Brief Communications

The Auxiliary Subunits Neto1 and Neto2 Reduce Voltage-Dependent Inhibition of Recombinant Kainate Receptors

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Kainate receptors can be subject to voltage-dependent block by intracellular polyamines, which causes inward rectification of the current–voltage relationship. Sensitivity to polyamine block is largely determined by the identity of a residue within the pore domain that can be altered through RNA editing. This process causes replacement of the encoded glutamine(Q) with a positively charged arginine(R), eliminating polyamine inhibition and thus inward rectification. In neurons, kainate receptors can associate with the auxiliary subunits Neto1 or Neto2. These transmembrane proteins alter the trafficking, channel kinetics, and pharmacology of the receptors in a subunit-dependent manner. We found that coexpression of Neto subunits with recombinant GluK2(Q) kainate receptors greatly reduced inward rectification without altering calcium permeability. This effect was separate from modulation of channel kinetics, as mutations within the extracellular LDLa domain of the Neto proteins completely eliminated their effects on desensitization but only reduced their effects on rectification. Conversely, deletion of the intracellular C-terminal domain of Neto1 or Neto2 or neutralization of positively charged residues within this domain prevented the reduction in rectification but did not alter effects on channel kinetics. These results demonstrate new roles for Neto1 and Neto2 in regulating kainate receptor function and identify domains within these auxiliary subunits important for mediating their effects.

Introduction

Kainate receptors are cation-permeable ionotropic glutamate receptors responsible for regulating excitatory neurotransmission both presynaptically and postsynaptically (Perrais et al., 2010). These tetrameric receptors are assembled from five different subunits (GluK1–GluK5) and the GluK1–3 subunits can form functional homomeric receptors. GluK1 and GluK2 subunits are subject to developmentally regulated RNA editing, causing exchange of an encoded glutamine(Q) for a positively charged arginine(R) residue in the pore domain (Sommer et al., 1991; Bernard and Khrestchatisky, 1994). This structural change has a number of functional effects, including alterations in single-channel conductance, calcium permeability, and sensitivity to block by intracellular polyamines (Rosenthal and Seeburg, 2012). Polyamines are present in the cytoplasm of most cells at millimolar concentrations, and inhibit GluK1–3(Q)-containing kainate receptors in a voltage-dependent manner (Bowie and Mayer, 1995; Perrais et al., 2009). As a result, the current–voltage relationship for these receptors in whole-cell recordings shows prominent inward rectification, whereas responses in the absence of polyamines or from receptors containing edited (R) subunits are outwardly rectifying.

Most ionotropic glutamate receptors appear to interact with auxiliary subunits, which are not necessary for channel function, but regulate membrane trafficking, channel kinetics, and pharmacological properties (Jackson and Nicoll, 2011). The TARP (transmembrane AMPAR regulatory proteins) subunits, among others, selectively interact with AMPA receptors, while in hippocampal and cerebellar neurons, postsynaptic kainate receptors were shown to coassemble with the auxiliary proteins Neto1 or Neto2 (Zhang et al., 2009; Tang et al., 2011). Interestingly, although the TARP and Neto proteins are structurally unrelated, they share some functional effects. Subtypes within both groups can slow the kinetics of desensitization and deactivation, change agonist profiles, and may alter surface expression and trafficking (Jackson and Nicoll, 2011). In addition, the TARP subunit stargazin(γ-2) reduces inhibition of AMPA receptors by intracellular polyamines, and therefore reduces inward rectification (Soto et al., 2007). To determine whether this function is also shared by the Neto proteins, we examined the effect of Neto1 or Neto2 on the voltage-dependent properties of recombinant, homomeric GluK2(Q) receptors.

Materials and Methods

Mammalian expression vectors encoding full-length cDNA for rat GluK2(Q) (in JG3.6, provided by S. Heinemann, Salk Institute, San Diego, CA), human Neto1 (in pCAGGS) and rat Neto2 (in pCDNA3, both Neto1 and Neto2 provided by S. Tomita, Yale University, New Haven, CT) were transiently transfected into HEK-293T (GenHunter) cells using calcium phosphate precipitation, as previously described (Mott et al.,
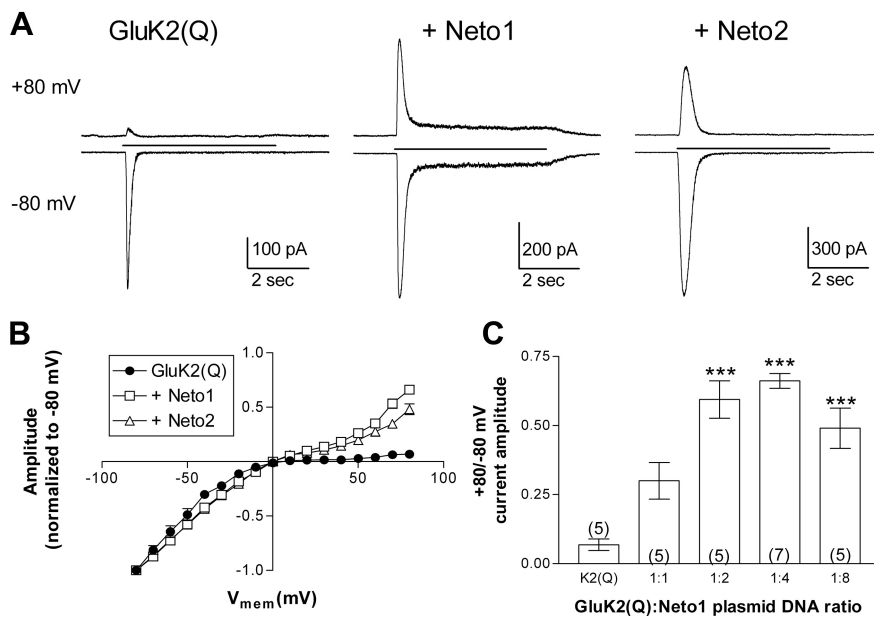
Figure 1. Coexpression with Neto1 or Neto2 reduces inward rectification of GluK2(Q) receptors. ***A***, Whole-cell current traces from HEK-293T cells transiently transfected with GluK2(Q) alone, or GluK2(Q) with either Neto1 or Neto2. Plasmids were transfected in a 1:4 (GluK2:Neto) ratio with 1 μg of GluK2 plasmid DNA. Glutamate (100 μM) was applied for 5 s (horizontal bar) to cells voltage clamped at +80 or −80 mV, as indicated. ***B***, Current–voltage relationships for cells transfected with GluK2(Q) alone or with Neto1 or Neto2. Transfection conditions as in ***A***. The peak current amplitude was normalized to the response at −80 mV for each cell. N = 5 cells for each subunit combination. ***C***, GluK2(Q)-encoding plasmid DNA (1 μg) was cotransfected with varying amounts of Neto1-containing plasmid. The peak current at +80 mV was divided by that at −80 mV. Error bars indicate mean ± SEM with the n given by the number in parentheses. ***Significant difference from GluK2(Q) ($p \leq 0.001$).

2010). Unless otherwise noted, cells were transfected with 1 μg of GluK2 and 4 μg of Neto1/2 plasmid DNA.

For whole-cell recordings, the external bath solution contained the following (in mM): 150 NaCl, 3 KCl, 10 HEPES, 1 CaCl$_2$, and 0.4 MgCl$_2$ adjusted to pH 7.4 and 295–305 mOsm. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments) on a two-stage puller (Narishige) to 5–10 MΩ and filled with a solution containing the following (in mM): 130 CsGluconate, 5 CsCl, 10 HEPES, 5 CsBAPTA, 2 MgCl$_2$, 2 MgATP, and 0.3 NaGTP with pH 7.3 and osmolarity 290–300 mOsm. Polyamines were not added to the internal solution. The +80/−80 amplitude ratio did not change over the course of the 20–30 min recording period, suggesting limited washout of endogenous polyamines. For measurement of Na$^+$/Ca^{2+} permeability, the intracellular solution contained the following (in mM): 140 CsCl, 1 MgCl$_2$, 10 EGTA, and 10 HEPES (pH 7.2 with CsOH). High Na$^+$ extracellular solution contained the following (in mM): 140 NaCl and 10 HEPES (pH 7.2 with NaOH). High Ca^{2+} extracellular solution contained the following (in mM): 110 CaCl$_2$ and 10 HEPES (pH 7.2 with CaOH$_2$).

Agonists were diluted into external solution and applied to cells using a computer-controlled exchanger with an exchange time of 20–30 ms (SF-77B; Warner Instruments). Current recordings were amplified (Axopatch 200B; Molecular Devices), filtered (1 kHz), and digitized at 10 kHz using a Digidata 1320 analog to digital board (Molecular Devices).

Whole-cell currents were analyzed using Clampfit (pClamp9.2 or 10 suite; Molecular Devices), Prism (Graphpad), and Origin7.5 (Origin-Lab). The onset of desensitization was fit with a single exponential function using Levenberg–Marquardt least-squares method. ANOVA and Tukey–Kramer multiple-comparisons tests were performed with Instat (Graphpad) using a significance level of $p < 0.05$.

Results

Neto1 and Neto2 reduce inward rectification of GluK2(Q) kainate receptors

Homomeric kainate receptors containing the unedited (Q) form of the GluK2 subunit respond to 100 μM glutamate with rapid

and complete desensitization and show strong inward rectification in whole-cell recordings (Fig. 1). We found that addition of either Neto1 or Neto2 altered both the desensitization kinetics and the amount of inward rectification (Fig. 1*A,B*). At this glutamate concentration, the effects of each Neto subunit were clearly distinct. The greatest impact of Neto1 was on the extent of desensitization. GluK2(Q) receptors produced no measurable current by the end of a 5 s application of 100 μM glutamate, but with Neto1 the steady-state current averaged 11.0 ± 1.0% ($n = 7$) of the peak current. In contrast, Neto2 primarily slowed the onset of desensitization (Fig. 1*A*). Although the whole-cell recording configuration did not permit accurate measurement of the fast components of desensitization, cotransfection with Neto2 clearly altered desensitization, slowing the average decay time constant from 100 ± 10 ms ($n = 5$) to 360 ± 30 ms ($n = 6$).

While Neto1 and Neto2 had distinct effects on the receptor's kinetic properties, both significantly reduced inward rectification. Because of voltage-dependent block by intracellular polyamines, little outward current was observed at positive membrane potentials in the absence of Neto and the +80 mV/−80 amplitude ratio averaged 0.07 ± 0.02. However, in the presence of either Neto1 or Neto2, the current–voltage relationship became more linear (with +80/−80 ratios averaging 0.66 ± 0.03 and 0.48 ± 0.05, respectively), without a change in the reversal potential (Fig. 1*A,B*). The effectiveness of Neto1 depended on the relative amount of plasmid DNA transfected. Previous studies used kainate receptor subunit:Neto ratios ranging from 1:3 to 1:30 (Zhang et al., 2009; Straub et al., 2011a). We varied the amount of Neto1 plasmid DNA cotransfected with 1 μg of DNA encoding GluK2(Q) and measured the current at +80 and −80 mV (Fig. 1*C*). A ratio of 1:4 appeared to be optimal under our conditions, with the greatest effect on rectification and reduced variability compared with a ratio of 1:2. We therefore used this DNA ratio for Neto1 and Neto2 in all further experiments.

Neto1 and Neto2 do not alter calcium permeability

In addition to effects on polyamine block and inward rectification, the Q/R modification also impacts ionic selectivity, with GluK2(Q)-containing receptors having significantly greater calcium permeability (Burnashev et al., 1995). To determine whether the Neto subunits influence this property, we examined the relative Na$^+$:Ca^{2+} permeability of GluK2(Q) receptors. To maintain a steady-state current for the duration of the voltage ramp, we used the weakly desensitizing agonist domoate. Starting at 5 s into the domoate application, when the current had reached steady state, the holding voltage was ramped from −100 mV to +70 mV over a period of 2 s (Fig. 2).

For GluK2(Q) receptors, the reversal potential in high Ca^{2+} extracellular solution shifted by −27.2 ± 4.2 mV ($n = 7$), similar to previous reports (Köhler et al., 1993; Burnashev et al., 1995). The addition of Neto1 or Neto2 did not alter this shift in reversal
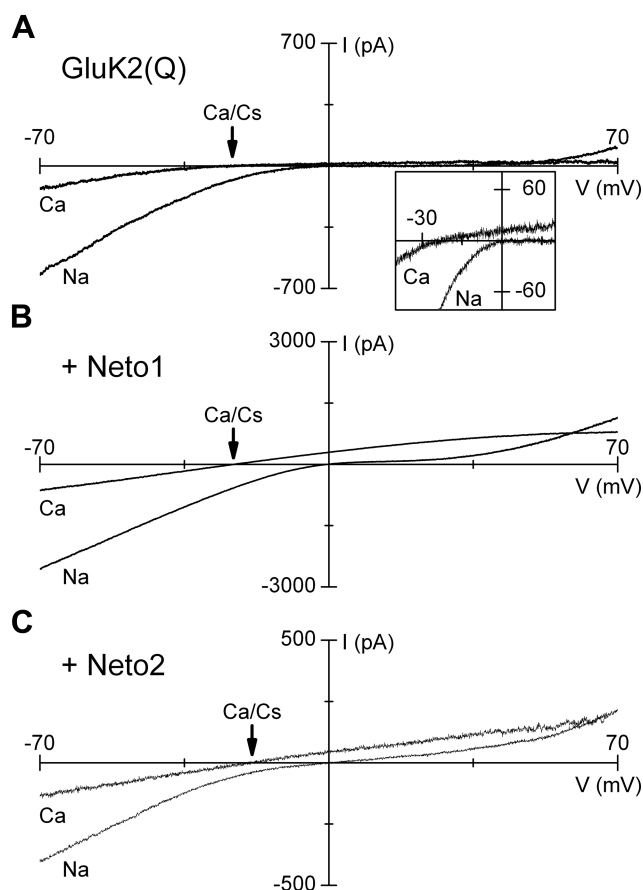
A

B

C

Figure 2. Effect of Neto subunits on Ca^{2+} permeability. **A–C**, Steady-state current voltage relationships in response to 10 μM domoate in either high Na^+ or high Ca^{2+} external solution for GluK2(Q) alone (**A**), with Neto1 (**B**), or with Neto2 (**C**). The inset shows GluK2(Q) reversal potentials at higher resolution. Voltage ramps in the presence and absence of domoate were subtracted to determine the reversal potential in each solution. The measured shift in reversal potential between Na^+/Cs^+ and Ca^{2+}/Cs^+ was corrected by the junction potential (9.3 mV). In high Na^+, the average reversal potential was close to 0 mV and was corrected to 0 in all experiments, with an equal correction applied to the Ca^{2+}/Cs^+ reversal potential. Arrows indicate the Ca^{2+}/Cs^+ reversal potentials that were −27 mV (GluK2), −24 mV (+Neto1), and −26 mV (+Neto2).

potential (V_{rev}) in high Ca^{2+} (Neto1: $V_{rev} = -24.4 \pm 0.8$ mV, $n = 5$; Neto2: $V_{rev} = -26.2 \pm 5.7$ mV, $n = 6$; Fig. 2), indicating that these proteins do not alter the Ca^{2+} permeability. This lack of effect on calcium permeability is similar to that of stargazin(γ-2), which also reduces inward rectification of AMPA receptors without affecting ion selectivity (Soto et al., 2007).

Mutations within the LDL-receptor class A repeat domain eliminate Neto effects on kinetics, but not on rectification
Little is known about the structural domains within the Neto proteins that confer their functional effects. It is possible that the same mechanisms that produce the changes in desensitization kinetics are also responsible for decreasing inward rectification, as conformational changes in the channel could alter both kinetics and access to a blocking site. For example, in addition to reducing block by intracellular polyamines, some TARP subunits also enhance block of AMPA receptors by extracellular polyamines (Jackson et al., 2011). This activity was correlated to an increase in channel conductance and was agonist-dependent, suggesting that both resulted from a change in pore structure.

To determine whether the effects of the Neto proteins on desensitization kinetics could be dissociated from those on inward rectification, we created point mutations at two cysteine residues within the LDL-receptor class A repeat (LDLa) domains of Neto1 and Neto2 (Fig. 3A). Previous work showed that these residues are necessary for Neto2 to enhance the glutamate-induced current in oocyte recordings (Zhang et al., 2009). We created mutated Neto1$_{(C270S/C304S)}$ and Neto2$_{(C271S/C305S)}$ subunits and coexpressed them with wild-type GluK2(Q) (Fig. 3B). In both cases, these mutations largely eliminated the effects of the Neto proteins on desensitization. For the Neto1$_{(C270S/C304S)}$ subunit, the steady-state current remaining at the end of the 5 s application was decreased to $0.79 \pm 0.18\%$ of the peak current ($n = 6$, $p \leq 0.001$ compared with wild-type Neto1). With the Neto2$_{(C271S/C305S)}$ mutations, the rate of onset of desensitization was similar to that of GluK2(Q) alone ($p > 0.05$) and significantly different from wild-type Neto2 ($n = 6$, $p \leq 0.001$; Fig. 3E). However, despite the loss of these kinetic effects, both Neto1$_{(C270S/C304S)}$ and Neto2$_{(C271S/C305S)}$ still significantly reduced the amount of rectification compared with GluK2(Q) alone (Fig. 3B,D).

The intracellular domains of Neto1 and Neto2 are responsible for their effects on inward rectification
While the LDLa domain mutations clearly had a larger impact on modulation of channel kinetics than on inward rectification, the ability to alter rectification was also significantly reduced. This might reflect a decrease in surface expression of the auxiliary subunits coassembled with the kainate receptor, as previously observed with these mutations in Neto2 (Zhang et al., 2009). However, others found that deletion of the LDLa domain in Neto2 did not reduce its interaction with GluK2 (Tang et al., 2011). Alternatively, this result could indicate that the two functional effects are correlated, possibly sharing a structural mechanism. To further address this question, we sought to identify structures responsible for the effect on rectification. We focused on the intracellular C-terminal domain of the Neto subunits, as this region might directly interact with the cytoplasmic polyamines. For both Neto proteins, these domains contain a substantial number of charged residues. The C-terminal domain of Neto1 is 168 aa in length, 47 of which are charged, while this domain in Neto2 contains 155 aa, with 44 charged residues. Earlier studies showed that deletion of the intracellular domain of Neto subunits did not impair their association with kainate receptor subunits (Tang et al., 2011).

To determine whether the intracellular domain could be responsible for reducing inward rectification, we inserted a stop codon near the putative transmembrane domain of Neto (Stöhr et al., 2002), truncating the protein after R347 in Neto1(c-del) or R348 in Neto2(c-del). These truncated constructs were coexpressed with GluK2(Q) and the response to 100 μM glutamate measured at −80 and +80 mV (Fig. 3C,D). Deletion of the C-terminal domain had no effect on the ability of the Neto proteins to modify the kinetic properties of the receptor, as Neto2(c-del) slowed the onset of desensitization to the same extent as wild-type Neto2 (Fig. 3C,E) and Neto1(c-del) produced a similar increase in the steady-state current (averaging $9.7 \pm 2.1\%$ of the peak current). However, for both Neto1 and Neto2, the reduction of inward rectification was completely eliminated by removal of the C-terminal domain (Fig. 3C,D).
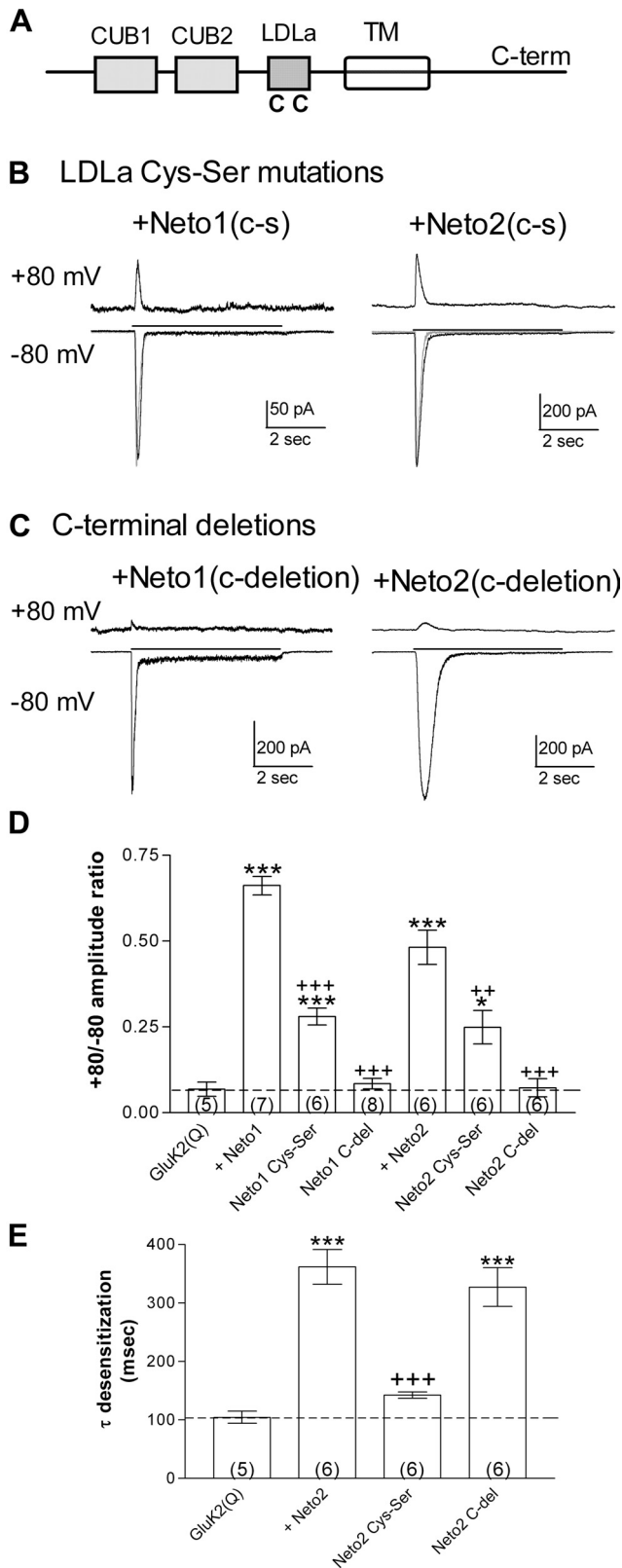
Role of charged residues within the C-terminal domain

These results demonstrate that the distinct effects of the Neto proteins on channel kinetics and inward rectification can be functionally and structurally separated, and that the intracellular C-terminal domain plays a critical role only for the latter effect. To identify the responsible sites within this domain, we created a series of progressive truncations of the Neto2 subunit (Fig. 4A,B). From the full-length domain of 155 aa, terminating at Phe499, we inserted stop codons to produce C-terminal domains terminating at Arg407 (63 residues), Leu377 (33 residues), or Asn359 (15 residues) (Fig. 4A,B). We found that even a Neto2 containing only 15 residues of the original C-terminal domain reduced inward rectification to the same extent as the full-length protein (Fig. 4A,B).

These findings implicated the region between R347 and N359 in the Neto2 intracellular domain. Within this sequence, Neto2 contains four positively charged residues. These four residues are conserved in Neto1, which also includes an additional arginine residue (Fig. 4C). To determine whether these residues are necessary for the effect of the Neto proteins, we mutated each of them to alanine (Fig. 4D–F). Individual mutations of single residues in Neto2 had no impact on its function (Fig. 4D). However, mutation of both R353 and K354 to alanine in the Neto1 subunit significantly reduced its effectiveness, and triple mutations of the RKK sequence within either Neto1 or Neto2 completely prevented their activity (Fig. 4E,F). Although the single mutations had no effect on the amount of rectification produced by endogenous levels of polyamines in the whole-cell recording configuring, excised patches could be used to determine whether each individual residue influences the concentration dependence of block. The triple mutations did not alter the impact of either Neto1$_{(R347A/K348A/K349A)}$ or Neto2$_{(R348A/K349A/K350A)}$ on receptor desensitization, with the extent of desensitization (Neto1: 10.9 $\pm$ 1.5%, $n = 5$) or onset of desensitization (Neto2: 370 $\pm$ 40 ms, $n = 5$) similar to that produced by the respective wild-type Neto subunits.

Discussion

We found that the auxiliary subunits Neto1 and Neto2 influenced both rectification and desensitization of recombinant GluK2(Q) kainate receptors. The effects on channel kinetics were generally consistent with previous studies, which reported that both Neto1 and Neto2 altered the onset and recovery from desensitization of kainate receptors in a subunit-dependent manner (Zhang et al., 2009; Copits et al., 2011; Straub et al., 2011a,b). In contrast, the

←

these mutated constructs; black traces show representative responses to 5 s applications of 100 μM glutamate (horizontal line) at membrane potentials of $+80$ or -80 mV. The underlying gray trace shows the response at -80 mV of GluK2(Q) receptors without Neto (same trace as Fig. 1A). C, The C-terminal domain of Neto1 or Neto2 was truncated after the fourth amino acid residue; these constructs were coexpressed with GluK2(Q). Current traces are in response to 5 s applications of 100 μM glutamate at $+80$ or -80 mV. D, The rectification ratio was calculated by dividing the amplitude of the peak response at $+80$ mV by that at -80 mV. The reversal potential was not affected by any of the mutations and was near 0 mV in all cases. Significant difference from GluK2(Q) receptors: ***$p \leq 0.001$, *$p \leq 0.05$; significant difference from the corresponding wild-type Neto protein: +++$p \leq 0.001$, ++$p \leq 0.01$. Error bars represent mean $\pm$ SEM and the number of cells is indicated by the number in parentheses. The dashed line shows the mean response of GluK2(Q) receptors in the absence of Neto. E, The onset of desensitization was measured by fitting the decay of whole-cell currents with a single exponential. Error bars represent mean $\pm$ SEM with the number of cells indicated by the number in parentheses. The dashed line shows the mean response of GluK2(Q) receptors in the absence of Neto. ***Significant difference from GluK2(Q) receptors ($p \leq 0.001$); +++Significant difference from wild-type Neto2 ($p \leq 0.001$).

Figure 3. Effects of Neto1/2 on channel kinetics and rectification are mediated by distinct structures. A, Schematic representation of the Neto subunit structure. The extracellular N-terminal sequence contains two CUB domains and one region with homology to the LDLa (Stöhr et al., 2002). This domain contains two cysteine residues (C) required for Neto2 to effectively enhance kainate receptor activity (Zhang et al., 2009). The single transmembrane domain (TM) is followed by an intracellular C-terminal domain. B, Two cysteine residues within the LDLa domain of Neto1 or Neto2 were mutated to serine. GluK2(Q) receptors were coexpressed with

A Neto2

B Neto2 truncations

C

Neto 2 ...K_{345}QP**RKK**VMAC**K**TAFN$_{359}$...
Neto 1 ...K_{344}QP**RKK**YVQ**RK**SDFD$_{358}$...

D Neto2 single point mutations

E

+Neto1
(R_{347}A,K_{348}A,K_{349}A)

+Neto2
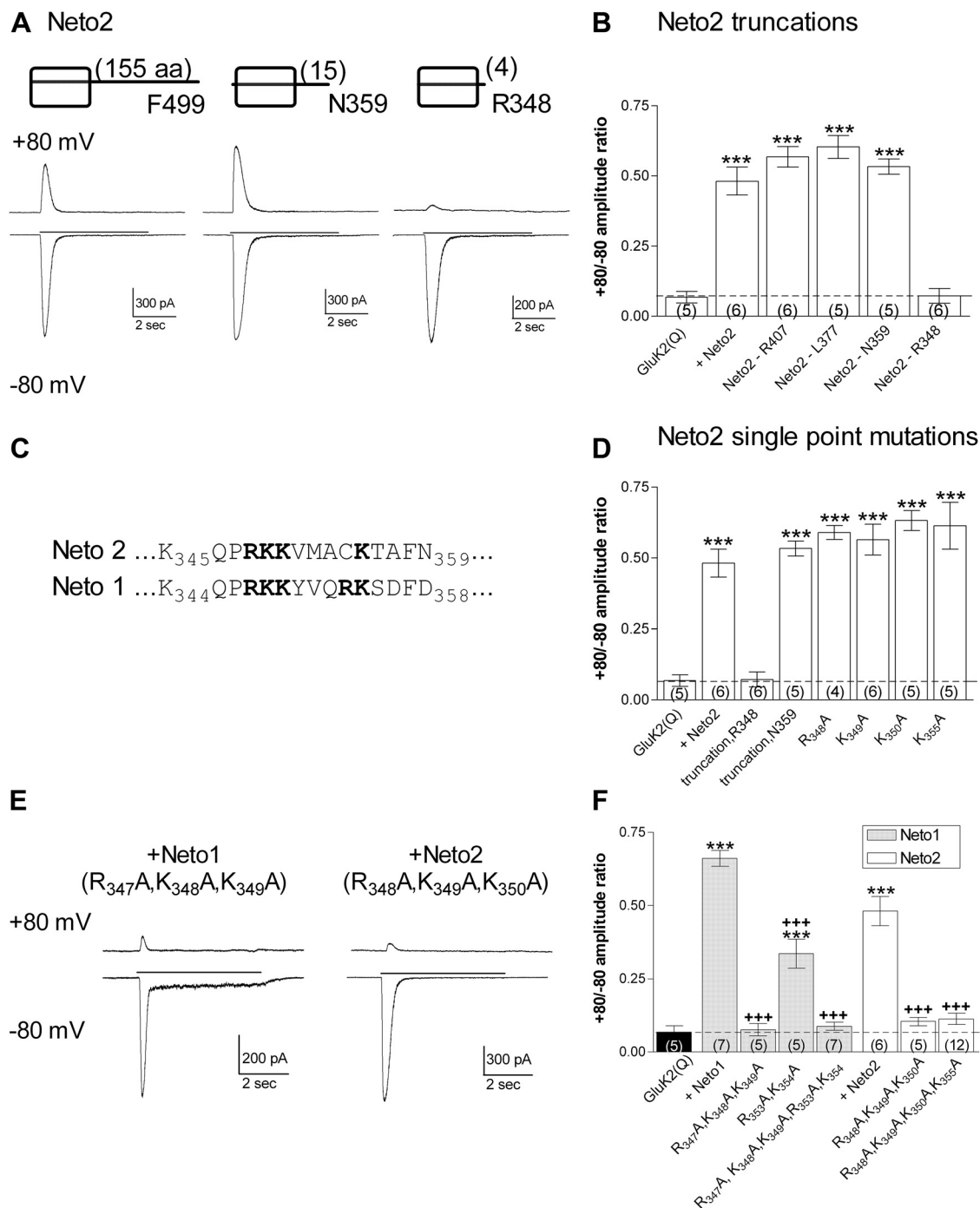(R_{348}A,K_{349}A,K_{350}A)

F

Figure 4. A series of positively charged residues near the transmembrane domain of Neto1/2 mediate their effects on GluK2(Q) rectification. ***A***, The intracellular C-terminal domain of Neto2 was progressively truncated to determine the minimum length necessary to reduce inward rectification of GluK2(Q). Schematics represent the wild-type subunit and two of the truncated subunits, showing the number of residues and the terminating amino acid for each construct. Representative current traces in response to 100 μM glutamate at +80 or −80 mV for receptors containing the indicated Neto2 subunit with GluK2(Q). ***B***, The ratio of the response at +80 and −80 mV is shown as mean ± SEM, with the number of cells indicated by the number in parentheses. The Neto2 truncation constructs are named by their terminating residues. The dashed line shows the mean response for GluK2(Q) alone. ***Significant difference from GluK2(Q) in the absence of Neto2 (p ≤ 0.001). ***C***, Amino acid sequences for Neto1 and Neto2 are aligned from the start of the intracellular domain through the site of the shortest truncation in Neto2 that permitted full function. Positively charged residues located in the functionally critical region between Arg348 and Asn359 of Neto2 are indicated in bold type. ***D***, Positively charged residues in Neto2 were individually mutated to alanine. Graph is constructed as described in ***B***. ***E***, Triple mutations were made in the intracellular domain of Neto1 or Neto2, converting the conserved RKK sequence in each subunit to AAA. Current traces show the response to 100 μM glutamate. ***F***, Multiple arginine or lysine residues in Neto1 or Neto2 were mutated to alanine. Graph is constructed as described in ***B***. ***Significant difference from GluK2(Q) receptors (p ≤ 0.001); $^{+++}$Significant difference from wild-type Neto1 or Neto2 (p ≤ 0.001).

present study is the first to describe a role for the Neto subunits in reducing inward rectification of kainate receptors. In addition to its modulation of kainate receptors, Neto1 also interacts with NMDA receptors, regulating their expression at synapses and playing an im-

portant role in synaptic plasticity (Ng et al., 2009). Since NMDA receptors are not regulated by cytoplasmic polyamines (Williams, 1997), it may be interesting to determine whether the C-terminal domain of Neto1 has a different role with these receptors.

Our results suggest that a series of three adjacent positively charged residues located near the intracellular side of the transmembrane domain in Neto1 and Neto2 are responsible for the decrease in inward rectification. These residues may act as a charge screen, reducing access of the positively charged polyamines to the pore of the channel. However, other possible mechanisms can also be imagined. For example, in the absence of Neto proteins, polyamine interaction with the kainate subunits is influenced not just by Q/R editing, but also by a nearby negatively charged residue, which is necessary for high affinity binding (Panchenko et al., 1999). The RKK sequence of the Neto proteins that we identified could neutralize the impact of this residue and thus reduce rectification. Hydrophobic residues surrounding the channel pore also influence block and contribute to the exceptionally high polyamine affinity associated with the GluK3 subunit (Panchenko et al., 2001; Perrais et al., 2009). In addition, extracellular structures have been found to regulate polyamine sensitivity, presumably by altering pore structure (Perrais et al., 2009). The Neto subunits could influence inward rectification through any of these mechanisms, although our results demonstrate a critical role for positively charged intracellular residues located near the membrane.

In addition to their voltage-dependent inhibition of outward current, polyamines also produce tonic inhibition of ionotropic glutamate receptors by binding to the channel's closed state. This tonic inhibition can be relieved by ion flow through the channel, producing an activity-dependent facilitation of glutamatergic synapses (Rozov et al., 1998; Rozov and Burnashev, 1999). The amount of facilitation could be influenced by both polyamines and by coassembly with auxiliary subunits (Soto et al., 2007). Polyamine levels are regulated during development (Shin et al., 2005) and can change in response to neuronal activity (Aizenman et al., 2002). Together, these studies suggest that the effects of Neto proteins on kainate receptor function could vary as a function of both development and behavioral state.

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