties of neuronal AMPA- and kainate-type iGluRs. Moreover, enhanced polyamine transport through iGluRs may have a role in regulating cellular polyamine levels.

The first documented evidence linking polyamines to an experimental observation can probably be attributed to the work of pioneering Dutch microscopist, Antonie Van Leeuwenhoek (1), who in 1678, while observing spermatozoa, inadvertently noted crystal formation of polyamines in a specimen of drying semen (2). The three naturally occurring polyamines, namely spermine (Spm)[2] (3, 4), spermidine (Spd) (5), and putrescine (Put), would not be isolated nor formally identified until several centuries later with much of the early seminal contributions, especially in the area of the microbial biosynthetic pathways, made by Dr. Herbert (or Herb) Tabor and his wife, Dr. Celia Tabor (6–11). Dr. Herb Tabor, who will celebrate his 100th birthday in November, 2018, could not have envisaged the many areas of research impacted by polyamines, although he and his wife appreciated early on, while working with Dr. Sanford Rosenthal at the National Institutes of Health, the ubiquitous cytoplasmic expression of polyamines and their potential role in disease (12–14). Dr. Herb Tabor also contributed significantly to the growth of the *Journal of Biological Chemistry* over many decades (6, 11); therefore, it is fitting that the Journal has commissioned a series of minireviews on the topic of polyamines to celebrate and reflect upon his life and career.

Our understanding of the impact of polyamines, particularly on membrane excitability, has advanced significantly in the last 2 decades beginning with a series of observations showing that cytoplasmic polyamines block the pore regions of several voltage- (15–18) and ligand-gated (17, 19–21) ion channel families that are all cation-selective. Because Spm, Spd, and Put are positively charged at physiological pH, they are preferentially attracted into the water-filled pore regions of cation-selective channels where, due to their larger cross-sectional diameter (*cf.* Refs. 22, 23), they obstruct the transport of smaller permeating ions, such as Na^+, K^+ and/or Ca^{2+} (24, 25). The degree of channel block is voltage-dependent; and as a result, the action of cytoplasmic polyamines can finely tune cellular excitability within the range of the resting membrane potential by regulating the number of channels available for activation. This property of cytoplasmic polyamines is exemplified by their modulation of G-protein–gated inward rectifier K^+ (GIRK) channels, which slows the cardiac action potential (26, 27). Additional studies have revealed other dynamic aspects of polyamine channel block that include the following: (i) the regulated synthesis of cellular polyamine levels through the activity of ornithine decarboxylase (28); (ii) the relief of channel block by activ-

This work was supported by grants from the Canadian Institutes of Health Research (to D. B.). This article is part of a series on "Polyamines," written in honor of Dr. Herbert Tabor's 100th birthday. The author declares that he has no conflicts of interest with the contents of this article.

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[2] The abbreviations used are: Spm, spermine; Spd, spermidine; iGluR, ionotropic glutamate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDAR, *N*-methyl-D-aspartic acid receptor; TARP, transmembrane AMPA receptor regulatory protein; NETO, neuropilin and tolloid-like protein; Put, putrescine; KAR, kainate; receptor; NASPM, 1-naphthyl acetyl spermine; CNIH, cornichon; CNS, central nervous system; pS, picosiemen; CTZ, cyclothiazide.

ity-driven unblocking of polyamines (29–31); and (iii) by trafficking polyamine-sensitive and -insensitive channels into and out of the plasma membrane (32–34). More recent work has identified an additional level of regulation of polyamine block of ionotropic glutamate receptors (iGluRs) through their association with several families of auxiliary proteins called transmembrane AMPA receptor regulatory proteins (or TARPs), cornichons (CNIHs), and neuropilin and tolloid-like proteins (NETOs) (35–39).

In this Minireview, I will examine what we have learned about the mechanism of polyamine block of iGluRs in the last 2 decades. A detailed treatise on the biophysical mechanism(s) of channel block has been reviewed elsewhere (25, 40–42). Consequently, more emphasis will be placed on recent work examining the structural make-up of the AMPAR and KAR ion channel pore and how different mechanisms, particularly via auxiliary proteins, modify their architecture to shape ion permeation and polyamine block of native channels.

What has emerged from this work is that most native AMPARs and KARs have evolved distinct mechanisms to circumvent polyamine channel block. The two distinct mechanisms that prevail in AMPARs include (i) the formation of an electrostatic repulsion site at the apex of the pore, called the Q/R site, and (ii) the co-assembly of AMPAR subunits with auxiliary proteins, such as TARPs and CNIHs. Kainate receptors have evolved three distinct mechanisms to attenuate polyamine block that include (i) electrostatic repulsion at the Q/R site, (ii) structural instability of pore helices via proline residues, and (iii) the modulatory effect of NETO proteins. Given the persistent presence of polyamines in the cytoplasm of almost all cells, these mechanisms ensure unfettered signaling by AMPARs and KARs in the mammalian CNS.

Ionotropic glutamate receptors are differentially sensitive to polyamine channel block

Ionotropic glutamate receptors mediate the vast majority of fast excitatory signaling in the mammalian brain through the activity of three major ion channel families that include AMPAR receptors (AMPARs), *N*-methyl-D-aspartic acid receptors (NMDARs), and kainate receptors (KARs) (Fig. 1) (42, 43). The iGluR family also includes an additional orphan-class family composed of δ1 and δ2 subunits (Fig. 1) that signal via a metabotropic pathway, rather than an ion channel pore (42), through the formation of a trans-synaptic scaffolding complex (44). Whereas AMPARs and KARs can be strongly regulated by cytoplasmic polyamines, NMDARs are relatively insensitive to intracellular channel block (25). Curiously, however, NMDARs are antagonized in a voltage-dependent manner by *external* Spm and polyamine spider toxins (45–50). Why NMDARs are insensitive to cytoplasmic polyamines is unclear, although it may relate to the narrower diameter (5.5 Å) and the multi-ion occupancy of the NMDAR pore (51) compared with the larger diameter (7–7.8 Å) (52) and the single ion-pore of AMPARs and KARs (50, 53) that may be more suitable for the blocker. Given the estimated radii of a sodium ion and a water molecule to be 1.02 and 2.75 Å, respectively (54), and the internuclear distance between sodium and oxygen to be 2.35 Å (55), these dimensions suggest that the 7–7.8 Å pore of AMPARs and KARs is capable of transporting hydrated alkali metal ions, such as Na^+. As discussed later, subtle changes in the geometry of the pore may be a critical factor in governing the degree of block of iGluRs by polyamines especially as it relates to the regulatory effect of auxiliary subunits on AMPARs and KARs.

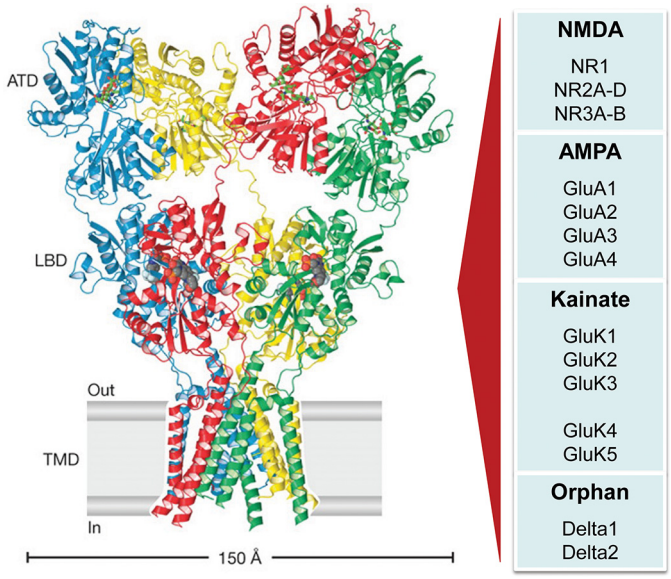

Figure 1. Ionotropic glutamate receptor families share a common tetrameric architecture. *Left,* X-ray crystal structure showing the tetrameric subunit arrangement of the homomeric GluA2 AMPAR, which consists of four distinct domains that include (i) an N-terminal domain (*ATD*), which directs subunit assembly and receptor clustering at synapses (189); (ii) four clamshell-like ligand-binding pockets (*LBD*) (190); (iii) a TMD that forms a central ion channel pore that transports Na^+ and Ca^{2+} ions in response to neurotransmitter binding (42, 43); and (iv) a cytoplasmic C-terminal domain (not shown) that directs receptor trafficking and synaptic anchoring (191). Image of structure was adapted from Ref. 192. *Right, table* of the four classes of iGluR arranged with each of their respective subunits.

Native AMPA receptor heteromers either possess or lack the GluA2 subunit

AMPARs are found at almost all glutamatergic synapses and in all brain regions (42, 43). Consequently, understanding how they are regulated, including block by cytoplasmic polyamines, has been an area of active investigation.

AMPARs are primarily responsible for the rapid millisecond response rise time to the neurotransmitter, L-glutamate (L-Glu) (40, 42). However, they also strengthen or weaken glutamatergic transmission by cycling into and out of synapses during periods of sustained patterned activity or altered homeostasis (56–59). AMPARs can, in principle, assemble as homomeric and/or heteromeric tetramers from any one of four possible receptor subunits, namely GluA1 to GluA4, that were cloned in the 1990s (60, 61). However, *in situ* hybridization (62) and single-cell PCR studies (63–65) established during that time that most brain regions and individual neurons express more than one AMPAR subunit that usually includes the GluA2 subunit. The current consensus is that neurons express a preferred family of AMPARs (40, 57, 66). For example, studies in the hippocampal and cortical brain regions suggest that most native AMPARs assemble as GluA1/GluA2 heteromers or GluA2/GluA3 heteromers (67–69). GluA1/A2 heteromers are proposed to traffic into central synapses during periods of pat-

terned activity triggered by the activation of Ca^{2+}/calmodulin-dependent protein kinase II, whereas GluA2/A3 receptors are thought to constitutively cycle into and out of synapses (69). Because EM studies estimate that the GluA3 subunit is expressed at about a tenth of the level of GluA1 and GluA2 (70), it suggests that a significant proportion of all AMPARs are earmarked for synaptic plasticity mechanisms (71). Other combinations are also possible, for example, migrating and mature cerebellar granule cells are thought to express GluA2/A4 heteromeric receptors (72, 73) as well as femtosiemens conductance channels that are consistent with GluA2 homomers (72).

There are, of course, exceptions to this general rule of the inclusion of GluA2. For example, several studies using single-cell PCR and/or antibody staining to document the expression level of different AMPAR subunits have identified some cells that either lack the GluA2 subunit entirely or express it at low levels. Cerebellar Bergmann glia cells (63, 74) and inhibitory interneurons of the basolateral amygdala (75, 76) are apparently devoid of GluA2, whereas dentate gyrus basket cells and some interneurons of the hippocampus, such as parvalbumin-positive (PV^+) basket cells (63, 65), and a fraction of neocortical fast-spiking nonpyramidal (64) and pyramidal layer V cells (77) all express low levels of GluA2. It is possible that other neurons and/or glial cells express GluA2 at diminished levels; however, their distribution has yet to be formally investigated. To complicate matters further, individual neurons may express both GluA2-lacking and GluA2-containing receptors which, in some cases, have been shown to be segregated to different synapses of the same neuron. For example, synapses of individual stratum lucidum hippocampal interneurons express GluA2-containing AMPARs when innervated by axon collaterals from CA3 pyramidal neurons and GluA2-lacking AMPARs when innervated by mossy-fiber axons of dentate gyrus granule cells (78). Interestingly, cells with reduced levels of GluA2 often express GluA1 and GluA4 receptor subunits. Whether GluA1 and GluA4 assemble as distinct populations of homomeric channels or heteromers in these cells is still not clear; although gene knockout studies reveal that both subunits contribute similarly to the ensemble AMPAR response of hippocampal PV^+ basket cells (79). GluA4-containing AMPARs are also one of the most prominent receptors expressed in the developing brain especially in inhibitory interneurons (80–82). Compared with the delivery mechanisms described for GluA1/A2 heteromers, GluA4-containing AMPARs appear to follow a different set of trafficking rules that is independent of Ca^{2+}/calmodulin-dependent protein kinase II and relies on spontaneous synaptic transmission rather than patterned neuronal activity (83). Consequently, receptor trafficking into and out of individual synapses is governed by the repertoire of AMPAR subunits that make up individual AMPAR heteromers. As explained below, the separation of native AMPAR populations into GluA2-containing or -lacking receptors not only affects receptor trafficking but also impacts the channel's functional behavior, including block by cytoplasmic polyamines.

GluA2 subunit determines ion-flow through the AMPA receptor pore

AMPARs have been grouped into two functionally distinct families based on the presence or absence of the GluA2 receptor subunit (25, 40, 41, 84). GluA2-lacking AMPARs possess ion channels with a large single-channel conductance (73), appreciable Ca^{2+} permeability (85–87), and high affinity to polyamine block (19, 88–90). In contrast, GluA2-containing receptors typically have much smaller unitary events (73), divalent impermeability (85–87), and insensitivity to polyamine block (19, 91). The exact copy number of GluA2 per AMPAR tetramer has been a matter of debate with some suggesting that the GluA2 content is variable (92) or fixed (93). However, a recent cryo-EM structure of the intact GluA2/A3 AMPAR suggests that they can assemble in a 2:2 fixed format with the GluA2 subunits occupying positions proximal to the pore (94). There is also evidence for the existence of a third class of AMPAR which, although similarly Ca^{2+}-permeable, is characterized by its near-insensitivity to internal and external channel block by polyamines (40). Although its exact molecular composition still remains unknown, AMPARs with these characteristics have been observed in the retina (95, 96), brain stem (97), cerebellum (33, 98), and glia progenitor cells (40, 99).

The critical role of the GluA2 subunit in determining sensitivity to polyamine block is due to RNA editing of an exon that encodes a critical residue in the GluA2 AMPAR pore region, called the Q/R site (Fig. 2) (100–102). The resulting codon change in GluA2 RNA transcripts, from an adenosine to inosine due to adenosine deaminase acting on RNA type 2 (ADAR2) activity (103–105), results in a switch in the amino acid sequence from a glutamine (Gln) to an arginine (Arg) (106, 107). Almost all transcripts of GluA2 are fully edited (>99% efficiency), which means that the Q/R site of all GluA2 receptor subunits contains a positively charged Arg (85, 101). The activity of ADAR2 is particularly remarkable in this regard because RNA editing of most other signaling proteins, such as potassium channels and sodium pumps, is much less efficient (106). In contrast, transcripts of GluA1, -A3, and -A4 are not subject to editing at this site; consequently, these subunits contain a Gln at the Q/R site instead. The specificity of ADAR2's actions on GluA2 is due to the cis-acting intron downstream of the unedited exonic site that is absent from GluA1, -A3, and -A4 and forms a dsRNA structure needed for editing (100, 104, 105, 107).

To complicate matters, unedited AMPARs possess at least 3–4 subconductance levels that range in amplitude from 5 to 7, 10 to 15, 20 to 25, and 30 to 40 pS (73, 108–111). In conditions of reduced AMPAR desensitization, the distinct sublevels correspond to the fractional occupancy of each of the four ligand-binding sites with agonist molecule (72, 108, 112). However, when desensitization is intact, the subconductance levels have been proposed to correspond to the number of desensitized subunits per tetramer (113). Under these conditions, the sublevels have been linked to conformations occurring within the ligand-binding domain dimer interface (114, 115) and/or the extracellular portion of the α-helix of transmembrane domain 3 (M3) (112). Based on what we know about other tetrameric ion

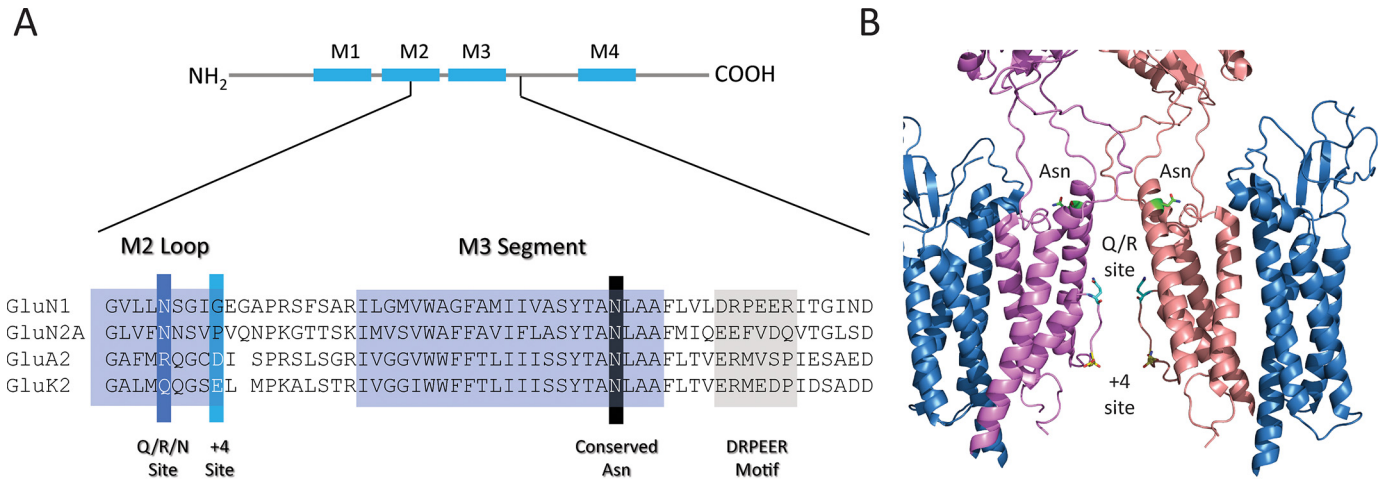

Figure 2. Sequence alignment of M2 re-entrant loop and M3 helix of different iGluR families. *A,* sequence comparison of the GluN1 and GluN2A NMDAR subunits with GluA2(R) AMPAR and the GluK2(Q) KAR subunits. The apex of the M2 pore loop of all iGluR families is capped by the Q/R/N site. In AMPARs and KARs, the Q/R site together with the +4 site (Asp in AMPARs and Glu in KARs) govern the degree of polyamine block. All iGluR families possess a conserved Asn residue in the M3 helix that contributes to the high-divalent permeability of the pore. *B,* cryo-EM structure of the GluA2(Q) AMPAR pore adapted from Protein Data Bank code 5WEO.

channels (116), it is unlikely that the distinct AMPAR open states of the channel correspond to significant structural re-arrangements of the pore region. It is possible, however, that each subconductance state may be distinguishable by their relative permeability to different cations. For example, in Shaker K^+ channels where inactivation has been slowed, the different sublevels observed during sequential channel activation have different selectively for alkali metal ions (117, 118). The smaller and intermediate open states are more permeable to Rb^+ ions, whereas the main open state is more permeable to K^+ (117, 118). Whether the different AMPAR subconductance levels are similarly distinguishable in their ability to transport different cations remains to be investigated.

In contrast, AMPARs containing four positively-charged Arg residues at the Q/R site, as would occur with homomeric GluA2(R) homomers, exhibit a dramatically reduced unitary conductance (73) and lose their selectivity for cations (52). Unitary events mediated by GluA2(R) AMPARs are too small to be resolved by conventional single-channel recordings and thus have been estimated to be 300 femtosiemens using stationary noise analysis (73). Experiments on homomeric GluA2(R) channels suggest that the channel is not exclusively cation-selective but also exhibits anion permeability with the relative permeability of Cl^- to Cs^+ estimated to be 0.17 (52). The impact of the inclusion of an Arg at the Q/R site on unitary conductance and/or cation *versus* anion selectivity cannot be explained by differences in the cross-sectional diameter of the pore that are similar for both Q- and R-forms (52). Instead, the positively-charged Arg ring structure at the selectivity filter is proposed to attract anion binding while repelling divalent ions (52). The electropositive nature of the pore would also disfavor monovalent cation transport accounting for the much-reduced unitary channel conductance (73).

Less is known about the impact of the Q/R site on recombinant heteromeric AMPARs, such as GluA1/A2 or GluA2/A3 receptors, due to the difficulty of obtaining single-channel recordings that are not contaminated by the expression of homomeric channels. The recent GluA2/A3 AMPAR structure suggests that the pore region of heteromers has a 2Q:2R arrangement (94), which would be expected to impact cation transport. In keeping with this, earlier work by Swanson *et al.* (73) had shown that the single-channel events of GluA2(R)/A4(Q) heteromers had a main conductance of 4 pS when compared with the 8-pS main open state of GluA2(Q)/A4(Q) heteromers, which is similar to observations reported for GluA1(Q)/A2(R) heteromeric channels (93). Heteromeric channels are strictly cation-selective (52) suggesting that, unlike homomeric GluA2(R) channels, the presence of only two Arg residues at the Q/R site is insufficient to attract anions into the pore.

Molecular architecture and block of the AMPA receptor pore

Prior to any direct structural insight, the Q/R site was already recognized as being at the apex of a re-entrant loop facing the extracellular vestibule of the channel (119–121). This structural arrangement is comparable with the pore architecture of other ion channels, such as K^+ channels, Na^+ channels, Ca^{2+} channels, and cyclic nucleotide-gated ion channels, which are now collectively known as pore–loop channels (122–125). The commanding position of the Q/R site makes it suitable to act as a selectivity filter of cations entering the extracellular or intracellular vestibules of the pore. When the pore region of AMPARs contains four neutrally-charged glutamine (Gln) residues at the Q/R site, such as GluA1/A4 heteromers, they are thought to form a single ion–binding site at the narrowest region of the pore that controls the transport of both monovalent and divalent cations (53). Because the permeability of all alkali metal ions, such as Na^+ and K^+, is similar for unedited Q-form AMPARs (52), cations are thought to be transported without losing their inner water shells, which is probably not the case for the NMDAR pore that possesses multiple ion–binding sites (53, 126). As mentioned above, this property is in keeping with the cross-sectional diameter of the AMPAR and KAR pores that have been estimated to be 7–7.8 Å (52). Exactly how the electrostatic environment of the AMPAR pore is

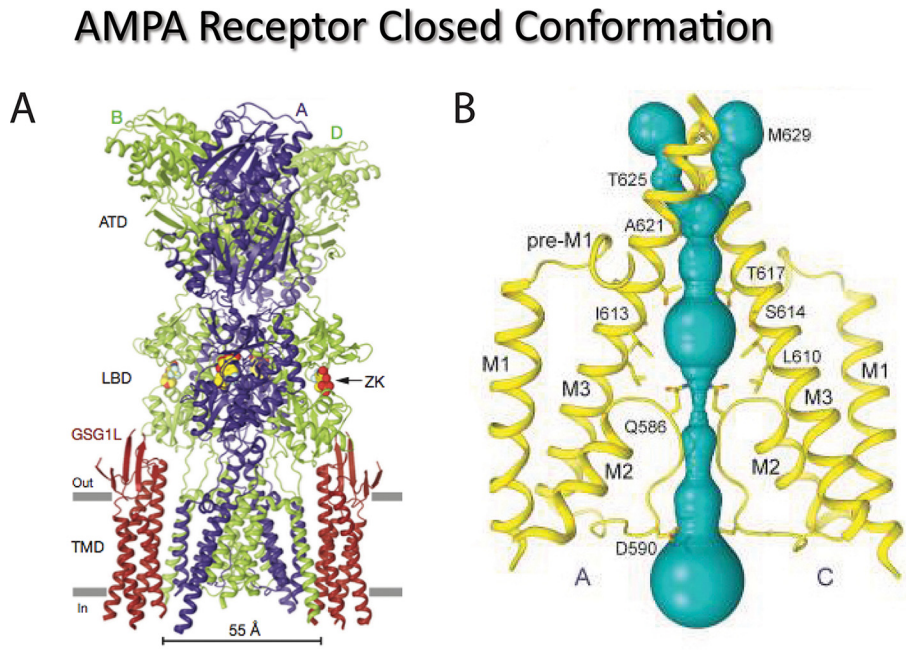

Figure 3. Closed conformation of the GluA2 AMPAR. *A,* cryo-EM structure of the GluA2 AMPAR in complex with the auxiliary protein, GSG1L, and the antagonist, ZK200775, which promote channel closure. *B,* closer view of the ion-conduction pathway (*violet*) with pore-lining residues in the M2 and M3 segments of subunits A and C shown in *yellow* highlighting the Q/R site (Gln-586) and +4 site (Asp-590). Adapted from Ref. 132 with permission.

altered when the Q/R residues contain both Gln and Arg residues, as would occur with GluA1/A2 or GluA2/A3, is not known.

The Q/R site is not the only residue that affects divalent permeability and polyamine block of AMPARs. Jatzke *et al.* (127) examined the role of several residues in the third transmembrane domain (M3) of AMPARs, including the conserved asparagine residue (N or Asn) of the SYTANLAAF motif and the highly-charged, extracellular vestibule formed by the DRPEER motif of the NR1 subunit, each of which have been linked to high Ca^{2+} permeability of NMDARs (Fig. 2) (128). As anticipated, mutation (Asn to Lys or Cys) of the conserved Asn in M3 of GluA2 AMPAR channels strongly attenuated divalent permeability establishing that this residue makes a significant contribution to Ca^{2+} influx in both AMPARs and NMDARs (127). Importantly, mutation of the Asn did not affect polyamine block (127) demonstrating that the ability of AMPARs to transport divalent ions can be uncoupled from their susceptibility to channel block. In contrast to NMDARs, the Glu and Arg residues C-terminal to M3 had only a minor effect on the divalent permeability of AMPARs and had no effect on KARs (127), although this region has been linked to regulation of KARs by auxiliary NETO proteins (129). Interestingly, another study also noted the uncoupling of Ca^{2+} permeability and polyamine block by mutating the negatively-charged Asp (to Asn) or Glu residue of AMPARs or KARs, respectively, which is four residues downstream of the Q/R site in the M2 (Fig. 2) (130). In this case, however, the +4 site attenuated polyamine channel block while apparently retaining high-divalent permeability (130). Panchenko *et al.* (121) explained this finding by proposing that there are multiple binding sites for the distributed charged amine groups in each polyamine molecule with the +4 site being located close to the cytoplasmic entrance of the channel pore. In keeping with this, mutation of the Q/R site (*e.g.* Gln to Asn (130)) was also shown to eliminate polyamine block while retaining divalent permeability (85, 130, 131) suggesting that polyamine molecules traverse multiple regulatory contact points in the pore.

To examine the structure of the ion-permeation pathway, Twomey *et al.* (132) compared the GluA2 AMPAR in conditions that promote the open or closed state of the channel. The closed conformation was favored by purifying the ZK200775 antagonist-bound GluA2 AMPAR in a covalent fusion construct with germline-specific gene 1-like (or GSG1L). GSG1L is an AMPAR auxiliary subunit that slows recovery from desensitization (133), and thus, the authors reasoned that it would promote the closed state of the channel (either resting and/or desensitized) (132, 134). The open conformation was achieved by purifying the agonist-bound GluA2 AMPAR in complex with the transmembrane AMPA receptor auxiliary protein (or TARP), stargazin, or γ2 (132), which, unlike GSG1l, promotes the channel open state (135, 136). The stability of the open state was further promoted by obtaining cryo-EM structures in the presence of the positive allosteric modulator, cyclothiazide (CTZ), which attenuates AMPAR desensitization (137). As elaborated below, these structural studies support the idea that the Q/R site, the +4 site, and conserved Asn of M3 are all ideally positioned to influence ion flow through the pore region of AMPARs (Figs. 3 and 4).

As can be readily appreciated from these structures, access of ions to the Q/R site is regulated by movement of the M3 bundle crossing composed of the Thr-617, Ala-621, Thr-625, and Met-629 residues (Figs. 3 and 4). In the closed position, the bundle crossing occludes access to the Q/R site (*i.e.* Gln-586, Fig. 3) whereas in the open conformation, it kinks out into a hydrophobic cavity in the middle of the channel pore (Fig. 4). A second constriction is formed below this cavity by the re-entrant loop of M2, which may operate as a lower gate much like other

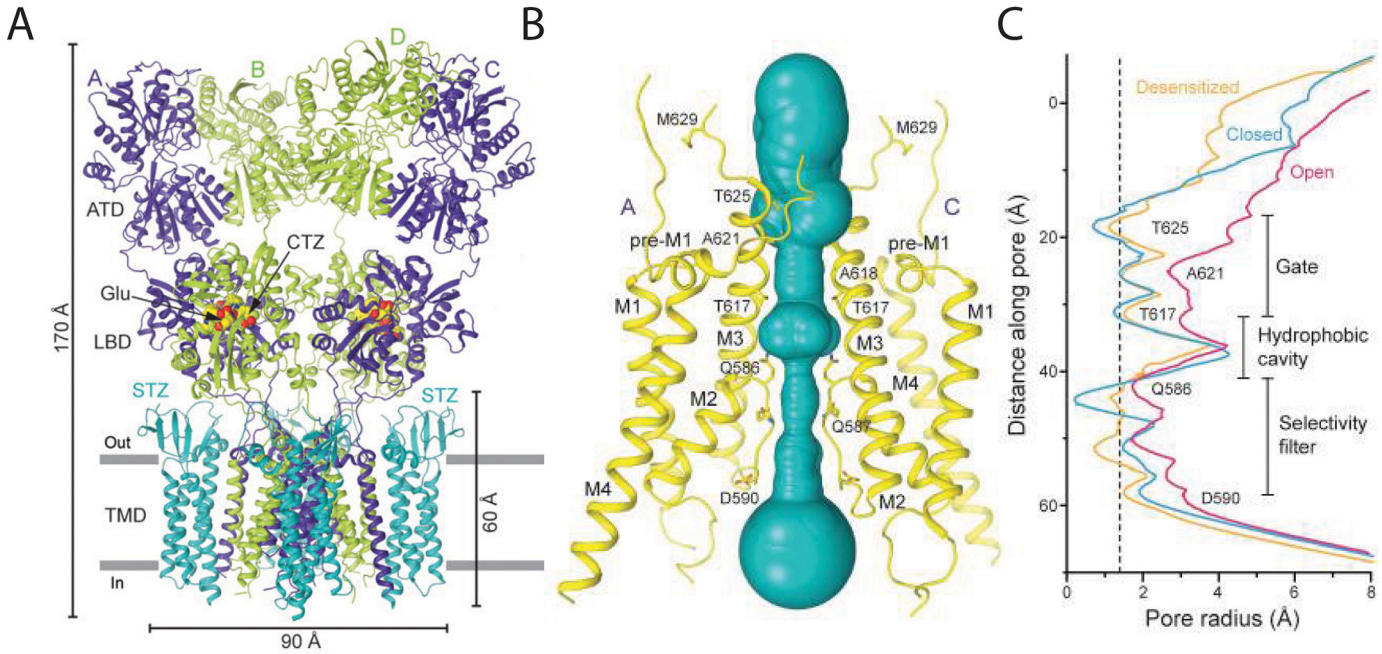

Figure 4. Open conformation of the GluA2 AMPAR. *A,* cryo-EM structure of the GluA2 AMPAR in complex with the auxiliary protein, γ2, the agonist, L-Glu, and the positive allosteric modulator, CTZ, which promote channel opening. *B,* closer view of the ion-conduction pathway (*violet*) with pore-lining residues in the M2 and M3 segments of subunits A and C shown in *yellow* highlighting the Q/R site (Gln-586) and +4 site (Asp-590). *C,* pore radius calculated using HOLE highlighting differences between the open state (*pink*), closed state (*blue*), and desensitized state (*orange*). Adapted from Ref. 132 with permission.

tetrameric ion channels, such as K^+ (138) and TRPV1 channels (139). In the closed conformation, the M2 helix below the Q/R site has an extensive network of hydrophobic interactions that are formed between M1 and M3 regions of the same subunit as well as M3 of the adjacent subunit (Figs. 3 and 4) (132). The open-pore dimensions shown in Fig. 4 fall short of the 7–7.8 Å measurement estimated for AMPARs and KARs (52), and therefore, our current model would not be expected to transport hydrated monovalent cations, such as Na^+ (see text above). Although molecular dynamics simulations of the open channel structure (140) and electron density measurements of the selectivity filter (see extended data, Fig. 5, *B* and *C,* of Ref. 132) suggest that the pore is in an open configuration, it is likely that more information is still needed to understand the molecular architecture and dimensions of the fully open AMPAR pore. Twomey *et al.* (132) also note that the pore loop is apparently more flexible in closed-state structures but more ordered in the open state to facilitate ion transport through the pore. As discussed below, flexibility of the pore loop is a recurring theme in iGluRs. The loss of polyamine block in GluK2/K5 KAR heteromers has also been proposed to be due to the destabilizing effect of proline residues on the M2 helix of GluK5 subunits (39).

The position of the polyamine blocker in the ion-permeation pathway was recently resolved by imaging cryo-EM structures of the GluA2(Q) AMPAR pore occupied by several known channel blockers. Specifically, the authors used the orb weaver spider toxin, argiotoxin 636 (AgTx-636) (141), the spider toxin analog, 1-naphthyl acetyl spermine (NASPM) (142), and the adamantane derivative, 1-trimethylammonio-5-(1-adamantane-methyl-ammoniopentane dibromide) (IEM-1460) (143), each of which block Ca^{2+}-permeable, GluA2(R)-lacking AMPARs in a use-dependent manner. To promote channel opening, Twomey *et al.* (132) tethered the AMPAR to the TARP, γ2, and bathed the samples in the agonist, L-Glu, and the positive allosteric modulator, CTZ (144). AgTX-636, NASPM, and IEM-1460 all contain extended polyamine tails and a bulky hydrophobic headgroup that gets trapped in the narrow constriction of the permeation pathway allowing the authors to identify which pore residues interact with the polyamine tail.

Using this approach, the overall GluA2 AMPAR structure with the pore blocker in place was revealed to be comparable with previous structures of the open-channel tetramer (132). The upper portion of the pore was characterized by an electroneutral surface and central cavity that sits atop a narrowing caused by the glutamines of the Q/R site, which then opens into the lower electronegative portion of the channel (Fig. 5). The electronegativity of this area most likely contributes to the cation selectivity of AMPARs, which occurs primarily through the backbone carbonyl oxygens of Gln-586, Gly-588, Cys-589, and Asp-590 (Fig. 5). These backbone residues presumably contribute to the membrane electric field that gives rise to the steep voltage dependence of polyamine block through the exit rates (unblock and permeation) of polyamine blocker from the pore (31, 38, 39). In keeping with this, Twomey *et al.* (132) suggest that the endogenous polyamines, Spm and Spd, block the pore at this point in the selectivity filter between residues Gln-586 and Asp-590. Because polyamines acting on AMPAR–TARP

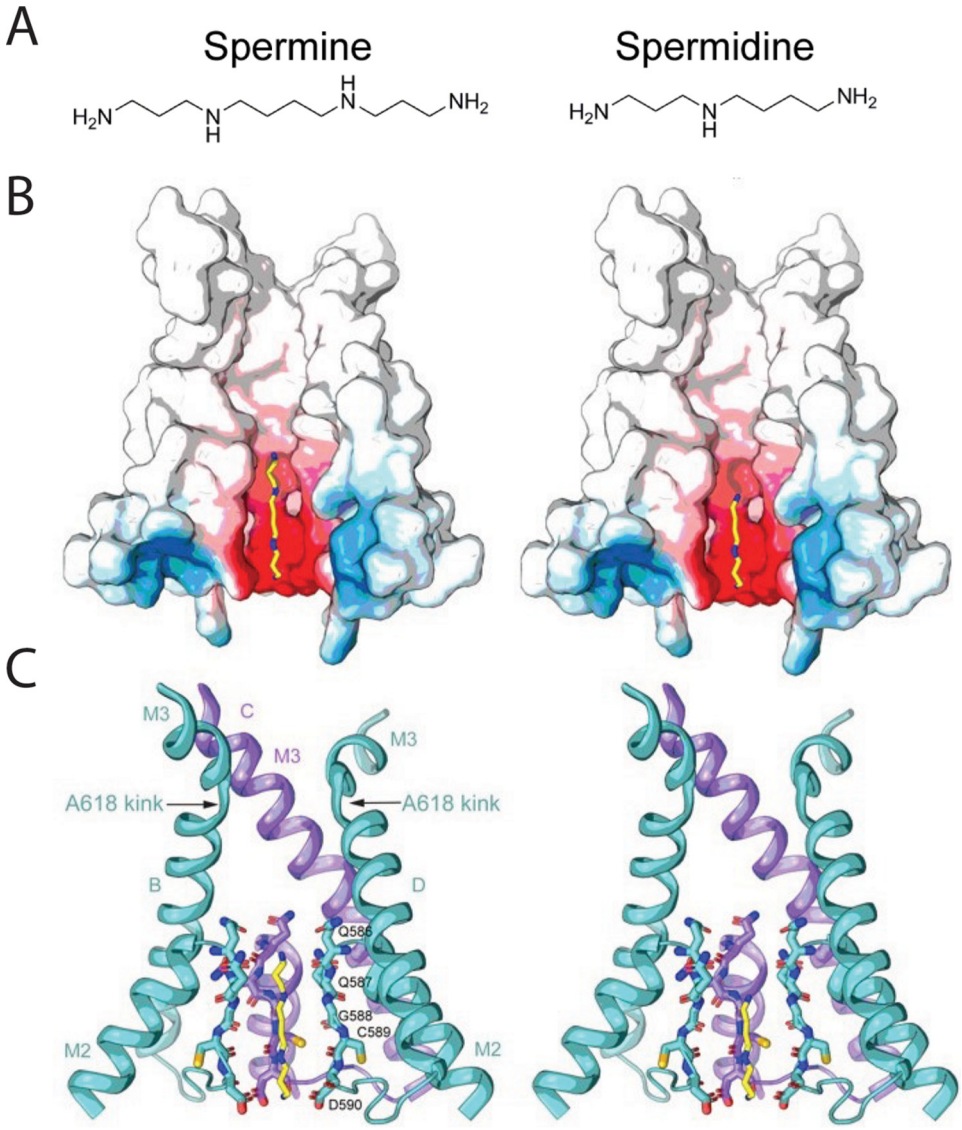

Figure 5. AMPAR pore occupied by polyamine channel blocker. *A,* extended structures of the endogenous polyamines, spermine and spermidine. *B,* cross-section of the GluA2(Q) AMPAR pore highlighting the electroneutral cavity (*white*) above the Q/R site and electronegative cavity (*red*) of the inner pore where endogenous polyamines, Spm and Spd (*yellow* for carbon and *blue* for nitrogen), are proposed to bind. *C,* more detailed structure of the pore highlighting the residues in M2 that contribute to polyamine binding and the position of the M3 helices in the open state. Adapted from Ref. 144 with permission.

complexes induce both channel block and appreciable blocker permeation, it has been speculated that the polyamine molecule may adopt two conformations in the pore, a linear structure, as noted here, that would favor permeation and a kinked structure that would lead to channel block (38). Whether polyamines entering the pore adopt two distinct structures, as also proposed for channel block of cyclic nucleotide-gated channels (21) or K^+ channels (145), awaits future investigation.

AMPA and kainate receptor auxiliary proteins attenuate polyamine channel block

An important development in the iGluR field has been the identification of a diverse family of auxiliary proteins that bind and modulate both AMPARs and KARs (146–151). TARP, stargazin (γ2), was the first auxiliary protein to be identified that was shown to be critical for the transport and synaptic targeting of native AMPARs (152). Additional studies uncovered that stargazin and other TARP isoforms modulate the gating properties of AMPARs (153) as well as their responsiveness to allosteric modulators (154) and antagonists (155, 156). Subsequent proteomic analyses then identified the cornichon family (157) as well as other auxiliary proteins such as GSG1L and CKAMP44 (158) that differentially associate with AMPARs in a developmental- and regional-specific manner (159). At about the same time, the neuropilin and tolloid-like proteins, NETO1 and NETO2, were also identified and revealed to bind to native KARs and to modulate their trafficking and gating behavior (149, 160–164). A feature common to both TARP, CNIH, and NETO auxiliary protein families has been their ability to attenuate the degree of polyamine block of AMPARs and KARs.

Both TARPs and CNIHs attenuate the polyamine block of AMPARs by causing a reduction in the onset of block and a greater relief of block observed at both negative and positive membrane potentials, respectively (35, 37, 38). NETOs attenuate the polyamine block of KARs in a comparable manner (36,

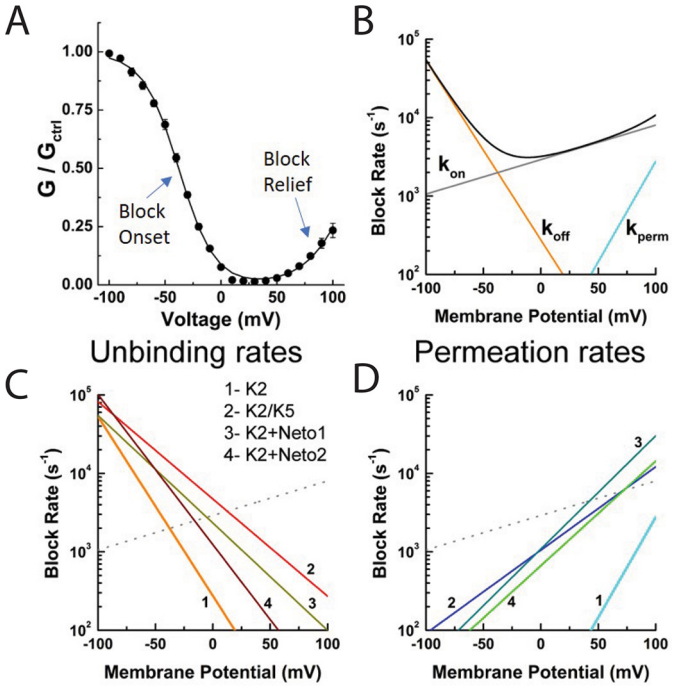

Figure 6. Auxiliary proteins speed up polyamine exit rates from the pore. *A,* conductance voltage plot of the voltage-dependent Spm block of GluK2 KARs. Note that the onset of block occurs at negative membrane potentials, whereas relief of block occurs at positive membrane potentials. *B,* plot showing how the rate of Spm binding (k_{on}), unbinding (k_{off}), and permeation (k_{perm}) to the GluK2 KAR pore changes at different membrane potentials. Note that although Spm-binding rate is fairly voltage-insensitive, exit rates from the pore (k_{off} and k_{perm}) are steeply voltage-dependent. The *solid black lines* correspond to the sum of all block rates at different membrane potentials. *C* and *D,* plots showing how the rate of Spm unbinding (*C*) and permeation (*D*) is shifted by NETO1 and NET2 auxiliary proteins as well as by heteromerization. Adapted from Ref. 39.

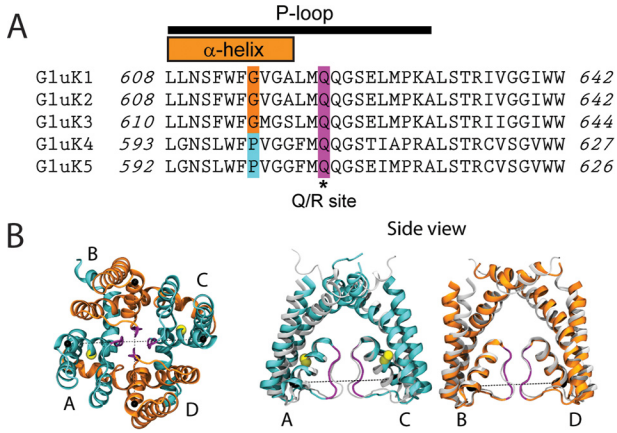

Figure 7. Asymmetric pore of heteromeric KAR channels. *A,* sequence alignment of the five KAR subunits highlighting the Gly residue of GluK1–3 subunits and the Pro of GluK4 and GluK5 found in the M2 helix. High nanomolar affinity block of GluK3 is conferred by the Met and Ser residues that are Val or Ala/Gly residues, respectively, in the other KAR subunits. *B, left,* NaK open channel structure (193) was used as a template for GluK2/K5 heteromers introducing the Pro residues on subunits A and C. *B, right,* side view of the A/C (*cyan*) and B/D (*orange*) subunits of the inverted NaK pore before (*gray*) and after (*colored*) 257-ns simulations reveal that the Pro residues introduce asymmetry into the pore. Adapted from Ref. 39.

39) suggesting that the underlying mechanism may be similar (Fig. 6). Unlike block of the multi-ion pore of K^+ channels (24, 27, 165, 166), the onset of block of AMPARs and KARs is caused by a single polyamine molecule entering and occluding the pore from the cytoplasm (31), whereas relief of block is due to polyamine permeation to the extracellular side of the pore (Fig. 6) (22, 25). At 0 mV, the apparent Spm affinity for unedited GluA2(Q) AMPARs is reduced by about 3- or 10-fold by association with CNIH3 or stargazin (38), respectively, whereas block of GluK2 KARs is attenuated by 8–20-fold through NETO1 and NETO2 association (36, 39). Because the rate of polyamine binding to the open AMPAR or KAR pore is unaffected by auxiliary proteins, the attenuation of block is caused almost entirely by faster exit rates of the blocker from the pore (Fig. 6) (36, 38, 39). Spm resides less time at its block site, and consequently, the blocker returns to the cytoplasm or permeates all the way through the pore more readily. In keeping with this, Brown *et al.* (38, 39) demonstrated that Spm permeates the AMPAR and KAR pores to a much greater extent when bound to auxiliary proteins. For example, the permeability of Spm relative to Na^+ was estimated to be 4–16-fold more permeable in GluA2 AMPARs associated with TARPs or CNIHs than GluA2 AMPAR alone (38). A similar observation was also noted when comparing Spm permeability of GluK2 KARs in the presence and absence of NETOs (39).

The structural mechanism by which AMPAR and KAR auxiliary proteins attenuate polyamine block is still unresolved. However, because their primary effect is to curtail the time the blocking molecule resides at its binding site (38, 39), it is likely that TARPs, CNIHs, and NETOs alter the pore architecture. An attractive possibility is that auxiliary proteins re-shape the electronegative cavity between the Q/R and +4 sites where Spm and other endogenous polyamines are proposed to reside (144). In keeping with this, Perrais *et al.* (167) have shown that the high nanomolar block affinity of GluK3 KARs is due to methionine (Met) and serine (Ser) residues in the M2 helix that are absent from GluK1 and GluK2 KARs, which contain Val and Ala residues instead (Fig. 7). The loss of nanomolar Spm affinity by replacement of the Met and Ser residues of GluK3 with Val and Ala, respectively, of GluK1 and GluK2 would not be expected to change the electrostatic environment of the pore; however, it may alter the structural flexibility of the M2 helix. In support of this, attenuation of polyamine block in GluK2/K5 KAR heteromers has been attributed to a conserved proline residue also found in the M2 helix, in this case of the GluK5 (and GluK4) subunit (Fig. 7) (39). Molecular dynamic simulations suggest that the two proline residues, which are arranged on opposing subunits of the GluK2/K5 tetramer (168), increase structural flexibility in the pore making it less favorable for polyamine block and more favorable for permeation (Fig. 7) (39). This is an important point because it reveals that GluK4- and/or GluK5-containing KAR heteromers attenuate polyamine block, not by an electrostatic repulsion mechanism, as is proposed for GluA2-containing AMPAR heteromers, but rather through a different mechanism that introduces structural flexibility in the pore helices. The fact that the near loss of polyamine block in GluK2/K5 heteromers is not accompanied by changes in divalent permeability (39) again suggests that the primary disruption in the ion-permeation pathway is at the level of the Q/R site and M2 helix rather than in the extended

M3 helix where the conserved Asn residue contributes to Ca^{2+} permeability (127). In keeping with this, TARPs acting on GluA4 AMPARs (37) and NETOs acting on GluK2 KARs (36) attenuate polyamine block without having any effect on divalent permeability.

The situation, however, is likely to be more complicated for several reasons. First, an Arg scan of the M3 helix atop of the Q/R site in GluK2 KARs identified residues that attenuate polyamine block (169) suggesting that alterations in this electroneutral cavity by auxiliary proteins may also affect the degree of channel block. A potential caveat, however, is that Arg replacement in the extracellular vestibule may create a local electrostatic environment that alters permeant ion flux in the pore, which will indirectly attenuate polyamine block (see Fig. 4*D* of Ref. 31), a possibility that may be in keeping with the enhanced anion permeability reported for the Arg mutants (169). Second, both CNIHs and TARPs attenuate polyamine block of GluA1 AMPARs while increasing divalent permeability (35) suggesting that auxiliary proteins may have a broader structural impact on both the inner electronegative cavity of the pore and the outer extracellular vestibule formed by the M3-S2 linker. The fact that the γ2 TARP enhances Ca^{2+} permeability of GluA1 (35) but not GluA4 (37) homomers is not understood, but it may suggest that the ability of TARPs to enhance divalent transport through the pore is AMPAR subunit-dependent. GSG1L also affects both ion permeation and channel block (133, 170). In this case, however, GSG1L acts to diminish AMPAR divalent permeability and to enhance polyamine block (albeit at membrane potentials $>+50$ mV) (170), which, although contrary to the effect of TARPs and CNIHs, is in keeping with auxiliary subunits having a broader impact on the structural integrity of the ion-permeation pathway. Third, and finally, two separate studies have identified that the cytoplasmic tails of NETOs and TARPs can attenuate polyamine block of KARs (36) and AMPARs (171), respectively. Fisher and Mott (36) observed that neutralization of three positively charged residues, namely Arg–Arg–Lys, which lie close to the transmembrane region of NETOs, eliminated the attenuation of polyamine block of KARs. Given this, the authors speculated that the C-tail of NETOs may act by a charge-screening mechanism to prevent polyamines entering the pore. A charge-screening mechanism, however, is unlikely because auxiliary subunits do not affect the rate of polyamine binding and therefore do not hinder their ability to access the pore (38, 39). Neutralization of the homologous residues in γ2 TARP, namely Arg–His–Lys, had no effect on polyamine block of AMPARs (171) suggesting that auxiliary proteins probably impact polyamine block differently between AMPARs and KARs and that other mechanisms are likely to be at play.

Linking neurodevelopmental disorders and cancer to polyamines and their transport

One of the most unexpected effects of auxiliary proteins is their ability to enhance the transport of polyamines across the plasma membrane (38, 39). This is particularly evident at AMPARs where the permeability of Spm relative to Na^+ at GluA2(Q) AMPARs ($P_{Spm}/P_{Na} = 0.3$) increased about 4- and 10-fold when GluA2 was co-assembled with γ2 TARP ($P_{Spm}/P_{Na} = 1$) and CNIH-3 ($P_{Spm}/P_{Na} = 4$), respectively (38). Given that other endogenous polyamines, such as Spd and Put, have fewer amine groups, they would be expected to be transported at a greater rate than Spm, as has already been shown at KARs (22, 39). Although it is not immediately clear how enhanced polyamine transport might be important to the mammalian CNS, it is worth noting that the developing brain is thought to express a greater abundance of GluA2-lacking AMPARs (172, 173), where polyamine transport may play a role. Outside the brain, polyamines are recognized for fulfilling many roles in cells, such as regulating protein and nucleic acid synthesis and structure, cell proliferation, differentiation, and apoptosis (174, 175). Consequently, it is likely that polyamines contribute to similar roles in the developing CNS. Interestingly, disrupted polyamine levels, due to the inactivation of the Spm synthase gene, give rise to the X-linked neurodevelopmental disorder called Snyder-Robinson syndrome that is associated with mental retardation (176). Although the potential role of polyamine-sensitive K^+ channels and AMPARs has been speculated upon (176), it is still not clear how deficits in cellular polyamines account for the CNS defects.

Polyamines and their transport by AMPARs may be more compellingly linked to their proposed roles in cancer (177). Cell growth and cancer have long been associated with elevated levels of polyamines (178) and with more recent studies establishing a connection to AMPAR activity. For example, pancreatic (179, 180) and kidney (181, 182) cancers have each been associated with the activation of AMPARs with tumor growth in breast and lung carcinoma, colon adenocarcinoma, and neuroblastoma cells shown to be diminished by AMPAR antagonists (183, 184). The elevated levels of extracellular polyamine levels reported in many cancers coupled with the prolonged activation of AMPARs (185) would provide the appropriate environment to permit the slow but steady transport of polyamines into cells. Although the mechanisms regulating polyamine biosynthesis and catabolism are well-understood (186, 187), the molecular events that lead to polyamine transport in mammalian cells are less clear (188). Consequently, whether the AMPAR in complex with auxiliary proteins, such as CNIHs, could contribute to this function would be an important question to consider in future studies.

Conclusion

Much has been learned about polyamine channel block of iGluRs since it was first identified over 2 decades ago. The discovery of auxiliary proteins and their regulation of iGluRs as well as breakthroughs in structural biology open new opportunities for the development of clinically-relevant compounds that might exploit these novel protein–protein interactions. Recent advances in gene-editing techniques, such as CRISPR, also make it possible to pinpoint how distinct families of auxiliary proteins shape iGluR signaling within neuronal circuits and how they may give rise to the aberrant activity that underlines neurological disease. Consequently, there is still much to be uncovered about how polyamine channel block contributes to membrane excitability and its potential role in disease.

Acknowledgments—I thank the present and former members of the Bowie lab for their ongoing discussions and careful reading of this manuscript. I also thank Yuhao Yan for providing the pore structure shown in Fig. 2. I am grateful to the two reviewers for their thoughtful critique that has helped improve the overall clarity and readability of the text.

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