Brief Communications

Neto2 Modulation of Kainate Receptors with Different Subunit Compositions

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Kainate receptors are less well understood than other glutamate receptors, and synaptic kainate receptors display properties that differ from recombinant receptors. In particular, the slow decay of kainate receptor synaptic currents contrasts with the rapid deactivation and desensitization of receptors expressed in heterologous cells. We recently identified Neuropilin and Tolloid like-2 (Neto2) as a novel accessory subunit of kainate receptors and showed that Neto2 modulates the gating kinetics of GluK2 receptors. However, the kainate receptor family consists of five different subunits (GluK1–5) that can form homomeric and heteromeric receptors with different functional properties. Here, we tested whether Neto2 modulation varies with subunit composition. Rapid application techniques were used to apply glutamate to outside– out patches that contained GluK1, GluK1/5, or GluK2/5 kainate receptors. Coexpression of Neto2 slowed desensitization to varying degrees. Responses to 1 ms pulses of glutamate were also slowed by Neto2, especially for receptors containing GluK5, as were postsynaptic currents in neurons expressing recombinant kainate receptors. In addition, Neto2 markedly increased the rate at which some receptors recovered from desensitization. These results suggest that Neto2 modulates the function of most kainate receptors.

Introduction

Glutamate is the main excitatory neurotransmitter in the brain, where it acts on three classes of ionotropic glutamate receptors: AMPA, NMDA, and kainate receptors (KARs) (Hollmann and Heinemann, 1994; Dingledine et al., 1999). Despite significant sequence homology and a shared architecture, the receptor subfamilies behave differently in response to glutamate and fulfill different roles in synaptic transmission. The role of KARs in synaptic transmission is less well understood than those of AMPA and NMDA receptors, and the receptors display several puzzling features (Huettner, 2003; Lerma, 2003). Notably, whereas synaptic KAR currents decay in tens or hundreds of milliseconds and are much slower than synaptic currents through AMPA receptors (Castillo et al., 1997; Vignes and Collingridge, 1997; Kidd and Isaac, 1999; Cossart et al., 2002), heterologously expressed KARs deactivate and desensitize rapidly with time constants similar to AMPA receptors (Dingledine et al., 1999; Erreger et al., 2004). The reasons for this discrepancy remain unclear, although GluK5-containing receptors display slow deactivation at nonsaturating concentrations of glutamate (Barberis et al., 2008).

The family of KARs consists of five subunits, the low-affinity subunits GluK1-3 and the high affinity subunits GluK4/5. GluK1-3 are capable of forming functional homomeric ion channels, while GluK4/5 have to coassemble with GluK1, 2, or 3, and endogenous KARs are believed to be mostly heteromers (Christensen et al., 2004; Ruiz et al., 2005; Nasu-Nishimura et al., 2006; Fernandes et al., 2009). We recently identified Neuropilin and Tolloid like-2 (Neto2) as a novel accessory subunit of KARs (Zhang et al., 2009). Neto2 interacts specifically with recombinant and native GluK1 and GluK2 and modulates KAR currents *in vitro* and *in vivo*. In our initial analysis, we demonstrated that Neto2 increases the open probability and burst length of individual GluK2 receptors and also speeds recovery from desensitization.

Since different KAR subunits and combinations form receptors with distinct properties and roles in synaptic transmission (Huettner, 2003; Lerma, 2003; Christensen et al., 2004; Erreger et al., 2004; Pinheiro and Mulle, 2006), we test here whether Neto2 also modulates the behavior of receptors formed from other KAR subunits. We focused on GluK1 and GluK2, both with and without GluK5. These three subunits are best studied, show the broadest expression in brain, and are generally thought to account for most of KAR-mediated synaptic transmission (Hollmann and Heinemann, 1994; Lerma, 2003; Pinheiro and Mulle, 2006). We found that Neto2 modulates all combinations of KARs tested, but the effects varied quantitatively with subunit composition. In total, our results imply that Neto2 is a ubiquitous modulator of KARs and suggest a role for Neto2 in shaping endogenous KAR-mediated synaptic currents.

Materials and Methods

Cell culture and transfection. tsA201 cells were plated onto 12 mm glass coverslips coated with 100 μg/ml poly-L-lysine, and the cells were maintained in humidified 95% H_2O/5% CO_2. Transfections were made with Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol.

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GluK5 and/or Neto2 were transfected in fourfold excess relative to GluK1/2. To identify transfected cells, eGFP was cotransfected at a ratio of 1:10.

Cerebellar granule cell cultures and transfections were performed as described previously (Zhang et al., 2009). In short, primary cultures from *stargazer* mice were made at postnatal day 7, transfected at DIV 5 using the calcium-phosphate method, and recorded from at DIV 7–10. *Stargazer* mice were obtained from the Jackson Laboratory and were maintained at the Yale animal facility under the guidelines of the Institutional Animal Care and Use Committee.

Electrophysiology. Recordings from outside–out patches were performed 2–3 d after transfection at room temperature as described previously (Robert et al., 2001). The holding potential was −70 mV or −100 mV, and series resistance compensation was set at 60–80%. The external solution was as follows (in mM): 150 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 2.5 glucose, and 10 HEPES, pH 7.4. Patch pipettes (open-tip resistance, 3–5 MOhm) were filled with a solution containing (in mM) 120 KF, 33 KOH, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, and 10 HEPES, pH 7.4. Glutamate was added to the external solution and was applied with theta glass pipettes mounted on a piezoelectric bimorph. The 10–90% rise times of agonist-evoked currents were 0.3–0.6 ms, and the rate of solution exchange estimated from open-tip potentials was 100–200 μs. The bath was superfused constantly with normal external solution at a rate of 1 ml/min. Agonist-evoked currents recorded in outside–out patches were analog low-pass filtered at 3 kHz and sampled at rates of 20–50 kHz.

Synaptic currents were recorded in whole-cell mode at room temperature and a holding potential of −70 mV. The external solution contained the following (in mM): 140 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 2.7 $MgCl_2$, 10 glucose, and 10 HEPES. The pipette solution contained the following (in mM): 130 Cs-methanesulfonate, 5 HEPES, 5 Mg-ATP, 0.2 Na-GTP, 20 TEA, 5 and EGTA.

Data analysis and statistics. Digitized records were transferred to IGOR software (Wavemetrics) and analyzed as described previously (Robert et al., 2001). In many cases, the decays of ensemble currents were biexponential. The time constants and relative amplitudes obtained from biexponential fits to these decays were used to calculate weighted tau values. Synaptic currents were aligned at the 50% rise-time and averaged. The frequency of events was low (<5 events per minute), and all events from an individual cell were used for analysis. Results are given as mean ± SEM. Mean values with and without Neto2 were compared with Student's *t* test, and $p < 0.05$ was considered statistically significant.

Results

The effect of Neto2 on desensitization of kainate receptors

We recently identified Neto2 as a novel accessory subunit of KARs and reported it modulated the kinetics of homomeric GluK2 receptors (Zhang et al., 2009). To determine whether Neto2 also modulates the kinetics of receptors formed from other widely expressed KAR subunits, we coexpressed Neto2 with GluK1, as well as GluK1 or GluK2 with GluK5.

We first analyzed desensitization kinetics. Coexpression of Neto2 slowed desensitization of GluK1 receptors sevenfold (weighted tau, 1.6 ± 0.2 ms and 11.2 ± 1.7 ms, respectively) (Fig. 1*a*), similar to what we observed before for GluK2 (Zhang et al., 2009). Whereas GluK1 desensitization was frequently fitted well by a single exponential in the absence of Neto2 (4 of 8 patches), in the presence of Neto2 desensitization was clearly biexponential (9 of 9 patches) (Fig. 1*a*,*f*). The second, slow component of decay was slower and more pronounced with Neto2 (Fig. 1*e*,*f*). In addition, coexpression of Neto2 resulted in a substantial increase in the steady-state current (Fig. 1*a*,*g*).

Receptors composed of GluK1 and GluK5 desensitized monoexponentially and more rapidly than GluK1 homomers (weighted tau, 0.7 ± 0.1 ms and 1.6 ± 0.2 ms, respectively) (Fig. 1*a*,*b*). Coexpression of Neto2 slowed desensitization of GluK1/5 heteromers several fold (weighted tau, 3.3 ± 0.5 ms for GluK1/5 + Neto2) (Fig. 1*b*). This effect was partially attributable a slower fast component of decay but mostly to the appearance of a second, very slow component that was not seen without Neto2 (Fig. 1*b*,*e*,*f*). In addition, Neto2 coexpression increased the steady-state current, which was nearly undetectable without Neto2 (Fig. 1*g*).

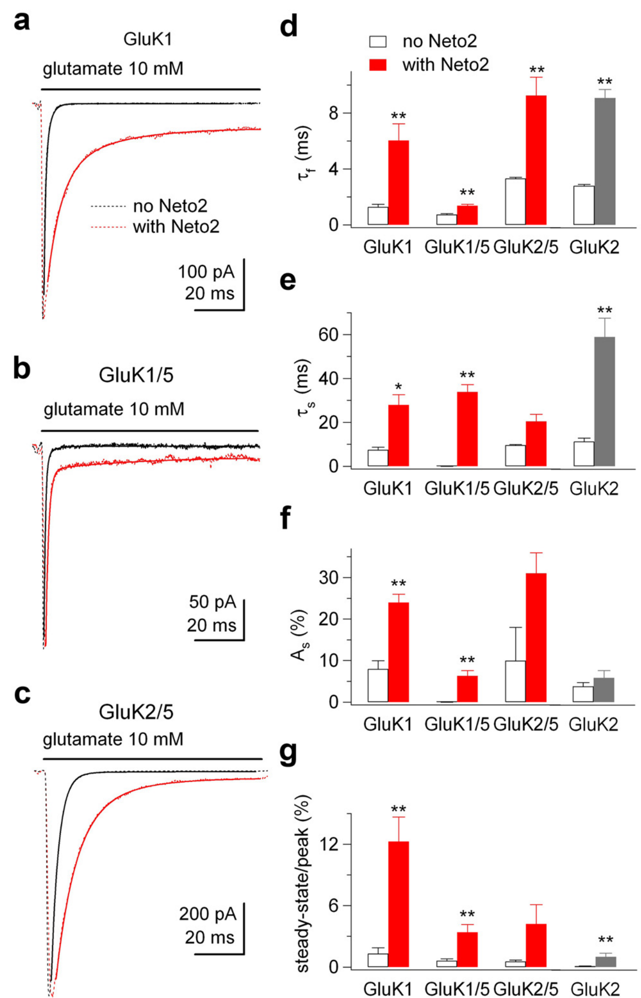

Figure 1. Neto2 modulates desensitization of different KAR subunits. ***a–c***, Currents evoked in outside–out patches from cells that were transfected with the indicated KAR subunits either without (black) or with (red) Neto2. Glutamate (10 mM) was applied for 100 ms (bars above traces). The monoexponential or biexponential fits to the decays of the currents (solid lines) are superposed on the data (dotted lines). The amplitude calibrations refer to the results without Neto2. The currents with Neto2 coexpression were scaled so that the peak currents were the same for each pair of results. ***d–g***, Bar graphs showing the mean results obtained for each subunit combination tested without (open bars) or with (red bars) Neto2 for the parameters obtained from fits to the desensitization decays: ***d***, time constant of the fast component of desensitization; ***e***, ***f***, time constant and relative amplitude of the slow component of desensitization (mean values from only those patches where a slow component was observed); ***g***, steady-state current as a percentage of the peak. The results we obtained before with GluK2 are also shown (gray) (Zhang et al., 2009). Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$; $n = 6$–9 for each group tested.

Heteromeric GluK2/5 receptors desensitized with kinetics similar to GluK2 homomers (Fig. 1*c*) (Zhang et al., 2009) and followed single-exponential kinetics. As for the other subunit combinations, Neto2 slowed the desensitization of GluK2/5 receptors (Fig. 1*c*) (weighted tau, 3.5 ± 0.2 vs 11.4 ± 1.2 ms). However, in contrast to results with GluK2 and Neto2, and the findings here with Neto2 and GluK1-containing receptors, some currents decayed monoexponentially (4 of 8 patches), and the slow component of desensitization was, when observed, not significantly accentuated by Neto2 coexpression (Fig. 1*e*,*f*). Steady-state currents for GluK2/5 receptors were

increased in the presence of Neto2, although the increase was not statistically significant (Fig. 1*c*,*g*).

Neto2 accelerates recovery from desensitization

The appearance of a large slow component in the desensitization decays of GluK2 currents was because of an effect of Neto2 to cause some GluK2 homomeric receptors to recover faster from desensitization (Zhang et al., 2009). Since coexpression of Neto2 also caused the appearance of a slow component in the desensitization decays of currents through GluK1, GluK1/5, and GluK2/5 receptors, and increased steady-state currents, we tested whether Neto2 also altered recovery from desensitization for these receptors.

Results obtained from two-pulse protocols to measure recovery from desensitization for GluK1, GluK1/5, and GluK2/5 receptors are shown in Figure 2. In the absence of Neto2, recovery followed a sigmoid time course (Bowie and Lange, 2002) and was slow for all three receptor types. Hodgkin–Huxley fits (Robert and Howe, 2003) to the mean results gave time constants for GluK1, GluK1/5, and GluK2/5 receptors of 2.9, 3.8, and 2.5 s, respectively. Coexpression of Neto2 dramatically increased recovery at short interpulse intervals, and recovery was evident for all receptor types at times when there was virtually no recovery without Neto2 (Fig. 2*a*–*c*). Hodgkin–Huxley fits to the data showed that Neto2 caused the appearance of a fast component of recovery (Fig. 2*d*–*f*), which had time constants of 50, 450, and 61 ms for GluK1, GluK1/5, and GluK2/5 receptors, respectively. For all three receptor types, the time course of the slow component of recovery was similar to that seen without Neto2 (Fig. 2*d*–*f*). The time constants of the slow component with Neto2 agreed within 5% with the corresponding values obtained without Neto2, results similar to findings for GluK2 homomeric receptors (Zhang et al., 2009).

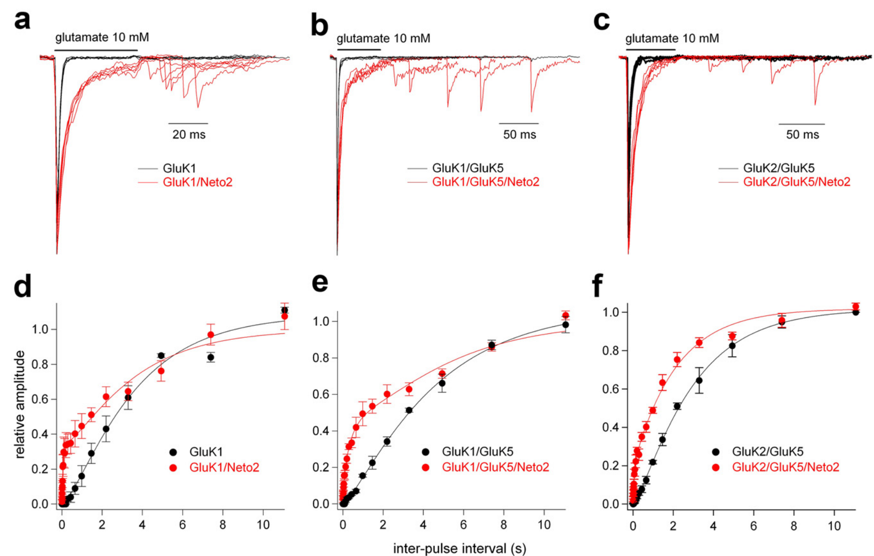

Figure 2. Neto2 accelerates recovery from desensitization. ***a*–*c***, Results from two-pulse experiments illustrating the early phase of recovery in outside–out patches from cells transfected with the indicated KAR subunits without (black) or with (red) Neto2. An initial 100 ms application of 10 mM glutamate was made and then repeated at various interpulse intervals. The envelop of the peak currents evoked by the second pulse gives the time course of recovery from desensitization. Note that substantial recovery is seen with Neto2 at interpulse intervals where there is virtually no recovery without Neto2 coexpression. The amplitude calibrations refer to the results without Neto2. The currents with Neto2 coexpression were scaled so that the peak currents were the same for each pair of results. ***d*–*f***, Mean results from 6–7 patches. The amplitude of the peak current evoked by the second application was expressed as a fraction of the peak current evoked by the first application with which it was paired. The results were fitted (solid lines) with equations consisting of one or two Hodgkin–Huxley components. Error bars indicate SEM.

The effect of Neto2 on KAR responses to brief pulses of glutamate

To test the effects of Neto2 on receptor responses to stimuli similar to those they are exposed to during synaptic transmission, we studied the effect of Neto2 on the response of the receptors to 1 ms pulses of 10 mM glutamate (we refer to the decay of these responses as deactivation).

Both with and without Neto2, deactivation decays for GluK1 receptors were in most cases adequately fitted by single exponential functions (Fig. 3*a*) (4 of 5 patches for GluK1 and 5 of 6 patches for GluK1 + Neto2). Coexpression of Neto2 slowed deactivation for GluK1 receptors (Fig. 3*a*,*d*), and weighted deactivation time constants were approximately doubled (weighted tau, 1.7 ± 0.4 ms vs 3.9 ± 0.7 ms). The deactivation of GluK1/5 receptors was fast and monoexponential in the absence of Neto2 (Fig. 3*b*,*d*,*f*). In the presence of Neto2, deactivation became clearly biexponential (Fig. 3*b*). The fast component of deactivation in the presence of Neto2 had a time constant close to the monoexponential decay in its absence (Fig. 3*d*); however, the second, additional component decayed much more slowly and accounted for 8.8 ± 1% of the overall amplitude (Fig. 2*e*,*f*).

Deactivation for GluK2/5 receptors was slightly slower than deactivation for GluK2 alone, and coexpression of Neto2 slowed deactivation of GluK2/5 receptors to a greater degree than for GluK2 homomers (approximately threefold) (Fig. 3*c*,*d*). However, the relative slowing of deactivation kinetics for GluK2/5 receptors was less profound than for GluK1/5 receptors, and a distinguishable slow component was observed only in one case.

Neto2 slows postsynaptic GluK1 currents

We next sought to determine whether, as for GluK2 (Zhang et al., 2009), Neto2 would modulate GluK1-containing receptors in a synaptic environment. Cerebellar granule cells express GluK1, GluK2, and GluK5 (Belcher and Howe, 1997; Ripellino et al., 1998). Whole-cell and unitary kainate receptor currents, but not EPSCs, can be recorded from these cells after reducing desensitization with concanavalin A (Pemberton et al., 1998; Smith et al., 1999), although the currents are small and difficult to isolate pharmacologically. Cerebellar granule cells from *stargazer* (stargazin-deficient) mice form functional glutamatergic synapses in culture but do not display AMPA receptor-mediated EPSCs (Hashimoto et al., 1999; Chen et al., 2000). We, therefore, used this system to examine the effect of Neto2 on synaptic KARs by over-expressing GluK1 in transfected neurons. NMDA and GABA receptors were blocked with AP-5 (100 μM) and picrotoxin (50 μM), respectively.

Cerebellar granule cells from *stargazer* mice that were transfected with GluK1 displayed small EPSCs (Fig. 4*a*) that were not observed after transfection with GFP alone. The EPSCs in GluK1-transfected neurons decayed twice as fast as AMPA receptor-mediated EPSCs in wild-type granule cells, or EPSCs in *stargazer* granule cells in which AMPA receptor transmission was rescued (Cho et al., 2007), and had time constants similar to those ob-

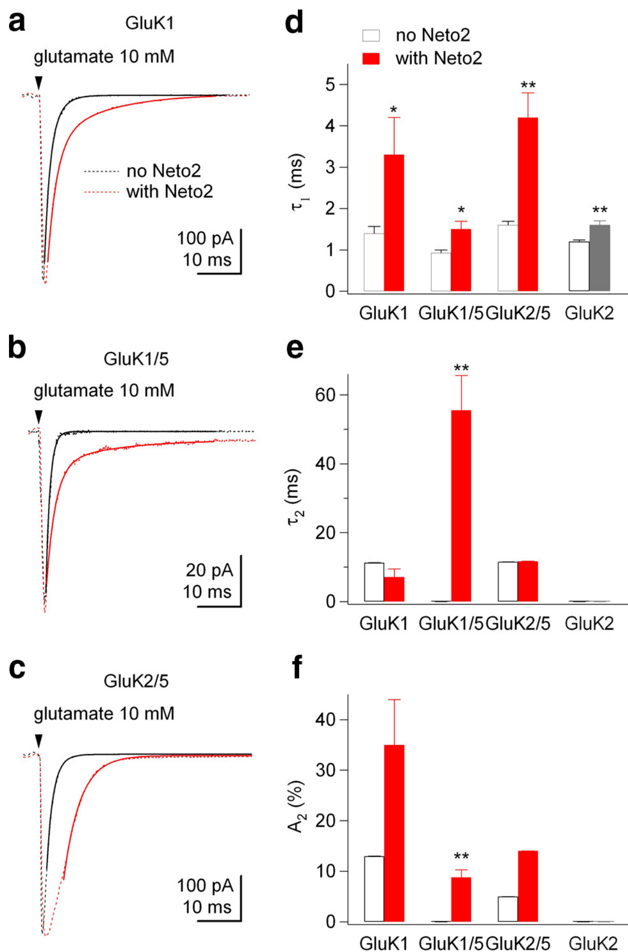

Figure 3. Neto2 modulates responses to brief pulses of glutamate. ***a–c***, Currents evoked in outside– out patches from cells that were transfected with the indicated KAR subunits either without (black) or with (red) Neto2. Glutamate (10 mM) was applied for 1 ms (arrowhead). The monoexponential or biexponential fits to the decays of the currents (solid lines) are superposed on the data (dotted lines). The amplitude calibrations refer to the results without Neto2. The currents with Neto2 coexpression were scaled so that the peak currents were the same for each pair of results. ***d–f***, Bar graphs showing the mean results obtained for each subunit combination tested without (open bars) or with (red bars) Neto2 for the parameters obtained from fits to the deactivation decays: ***d***, time constant of the fast component of deactivation; ***e***, ***f***, time constant and relative amplitude of the slow component of deactivation (mean values from only those patches where a slow component was observed). The results we obtained before with GluK2 are also shown (gray) (Zhang et al., 2009). Error bars indicate SEM. For GluK2/5 receptors, and GluK1 without Neto2, a slow component was only observed in one patch. $^{*}p < 0.05$, $^{**}p < 0.01$; $n = 5–7$ for each group tested.

served for GluK1/5 heteromeric receptors in deactivation protocols (compare Figs. 4*c*,*e* and 3*b*,*d*). Cotransfection of GluK1 and Neto2 resulted in a significant, nearly threefold, slowing of synaptic currents (Fig. 4*d*,*e*), confirming that Neto2 modulation of KAR gating remains intact for GluK1-containing receptors that are localized to synapses.

Discussion

The results reported here extend our previous work on modulation of GluK2 gating by Neto2 (Zhang et al., 2009) and show that Neto2 also modulates the gating of GluK1 receptors, as well as receptors containing GluK5 in combination with GluK1 and GluK2. While Neto2 modulation varies quantitatively with subunit composition, the effects of Neto2 are qualitatively similar for all receptors studied, and our results suggest that the coexpression of Neto2 likely modulates the gating behavior of most native kainate receptors in brain.

Neto2 modulation of desensitization

As was the case for GluK2 receptors, the primary effect of Neto2 on the KARs studied here appears to be on conformational changes associated with entry into and recovery from desensitization. Although Neto2 significantly slowed the decay of currents through GluK1 receptors evoked by a 1 ms pulse of glutamate, the similar deactivation and desensitization time constants for GluK1 receptors in the absence of Neto2 suggest that desensitization contributes substantially to the decay of GluK1 receptor currents, even when very brief pulses of glutamate are applied. The effects of Neto2 on deactivation kinetics were larger for receptors that contained GluK5 subunits. However, the deactivation and desensitization time constants were also similar for these receptors, both with and without Neto2. Presumably, inclusion of the high-affinity GluK5 subunit in receptor assemblies slows the rate at which glutamate dissociates (Barberis et al., 2008), and receptor gating is terminated not by glutamate unbinding but by desensitization, at least at saturating concentrations of glutamate.

The coexpression of Neto2 had a large effect on the early phase of recovery from desensitization for all receptors tested and resulted in the appearance of a fast component of recovery, as we found before for GluK2 receptors (Zhang et al., 2009). In our work on GluK2, we were able to transfect Neto2 at large excess and demonstrate that a 10:1 cDNA ratio (Neto2:GluK2) produced maximal effects on GluK2 gating, suggesting that the receptors with unaltered recovery kinetics were unlikely to represent receptors not associated with Neto2. Because GluK1 and GluK5 did not express as well as GluK2, we were unable to express Neto2 at greater than a 4:1 excess. It is, therefore, possible that the patches studied here contained a mixed population of receptors and that the receptors with unaltered recovery kinetics were receptors with incomplete inclusion of Neto2. Alternatively, Neto2 may promote gating behavior with distinct recovery kinetics, and individual receptors associated with Neto2 may switch between this behavior and the gating behavior seen in the absence of Neto2.

For GluK2, Neto2 also increased the amplitude of peak currents (Zhang et al., 2009). Whether this is true for the receptors studied here is unclear. Although we expect this is the case, confirmation of this expectation would require single-channel recordings in patches containing only a few receptors.

Neto2 modulation and subunit composition

Coexpression of GluK5 with and without Neto2 had different effects on the properties of receptors containing GluK1 and GluK2. GluK1/5 heteromeric receptors showed extremely rapid desensitization and deactivation, consistent with initial reports on GluK5 (Herb et al., 1992). The slower kinetics of GluK1/5 receptors when coexpressed with Neto2 was mostly attributable to the appearance of an additional slow component of decay that accounted for ~10% of the overall amplitude. In contrast, GluK5 only slightly slowed desensitization and deactivation for GluK2 receptors, although the effect of Neto2 on deactivation was greater for GluK2/5 heteromers. This finding is consistent with *in vivo* data for GluK2-dependent EPSCs at mossy fiber–CA3 synapses in the hippocampus (Mulle et al., 1998), which decay faster in GluK5-deficient mice (Contractor et al., 2003).

NETO2 modulates KAR-mediated synaptic currents

Synaptic transmission is generally thought to reflect a situation similar to a deactivation protocol (Clements et al., 1992). Interestingly, Neto2 enhanced the slow component of decay in deactivation protocols, especially for GluK1/GluK5 receptors, indicating that glutamate stays bound long after the removal of

glutamate. We, therefore, tested the effect of Neto2 on synaptic GluK1 currents directly, using cerebellar granule cells from *stargazer* mice. Overexpression of GluK1 resulted in small low-frequency EPSCs that decayed extremely rapidly, suggesting an association with endogenous GluK5. While coexpression of Neto2 slowed the decay of these EPSCs approximately threefold, we did not observe a second, very slow, component as we did in a deactivation protocol. However, it should be noted that the currents we recorded were very small (10–25 pA), and a slow component that was only 10% or so of the peak amplitude could easily be missed. It is also important to emphasize that the system used here is artificial, and other endogenous binding partners or morphological features that are not present in culture might influence physiological synaptic currents.

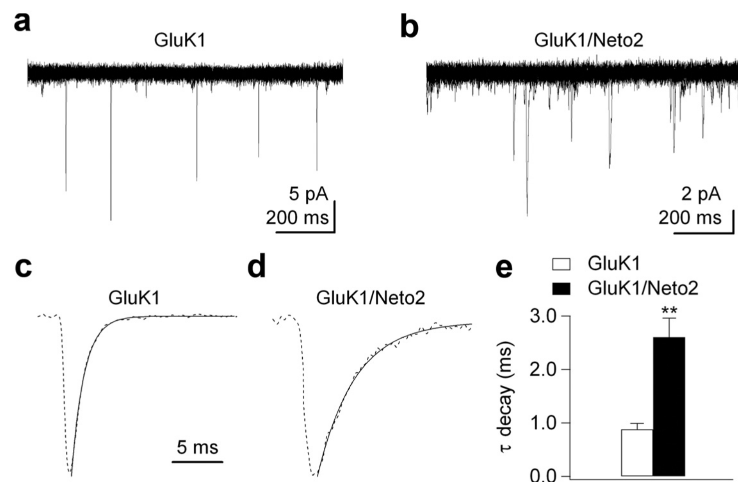

Figure 4. Neto2 slows postsynaptic GluK1 currents. Primary cultures of cerebellar granule cells from *stargazer* mice were transfected with GluK1 alone or GluK1 and Neto2. Expression of eGFP was used to identify transfected neurons. Neurons transfected with eGFP alone did not show synaptic activity, but spontaneous synaptic currents were consistently observed in neurons transfected with GluK1 (***a***) or GluK1 and Neto2 (***b***). The examples shown in ***a*** and ***b*** are five superposed segments of data and do not reflect the frequency of the synaptic events. The decays of the EPSCs were clearly slower in neurons cotransfected with Neto2. ***c***, ***d***, Examples of ensemble averages obtained from cells transfected with GluK1 (***c***) or GluK1 and Neto2 (***d***). ***e***, Bar graph showing mean results for the time constant obtained from monoexponential fits to the decays of ensemble EPSCs without (open bar) and with (filled bar) Neto2. Error bars indicate SEM. $^{**}p < 0.01$; $n = 4$ and 5.

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