Title: Neto proteins regulate gating of the kainate-type glutamate receptor GluK2 through two binding sites

Running Title: GluK2 gating by Netos

Authors: Yan-Jun Li¹#, Gui-Fang Duan¹#, Jia-Hui Sun¹#, Dan Wu¹, Chang Ye¹, Yan-Yu Zang¹, Gui-Quan Chen¹², Yong-Yun Shi³, Jun Wang⁴, Wei Zhang⁵*, and Yun Stone Shi¹²⁶*

¹State Key Laboratory of Pharmaceutical Biotechnology, Department of Neurology, Affiliated Drum Tower Hospital of Nanjing University Medical School, and Minister of Education Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, Nanjing 210032, China
²Institute for Brain Sciences, Nanjing University, Nanjing 210032, China
³Department of Orthopaedics, Luhe People's Hospital Affiliated to Yangzhou University, Nanjing 211500, China
⁴Minister of Education Key Laboratory of Modern Toxicology, Department of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China
⁵Institute of Chinese Integrative Medicine, Hebei Medical University, Shijiazhuang 050017, China
⁶Chemistry and Biomedicine Innovation Center, Nanjing University, Nanjing 210032, China

#These authors contributed equally to this work.
¹To whom correspondence may be addressed. Email: yunshi@nju.edu.cn and weizhang@hebm.edu.cn

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ABSTRACT

The neuropilin and tolloid-like (Neto) proteins Neto1 and Neto2 are auxiliary subunits of kainate-type glutamate receptors (KARs) that regulate KAR trafficking and gating. However, how Netos bind and regulate the biophysical functions of KARs remains unclear. Here, we found that the N-terminal domain (NTD) of glutamate receptor ionotropic kainate 2 (GluK2) binds complement C1r/C1s-Uegf-BMP (CUB1) domains of Neto proteins (i.e. NTD–CUB1 interaction), and that the core of GluK2 (GluK2\(\Delta\)NTD) binds Netos through domains other than CUB1s (core–Neto interaction). Using electrophysiological analysis in HEK293T cells, we examined the effects of these interactions on GluK2 gating, including deactivation, desensitization, and recovery from desensitization. We found that NTD deletion does not affect GluK2 fast gating kinetics, the desensitization and the deactivation. We also observed that Neto1 and Neto2 differentially regulate GluK2 fast gating kinetics which largely rely on the NTD–CUB1 interactions. NTD removal facilitated GluK2 recovery from desensitization, indicating that the NTD stabilizes the GluK2 desensitization state. Co-expression with Neto1 or Neto2 also accelerated GluK2 recovery from desensitization, which fully relied on the NTD–CUB1 interactions. Moreover, we demonstrate that the NTD–CUB1 interaction involves electric attraction between positively charged residues in the GluK2_NTD and negatively charged ones in the CUB1 domains. Neutralization of these charges eliminated the regulatory effects of the NTD–CUB1 interaction on GluK2 gating. We conclude that KARs bind Netos through at least two sites and that the NTD–CUB1 interaction critically regulates Neto-mediated GluK2 gating.

INTRODUCTION

In the central nerve system, excitatory synaptic transmission is primarily mediated by glutamate. Glutamate released from presynaptic terminals excites three types of ionotropic glutamate receptors, which are pharmacologically classified as AMPA (amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors (AMPArs), NMDA (N-methyl-D-aspartic acid) receptors (NMDARs), and kainate receptors (KARs) (1). AMPARs mediate the majority of fast transmission while NMDARs are responsible for synaptic plasticity. KARs are expressed in subsets of neuronal types in the brain. They not only contribute to EPSCs on postsynaptic cell, but also regulate neurotransmitter release on the presynaptic terminal (2). Additionally, KAR activity is involved in synaptic plasticity (3). Dysfunction of KARs causes neurologic diseases such as epilepsy, schizophrenia, and autism (2,4).

Ionotropic glutamate receptors are tetrmeric. Each subunit contains a large N-terminal domain (NTD), accounting for about 40% of the full length, followed by a ligand binding domain (LBD), and a transmembrane domain (TMD) forming the ion channel pore, then an intracellular C-terminal tail associating
with scaffold proteins. Among these domains, the NTD is the largest, but its function, until recently, has been poorly understood. The NTDs of NMDARs are important for their gating and regulation by allosteric modulators including $\text{Zn}^{2+}$, $\text{H}^+$, ifenprodil etc (1,5,6). The NTDs of AMPARs play little role in receptor fast gating but instead contribute to the stabilization of desensitization (7). Additionally, NTD truncated AMPAR subunits display increased mobility on synapse and lose their ability to sustain long term potentiation (8,9). Similar to AMPARs, removal of GluK2_NTD does not change the rates of deactivation and desensitization in heterologous systems (10), whereas NTDs of KARs are crucial for synaptic localization (11,12).

Beside the pore-forming subunits, native KARs and AMPARs associate with auxiliary proteins (13). Neto proteins bind KARs and regulate KAR deactivation, desensitization, rectification, synaptic trafficking similar to the effects of TARPs on AMPARs (14-16). Furthermore, Netos play critical roles in determining the axonal distribution of KARs in neurons (17,18). Netos are also proposed to play a role in neural circuit development (19-21) and be required for normal fear expression (22,23). Netos are single-pass transmembrane proteins with a long extracellular N-terminal sequence containing two Cir/C1s-Uegf-BMP (CUB) domains and a low density lipoprotein class A (LDLa) domain, and a short intracellular C-terminal tail (24). Neto2 mutations in LDLa eliminate its effects on desensitization (25), and mutations in the intracellular C-terminal tail prevent the effects of Neto1/2 on rectification (26). Biochemical studies indicate that the CUB2 domain is critical for Neto protein binding to GluK2 (27). Nevertheless, the exact interaction between Netos and KARs remains elusive.

We previously reported that synaptic targeting of GluK1 in hippocampal CA1 neurons relies on Neto proteins while that of GluK2 does not (11,28). The differential trafficking properties and dependence on Netos between GluK1 and GluK2 rely on their NTDs (11,29). We thus suspect that the NTDs of KARs might directly bind Neto proteins. Here we find that GluK2NTD specifically binds the CUB1 domain of Neto proteins. In addition, GluK2 without NTD (GluK2_core) can still bind to Neto proteins with or without CUB1 domain. The effects of NTD-CUB1 and core-Neto interactions on GluK2 gating, including desensitization, deactivation and recovery from desensitization, are systematically studied. Our data suggest a two-step model for Neto regulation of KAR gating and emphasize an important regulatory role for the NTD-CUB1 interaction.

**RESULTS**

**Netos interact with GluK2 through multiple sites**

Previously we found the synaptic targeting property of GluK1 and GluK2 is differentially regulated by Netos in an NTD-dependent manner (11,28,29), indicating that NTDs of KARs might directly interact with Netos. To test this hypothesis, we expressed the NTD of GluK2 by introducing a stop codon at
position 401, thus the NTD (residues 31-400) will be synthesized through the secretory ER pathway under the guidance of the signal peptide. To facilitate its detection, an HA tag was inserted after the signal peptide and a FLAG epitope was inserted at the C-termini of Netos. Consistent with our prediction, GluK2NTD was co-immunoprecipitated with Neto proteins (Fig. 1, A and B). We also observed that GluK2ΔNTD, in which the NTD was deleted, co-immunoprecipitated with Netos (Fig. 1, A and B), indicating that GluK2 interacts with Netos through multiple sites. In Netos, two CUB domains and an LDLα domain are located extracellularly. Which of these domains might interact with GluK2NTD? Since the NTD is the distal extracellular domain of KARs and is about 80 Å above the membrane plane (30), it is reasonable to suspect the very distal CUB1 domain of Netos might interact with GluK2NTD. Indeed, GluK2NTD was efficiently co-immunoprecipitated with Neto1CUB1 or Neto2CUB1 (Fig. 1C). Meanwhile, GluK2ΔNTD was pulled-down by Neto1/2ΔCUB1 (Fig. 1D), indicating a second interaction which we named as core-Neto interaction. Furthermore, deletion of CUB1 domains largely diminished Neto interaction with GluK2NTD (Fig. 1, E-G), suggesting that GluK2NTD specifically interacts with the CUB1 domains of Neto proteins. Thus, our observation suggested that GluK2 bind Netos through at least two interaction sites, the NTD-CUB1 interaction and core-Neto interaction (Fig. 1H).

**NTD truncation does not affect GluK2 desensitization and deactivation**

Previous work demonstrated that NTD truncated GluK2 receptors are functional (10). We thus used GluK2ΔNTD to study Neto related gating (Fig. S1A). Western blot analysis from cell lysate showed similar expression level of GluK2ΔNTD and intact GluK2 receptors (Fig. S1B). Surface biotinylation revealed that the surface expression of GluK2 receptors was unaffected by NTD truncation (Fig. S1B). Furthermore, immunofluorescence experiments examining an HA tag inserted at N-termini of full-length and NTD-deleted GluK2 showed that GluK2ΔNTD could express and traffic to plasma membrane like full-length GluK2 receptors (Fig. S1C). Together, these observations suggest that the NTD is not required for GluK2 expression and membrane trafficking.

We then tested the effects of NTD truncation on gating kinetics by applying saturating concentration of glutamate (10 mM) to outside-out patches excised from the transfected HEK cells using a fast piezoelectric system (25). The desensitization kinetics recorded by 500 ms application of glutamate displayed no difference between full-length and NTD-deleted GluK2 receptors (Fig. 2A and Fig. S2A). Similarly, the deactivation kinetics recorded by brief application (1 ms) of 10 mM glutamate was unchanged by NTD truncation (Fig. 3A and Fig. S2B). These observations are consistent with previous reports (10), suggesting that the NTD itself has little effect on the deactivation and desensitization kinetics of KARs.
NTD-CUB1 interactions distinguish Neto1 and Neto2 on desensitization

Previous works show that Neto1 speeds and Neto2 slows GluK1 desensitization in recombinant system or in neurons (28,31). Similarly Neto1 speeds and Neto2 slows GluK2 desensitization when overexpressed in hippocampal CA1 neurons (11). Here we found in HEK cells that Neto2 dramatically slows the desensitization of GluK2 by ~4 times while Neto1 has little effects (Fig. 2B and Fig. 2A), indicating that Neto1 and Neto2 differentially regulate GluK2 desensitization. Interestingly, in the absence of NTD, GluK2ΔNTD was slightly slowed by both Neto1 and Neto2 (Fig. 2C) to a similar extent. When CUB1 domains of Netos were removed, GluK2 desensitization was not changed by Neto1ΔCUB1 or Neto2ΔCUB1 (Fig. 2D). Furthermore, GluK2ΔNTD desensitization was similarly slowed by Neto1ΔCUB1 and Neto2ΔCUB1 (Fig. 2E). These results thus demonstrated that the differential regulation of GluK2 desensitization by Neto1 and Neto2 relies on the NTD-CUB1 interaction. In addition, in the cases of ΔNTD (Fig. 2C), ΔCUB1 (Fig. 2D) and ΔNTD+ΔCUB1 (Fig. 2E), conditions in which NTD-CUB1 interactions were disrupted, KAR desensitization was generally slowed by Netos, indicating that the core-Neto interactions slow desensitization. Comparing the Neto effects on desensitization in Figure 2B and those in Figure 2C-E, leads to the conclusion that NTD-Neto1CUB1 speeds KAR desensitization and NTD-Neto2CUB1 slows it.

NTD-CUB1 interactions distinguish Neto1 and Neto2 on deactivation

We further studied the Neto regulatory effects on GluK2 deactivation. Neto2 but not Neto1 slowed GluK2 deactivation (Fig. 3A and Fig. S2B). While the NTD was removed, GluK2ΔNTD was moderately slowed by Neto1 and Neto2 to similar extent (Fig. 3C). When CUB1 domains were deleted, GluK2 deactivation was slightly slowed by Neto1ΔCUB1 and not by Neto2ΔCUB1 (Fig. 3D), but the effects of Neto1ΔCUB1 and Neto2ΔCUB1 were not significantly different. Furthermore, GluK2ΔNTD deactivation was slowed by Neto1ΔCUB1 and Neto2ΔCUB1 to similar extent (Fig. 3E). The results thus revealed that the different effects of Neto1 and Neto2 on GluK2 deactivation rely on the NTD-CUB1 interaction. Also like the desensitization data, it can be concluded that the core-Neto interactions slow deactivation in general.

Taken together, these data revealed that the NTD-CUB1 interaction is critical for Neto1/2 modulation of GluK2 fast gating kinetics.

NTD deletion facilitates GluK2 recovery from desensitization

Deletion of NTDs of AMPA receptors facilitates their recovery from desensitization (7). We wondered whether this is the case for KARs. The recovery rate of full-length and NTD-deleted GluK2 receptors was monitored through a pair of glutamate (10 mM for 50 ms) applications with variable intervals. GluK2 receptors completely recovered from desensitization in
seconds with \( \tau_{\text{rec}} \) value of 2.62 ± 0.54 s, similar to previous reports (25,32). NTD truncation dramatically sped up this process by about 3 times (\( \tau_{\text{rec}} = 0.91 ± 0.23 \) s) (Fig. 4A, 4B and Fig. S3). These data thus suggest that the NTDs of KARs strongly inhibit the recovery from desensitization.

**Netos speeds the recovery rate of GluK2 but not GluK2ΔNTD**

When GluK2 was coexpressed with Neto1 or Neto2, the recovery from desensitization were facilitated (Fig. 4C), consistent with previous reports (25,31-33) that Netos speed KAR recovery. Thus, removal of NTD and coexpression with Netos had similar effects on GluK2 recovery from desensitization. Could these two manipulations have synergistic effects on the recovery rate? Very surprisingly, we found the recovery rate of GluK2ΔNTD was notably slowed by Neto2 with \( \tau_{\text{rec}} \) doubled but not by Neto1 (Fig. 4D). Neto2ΔCUB1 also slowed the recovery of GluK2 (Fig. 4E) and GluK2ΔNTD (Fig. 4F) by doubling the \( \tau_{\text{rec}} \) while Neto1ΔCUB1 had no effects (Fig. 4, E and F). Thus, data in Figure 4D-F indicated that core-Neto1 and core-Neto2 differentially regulate GluK2 recovery; core-Neto2 slows recovery while core-Neto1 has no effects. Furthermore, the left shifting of the recovery curve by Netos in Figure 4C compared to the generally right shifting in the absence of NTD-CUB1 interaction (Fig. 4, D-F), indicated NTD-CUB1 interaction speeds KAR recovery from desensitization.

**The residues in Netos responsible for NTD-CUB1 interactions**

Thus far we have found that Neto proteins interact with GluK2 through at least two sites, the NTD and the core. NTD-CUB1 interaction plays important role in regulating GluK2 gating. We thus wondered what exact sites are responsible for this interaction. We first made a model of Neto2CUB1 domain by Homology Modeling using Deepview software. The spindle-shaped CUB1 domain was polarized according to its charge distribution (Fig. 5A). On one end of the molecule, positively charged arginine residues including Arg50, Arg81, Arg83, Arg131 and Arg135 are clustered to make the positive charge pole. On the other end, negatively charged Asp144, Glu145, Glu146 and Glu148 composed of the negative charge pole. We then examined whether these charges play a role in the interaction with GluK2NTD by mutating them to alanine residues. When the 4 negatively charged residues were mutated (DE4A), the Neto2CUB1 domain failed to pull down GluK2NTD (Fig. 5B, arrow), while mutation of the 5 positively charged residues (R5A) did not affect the pull-down efficiency. There are 3 negatively charged residues in the corresponding region in the Neto1CUB1 domain (Fig. 5A, right panel). Mutation on these negatively charged residues in the Neto1CUB1 domain (DE3A) also largely diminished the interaction with GluK2NTD (Fig. 5C, arrow). We further examined whether these negatively charged residues were responsible for the function of the NTD-CUB1 interaction. When the 4 negatively charged residues in Neto2 were mutated to alanines, the slowing of GluK2 desensitization by Neto2 was largely impaired (Fig. 5D and
The residues in GluK2NTD responsible for NTD-CUB1 interactions

Since the negatively charged pole on the CUB1 domains are responsible for the interaction with GluK2NTD, we suspect that a positively charged patch on the GluK2NTD surface might interact with CUB1s. We then searched the surface of GluK2NTD for highly positively charged regions. The GluK2NTD dimer was adapted from the full length GluK2 Cryo-EM structure (PDB ID: 5kuf). We identified 6 positively charged clusters which contain at least 2 positively charged residues (in blue, Fig. 6A) from one subunit of the NTD dimer. The positively charged regions were neutralized by mutating lysine and arginine residues to alanine residues (Fig. 6A). Other positively charged residues scattered on the NTD surface were not touched (in pink, Fig. 6A). Pull-down experiments showed that when group 1 positively charged residues in GluK2NTD were mutated, the interaction with Neto2CUB1 was largely diminished, while mutations on groups 2-6 did not affect NTD interaction with Neto2CUB1 (Fig. 6B). The group 1 residues contain Arg50, Lys82, Lys93 and Lys94. Neither single mutation on these residues nor double mutations on Arg50 and Lys82 affected the pull-down efficiency (Fig. 6C). These data suggest the positively charged residues in group 1 are redundant. We then examined the functional effects of mutating these positively charged residues. GluK2(RK4A), in which 4 positively charged residues (Arg58, Lys82, Lys93 and Lys94) were mutated to alanines, was modestly slowed by Neto1 and Neto2 (Fig. 6D), resembling that of NTD deletion (Fig. 2D). Netos failed to facilitate the recovery of GluK2(RK4A) from desensitization (Fig. 6E), indicating the NTD-CUB1 interaction was impaired with these mutations, resembling that of GluK2ΔNTD (Fig. 4D).

Differential regulation of desensitization does not simply rely on CUB1s

Neto1 and Neto2 have significant differential effects on GluK2 fast gating especially on the desensitization kinetics. Our deletion and mutation experiments indicate that the CUB1 domains might account for the difference. To test this hypothesis, we made chimeric constructs of Netos by swapping the CUB1 domains (Fig. S4A). Indeed, Neto1(Neto2CUB1), Neto1 harboring Neto2CUB1 slowed GluK2 desensitization in comparison to Neto1, but the slowing effect was much less than Neto2 (Fig. S4B and C). On the other hand, Neto2(Neto1CUB1) slowed
GluK2 desensitization just like Neto2 (Fig. S4B and C). These data thus indicate that the difference in CUB1 sequences, at most, only partially accounts for the differential regulatory effects on GluK2 fast gating by Netos.

**NTD-CUB1 interactions do not affect rectification**

Another biophysical property of KARs affected by Netos is voltage-dependent blockage by polyamines (26,34). We examined the rectification property of GluK2 receptors after NTD truncation in the presence of ConA to prevent desensitization (35). NTD deletion enhanced rectification (Supporting Fig. S5, A and B), reducing the rectification index (Fig. S5C). Both Neto1 and Neto2 markedly reduced the inward rectification of full-length GluK2 receptor (Fig. S5, A and C) and GluK2ΔNTD (Fig. S5, B and C). Neto1 effects were relatively stronger than Neto2. These data thus suggested the GluK2 NTD is not involved in Neto modulation of receptor rectification.

**DISCUSSION**

In the present study, we have experimentally defined two interactions between GluK2 receptor and auxiliary Neto proteins. The GluK2NTD directly interacts with Neto proteins through binding to CUB1 domains, defining the NTD-CUB1 interactions. The core of GluK2 interacts with Neto domains other than CUB1, defining a second core-Neto interaction. The NTD-CUB1 interaction involves the static electric attraction between a negatively charged cluster in CUB1 domains and a positively charged patch on the surface of GluK2NTD.

By coexpression of NTD truncated GluK2 and CUB1 truncated Netos, we have systemically examined 1. the NTD alone, 2. the core-Neto interaction, 3. the NTD-CUB1 interaction on KAR gating, including desensitization, deactivation and recovery from desensitization. The three factors have different effects on GluK2 fast gating (deactivation and desensitization) and slow gating (recovery from desensitization). To facilitate the understanding of our data, we made schematic models (Fig. 7) and started from the smallest functional receptor GluK2ΔNTD. For fast gating such as desensitization, comparison 1 in Figure 7A suggests that NTD alone has no effects on GluK2 desensitization. Comparison 2 in Figure 7A suggests core-Neto interactions slow KAR desensitization. Comparison 3 in Figure 7A suggests NTD-Neto1CUB1 interaction speeds desensitization while NTD-Neto2CUB1 slows desensitization. For recovery from desensitization, comparison 1 in Figure 7B suggests NTD has strong inhibitory effects on GluK2 recovery. Comparison 2 and 2’ in Figure 7B suggest core-Neto1 has no effects on KAR recovery from desensitization while core-Neto2 slows the recovery. Comparison 3 in Figure 7B suggest NTD-CUB1 interactions facilitate GluK2 recovery.

Following the discovery of Netos as KAR auxiliary subunits, numerous studies have explored Neto regulation on KAR kinetics, mostly through recombinant systems or through overexpression in neurons. A general
conclusion is that the Neto regulation of KARs is receptor subunit specific and Neto-isoform specific. Neto1 speeds GluK1 desensitization in recombinant systems as well as overexpression in hippocampal CA1 neurons (28,31). Neto1 speeds GluK2 desensitization in hippocampal CA1 neurons (11) but not in HEK cells (this study). Neto1 has relatively small or no effects on GluK1 or GluK2 deactivation in neurons (11,28). In contrast, Neto2 significantly slows the desensitization and deactivation of GluK1 and GluK2 in either recombinant systems or neurons (11,25,28,36). We find that deleting the NTD has no effect on GluK2 decay kinetics. Interestingly, the core-Neto interactions by Neto1 and Neto2 similarly slow the desensitization and deactivation of GluK2 under 5 conditions when NTD-CUB1 interactions are disrupted: deletion of the NTD, deletion of the CUB1, deletion of both, mutation on the CUB1 negative charges, or mutation on GluK2NTD positive charges (Fig. 2C-E, 3C-E, 5D and 6D). Only when full-length wt Neto1/2 are co-expressed with GluK2, are the desensitization and deactivation dramatically different between Neto1 and Neto2 (Fig. 2B and 3B). Therefore, the differential modulatory effects of Neto1 and Neto2 on GluK2 desensitization and deactivation rely on the CUB1 interaction with the NTD of KARs. However, the sequence difference in CUB1 domains can't explain the differential regulatory effects of Neto1 and Neto2 on GluK2 fast gating kinetics. By switching the CUB1 domains, the desensitization kinetics are not switched (Fig. S4). Fisher (32) found switching both CUB1 and CUB2 domain between Neto1/2 can largely (yet incompletely) switch the differential gating properties on GluK1. Therefore, the Neto isoform specific modulation on KAR fast gating might require a more sophisticated stereoscopic interaction between NTD and CUB1. In addition, a more complicated allostERIC modulation between NTD-CUB1 and core-Neto interactions might exist. To fully understand the differential modulatory effects by Neto1 and Neto2 require the structural picture of GluK/Neto complex in future.

One interesting property of the NTDs of glutamate receptors is their effect on the recovery from desensitization. NMDARs have little desensitization, while non-NMDARs, AMPARs and KARs, desensitize soon after activation and recover in variable time intervals (37). GluK2 fully recovers from desensitization in seconds, while AMPARs recover in hundreds of milliseconds (25,38,39). During desensitization, the association of LBD dimers ruptures, and rearranges into quasi-fourfold architecture (6,37,40). Additionally, NTD dimers of GluA2 receptors appear to separate, whereas the NTD dimers of GluK2 receptors remain undisrupted. This may explain why GluK2 is much more stable in the desensitized state compared to GluA2 receptors (40-42). NTD truncated GluK2 receptors desensitize at the same rate as intact receptors (Fig. 2A), but recover three times faster (Fig. 4B). These results are consistent with the structural observations, suggesting that NTDs stabilize the desensitized state of AMPARs (7) or KARs.
Netos were reported to facilitate the recovery from desensitization of variable homomeric and heteromeric KARs (14, 25, 31-33, 36). We also observed the facilitation of GluK2 recovery by Netos which completely rely on the NTD-CUB1 interactions. Under all conditions that NTD-CUB1 interactions are disrupted, including deletion of the NTD and/or CUB1, and mutations on the interface, the core-Neto interactions generally stabilizes the desensitized states and slows the recovery. For this effect, Neto2 is much stronger than Neto1.

A large amount of studies suggest that KARs interact with Netos at multiple sites. From the Neto side, CUB domains, LDLα, TMD and CTD are involved (25, 26, 32). However, which domains of KARs that are involved in the interaction remain largely unclear. A previous study suggested that the linker between M3 and S2 is important for Neto-related gating (43). Our biochemical results demonstrate that both the isolated NTD and the GluK2ΔNTD are able to bind Netos, consistent with the notion that KARs bind Netos through multiple sites. Functionally, the NTD-CUB1 interactions and core-Neto interactions have differential effects on GluK2 deactivation, desensitization and recovery from desensitization, suggesting a two-step model for Neto regulation of KAR gating (Fig. 7).

**MATERIALS AND METHODS**

**Molecular Biology**

GluK2 (Q form) and Neto2 from rat and Neto1 from mouse were used in this study. To ensure the co-expression of Neto proteins and GluK2 receptor in the same HEK293T cells, the cDNA of GluK2 was subcloned into vector pCAGGS-IRES-EGFP, while Neto1 and Neto2 were subcloned into vector pCAGGS-IRES-mCherry(28). Mutations in GluK2 and Netos were made by overlapping PCR. Specifically, GluK2ΔNTD was made by deletion of Thr32-His400 of GluK2 without disruption of the signal peptide (residues 1-31). Additionally, we subcloned the NTD together with the signal peptide into vector pCAGGS-IRES-EGFP to construct isolated NTD. For pull-down experiments, an HA tag was inserted into GluK2 constructs after the sequence of signal peptide, and a FLAG tag was inserted into C-termini of Neto1 and Neto2 constructs. All the constructs were confirmed using sequencing over the entire length of the coding region.

**Western Blots**

HEK293T cells were cultured using DMEM with 10% FBS, and passaged every two days. In western blot experiment, HEK293T cell were transiently transfected using Lipofectamine 2000 Reagent (Invitrogen) following manufacturer’s instructions. The vectors used in transfection was GluK2(mutation) : Neto(mutation) = 1:1 for biochemical experiments. 4 h later, medium was changed and 100 μM DNQX (Abcam) was added to block GluK2 receptor currents. Cells were lysed in RIPA buffer 48 h later. The cell lysates were kept on the ice for 30 min, and then centrifuged at 13,800 x g, 4°C for 30 min. After centrifugation, the supernatant was transferred to a new
tube and completely mixed with 4 X loading buffer. Then the mix was immediately loaded into 8% SDS-PAGE gels in the presence of DTT. The protein bands were transferred to PVDF membranes (Millipore) at 100 V for 2 h, and then blocked in 5% non-fat milk dissolved in TBST at room temperature for 1 h. Finally, the level of GluK2 receptor and Neto proteins were probed with anti-HA antibody (Sigma, H3663), anti-FLAG antibody (Sigma, F3165) or anti-C-terminal GluR6/7 (Merk Millipore, 04-921) respectively, and detected using the ECL substrate (Thermo) before exposure.

**Cell-Surface Biotinylation**

Cells were washed three times with ice-cold PBS before 1 mM solution of Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific Life Sciences, catalog no. 21335) in PBS for biotinylating cell surface proteins. After incubating at 4°C for 30 min, reactions were quenched with 50 mM glycine, followed by rinsing three times with ice-cold TBS. Cells were then scraped in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium phosphate, 2 mM EDTA, and 0.2% sodium vanadate) supplemented with a mixture of protease inhibitors (Roche Applied Science) and solubilized for 1 h at 4°C. Nonsolubilized particles was removed by centrifugation at 13,800 x g for 10 min at 4°C. The solubilized protein concentration was determined by BCA assay and mixed with monomeric avidin agarose beads (Thermo Fisher Scientific Life Sciences, catalog no. 20228). The mixture was incubated for 1 h with rotation at room temperature. Beads were subsequently washed three times with PBS. Finally, proteins were eluted by boiling in Laemmli buffer and then separated by electrophoresis on 8% SDS-PAGE gels.

**Immunocytochemistry and Confocal Microscopy**

Cell surface receptors were detected by non-permeabilized immunocytochemistry. HEK293T cells were washed in PBS and fixed in 4% PFA in PBS. After blocking in normal goat serum, cell-surface GluK2 or GluK2ΔNTD staining was examined using mouse anti-HA antibody (Sigma, H3663), followed by goat anti-mouse Alexa 549 secondary antibody. Samples were then permeabilized with 0.1% Triton X-100, and total GluK2 content was determined by staining with rabbit anti-C-terminal GluR6/7 (Merk Millipore, 04-921) and goat anti-rabbit Alexa 488. After the secondary antibody was washed by PBS for three times, the cells were additionally incubated with Hoechst 33258 for nuclear staining. Samples were examined and analyzed through a 63 X oil immersion lens on a Zeiss LSM880 microscope.

**Immunoprecipitation**

Transfected cells were washed three times with PBS, and harvested and solubilized in Lysis buffer (50 mM Tris-Cl, PH 7.2, 150 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100), supplemented with a mixture of protease inhibitors (Roche Applied Science) and solubilized for 1h at 4°C. After centrifuged at 13,800 x g for 10 min, the pellet was discarded. Lysates...
were then incubated with antibodies at 4°C overnight. Then lysates were incubated with Protein G beads (GE Healthcare) for 2 h at 4°C on a rotating platform. After incubation, beads were washed 4 times with Lysis buffer and boiled in 40 ul 2 X Laemmli buffer. The mixtures were then centrifuged at 13,800 x g and the supernatant was used for detection by western blot. For all samples, 1% of that used for IPs was used for input in gel analysis.

Electrophysiology

**Whole Cell Electrophysiology Recording** Whole cell recording was performed on transfected HEK293T cells as described previously (44). The vectors used in transfection for electrophysiology recording was GluK2(mutation): Neto(mutation) = 1:2 to ensure that Netos were expressed with sufficient amount compared to GluK2. The cells were bathed in the extracellular solution (in mM): 145 NaCl, 2.5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). The positively transfected cells were identified by fluorescence via epifluorescence microscopy. Whole cell patches were performed with glass pipettes (3 to 5 MΩ) filled with intracellular solution (in mM): 140 CsCl, 4 MgCl₂, 1 EGTA, and 10 HEPES (pH 7.4). The current data were analyzed using Clampfit software.

**Outside-Out Patch Recording** The outside-out patches was pulled from transfected HEK293T cells and recorded as previously reported (25). The external solution was (in mM): 140 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 5 glucose, and 10 HEPES (pH 7.4). Patch pipettes (resistance 3 to 5 MΩ) were filled with a solution containing (in mM): 130 KF, 33 KOH, 2 MgCl₂, 1 CaCl₂, 11 EGTA, and 10 HEPES (pH 7.4). 10 mM glutamate diluted into the external solution was applied with theta glass pipettes mounted on a piezoelectric bimorph. The deactivation and desensitization were recorded by 1 ms and 500 ms glutamate application respectively, and analyzed by fitting with a single exponential function \(A = A_0 \exp(-t/\tau) + C\) or a double exponential function \(A = A_0 (f_1 \exp(-t/\tau_1) + f_2 \exp(-t/\tau_2)) + C\). In these functions \(t\) is the time. The currents amplitude \(A\) starts at \(A_0\) and decays down to \(C\). In our recording, the steady state currents \(C\) was generally undetectable. \(f_1\) and \(f_2\) are the fractions of respective components as percent \((f_1 + f_2 = 1)\), and \(\tau_1\) and \(\tau_2\) are decay kinetics of fast and slow components. The weighted \(\tau\) was calculated using the formula: weighted \(\tau = f_1 \tau_1 + f_2 \tau_2\). The recovery from desensitization was examined by pairs of 50 ms applications of 10 mM glutamate, with intervals ranged from 5 ms to 8000 ms. The recovery ratio was calculated...
via dividing the second peak amplitude by the first peak and analyzed by fitting with a single exponential function: 
\[ f = (f_{\text{max}} - C) \exp\left(-\frac{t}{\tau_{\text{rec}}} \right) + C, \]
where \( t \) is the time; \( f_{\text{max}} \) is the maximal recovery; \( C \) is non-desensitized steady state fraction at the end of 50 ms glutamate application; \( \tau_{\text{rec}} \) is the recovery constant.

Homology Modeling

A 3D model of Neto2CUB1 was made by Homology Modeling using Deepview software. The amino acid sequence of Neto2CUB1 was loaded into the workspace and BLAST against ExPDB database for searching appropriate templates. Cubilin (PDB ID: 3kq4) was chosen as an optimal template because of the high coverage rate (98%) and sequence identity (39%). Homologous sequence in Cubilin was residues 234-346. The model was computed and built by the SWISS-MODEL server. This model and GluK2 NTD dimer structure (adapted from PDB ID: 5kuf) were viewed and depicted using Pymol software.

Statistical Analysis

Data were presented as mean ± SD from three or more independent experiments. Statistical analyses were carried out using GraphPad Prism 7 software and analyzed using one-way ANOVA test, two-way ANOVA or unpaired t-test if not otherwise stated. All p < 0.05 was considered significant and labeled as *, p < 0.01 was labeled as **, and p < 0.001 was labeled as ***.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J., and Dingledine, R. (2010) Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* **62**, 405-496

2. Lerma, J., and Marques, J. M. (2013) Kainate receptors in health and disease. *Neuron* **80**, 292-311

3. Schmitz, D., Mellor, J., and Nicoll, R. A. (2001) Presynaptic kainate receptor mediation of frequency facilitation at hippocampal mossy fiber synapses. *Science* **291**, 1972-1976

4. Volk, L., Chiu, S. L., Sharma, K., and Huganir, R. L. (2015) Glutamate synapses in human
cognitive disorders. *Annu Rev Neurosci* **38**, 127-149

5. Zhu, S., and Paoletti, P. (2015) Allosteric modulators of NMDA receptors: multiple sites and mechanisms. *Curr Opin Pharmacol* **20**, 14-23

6. Karakas, E., Regan, M. C., and Furukawa, H. (2015) Emerging structural insights into the function of ionotropic glutamate receptors. *Trends in biochemical sciences* **40**, 328-337

7. Moykkynen, T., Coleman, S. K., Semenov, A., and Keinanen, K. (2014) The N-terminal domain modulates alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor desensitization. *J Biol Chem* **289**, 13197-13205

8. Watson, J. F., Ho, H., and Greger, I. H. (2017) Synaptic transmission and plasticity require AMPA receptor anchoring via its N-terminal domain. *eLife* **6**

9. Diaz-Alonso, J., Sun, Y. J., Granger, A. J., Levy, J. M., Blankenship, S. M., and Nicoll, R. A. (2017) Subunit-specific role for the amino-terminal domain of AMPA receptors in synaptic targeting. *Proc Natl Acad Sci U S A* **114**, 7136-7141

10. Plested, A. J., and Mayer, M. L. (2007) Structure and mechanism of kainate receptor modulation by anions. *Neuron* **53**, 829-841

11. Sheng, N., Shi, Y. S., and Nicoll, R. A. (2017) Amino-terminal domains of kainate receptors determine the differential dependence on Neto auxiliary subunits for trafficking. *Proc Natl Acad Sci U S A* **114**, 1159-1164

12. Matsuda, K., Budisantoso, T., Mitakidis, N., Sugaya, Y., Miura, E., Kakegawa, W., Yamasaki, M., Konno, K., Uchigashima, M., Abe, M., Watanabe, I., Kano, M., Watanabe, M., Sakimura, K., Aricescu, A. R., and Yuzaki, M. (2016) Transsynaptic Modulation of Kainate Receptor Functions by C1q-like Proteins. *Neuron* **90**, 752-767

13. Jackson, A. C., and Nicoll, R. A. (2011) The expanding social network of ionotopic glutamate receptors: TARPs and other transmembrane auxiliary subunits. *Neuron* **70**, 178-199

14. Howe, J. R. (2015) Modulation of non-NMDA receptor gating by auxiliary subunits. *J Physiol* **593**, 61-72

15. Straub, C., and Tomita, S. (2012) The regulation of glutamate receptor trafficking and function by TARPs and other transmembrane auxiliary subunits. *Curr Opin Neurobiol* **22**, 488-495

16. Copits, B. A., and Swanson, G. T. (2012) Dancing partners at the synapse: auxiliary subunits that shape kainate receptor function. *Nat Rev Neurosci* **13**, 675-686

17. Wyeth, M. S., Pelkey, K. A., Yuan, X., Vargish, G., Johnston, A. D., Hunt, S., Fang, C., Abebe, D., Mahadevan, V., Fisahn, A., Salter, M. W., McInnes, R. R., Chittajallu, R., and McBain, C. J. (2017) Neto Auxiliary Subunits Regulate Interneuron Somatodendritic and Presynaptic Kainate Receptors to Control Network Inhibition. *Cell reports* **20**, 2156-2168
18. Orav, E., Atanasova, T., Shintyapina, A., Kesaf, S., Kokko, M., Partanen, J., Taira, T., and Lauri, S. E. (2017) NETO1 Guides Development of Glutamatergic Connectivity in the Hippocampus by Regulating Axonal Kainate Receptors. *eNeuro* 4

19. Orav, E., Dowavic, I., Huupponen, J., Taira, T., and Lauri, S. E. (2019) NETO1 Regulates Postsynaptic Kainate Receptors in CA3 Interneurons During Circuit Maturation. *Molecular neurobiology*

20. Jack, A., Hamad, M. I. K., Gonda, S., Gralla, S., Pahl, S., Hollmann, M., and Wahle, P. (2019) Development of Cortical Pyramidal Cell and Interneuronal Dendrites: a Role for Kainate Receptor Subunits and NETO1. *Molecular neurobiology* 56, 4960-4979

21. Vernon, C. G., and Swanson, G. T. (2017) Neto2 Assemblies with Kainate Receptors in DRG Neurons during Development and Modulates Neurite Outgrowth in Adult Sensory Neurons. *J Neurosci* 37, 3352-3363

22. Sargin, D. (2019) Heightened fear in the absence of the kainate receptor auxiliary subunit NETO2: implications for PTSD. *Neuropsychopharmacology*

23. Mennesson, M., Rydgren, E., Lipina, T., Sokolowska, E., Kulesskaya, N., Morello, F., Ivakine, E., Voikar, V., Risbrough, V., Partanen, J., and Hovatta, I. (2019) Kainate receptor auxiliary subunit NETO2 is required for normal fear expression and extinction. *Neuropsychopharmacology*

24. Stohr, H., Berger, C., Frohlich, S., and Weber, B. H. (2002) A novel gene encoding a putative transmembrane protein with two extracellular CUB domains and a low-density lipoprotein class A module: isolation of alternatively spliced isoforms in retina and brain. *Gene* 286, 223-231

25. Zhang, W., St-Gelais, F., Grabner, C. P., Trinidad, J. C., Sumioka, A., Morimoto-Tomita, M., Kim, K. S., Straub, C., Burlingame, A. L., Howe, J. R., and Tomita, S. (2009) A transmembrane accessory subunit that modulates kainate-type glutamate receptors. *Neuron* 61, 385-396

26. Fisher, J. L., and Mott, D. D. (2012) The auxiliary subunits Neto1 and Neto2 reduce voltage-dependent inhibition of recombinant kainate receptors. *J Neurosci* 32, 12928-12933

27. Tang, M., Pelkey, K. A., Ng, D., Ivakine, E., McBain, C. J., Salter, M. W., and McInnes, R. R. (2011) Neto1 is an auxiliary subunit of native synaptic kainate receptors. *J Neurosci* 31, 10009-10018

28. Sheng, N., Shi, Y. S., Lomash, R. M., Roche, K. W., and Nicoll, R. A. (2015) Neto auxiliary proteins control both the trafficking and biophysical properties of the kainate receptor GluK1. *eLife* 4, e11682

29. Duan, G. F., Ye, Y., Xu, S., Tao, W., Zhao, S., Jin, T., Nicoll, R. A., Shi, Y. S., and Sheng, N. (2018) Signal peptide represses GluK1 surface and synaptic trafficking through binding to amino-terminal domain. *Nature communications* 9, 4879

30. Meyerson, J. R., Chittori, S., Merk, A., Rao, P., Han, T. H., Serpe, M., Mayer, M. L., and
Subramaniam, S. (2016) Structural basis of kainate subtype glutamate receptor desensitization. *Nature* **537**, 567-571

31. Copits, B. A., Robbins, J. S., Frausto, S., and Swanson, G. T. (2011) Synaptic targeting and functional modulation of GluK1 kainate receptors by the auxiliary neuropilin and tolloid-like (NETO) proteins. *J Neurosci* **31**, 7334-7340

32. Fisher, J. L. (2015) The auxiliary subunits Neto1 and Neto2 have distinct, subunit-dependent effects at recombinant GluK1- and GluK2-containing kainate receptors. *Neuropharmacology* **99**, 471-480

33. Fisher, J. L., and Mott, D. D. (2013) Modulation of homomeric and heteromeric kainate receptors by the auxiliary subunit Neto1. *J Physiol* **591**, 4711-4724

34. Brown, P. M., Aurousseau, M. R., Musgaard, M., Biggin, P. C., and Bowie, D. (2016) Kainate receptor pore-forming and auxiliary subunits regulate channel block by a novel mechanism. *J Physiol* **594**, 1821-1840

35. Fay, A. M., and Bowie, D. (2006) Concanavalin-A reports agonist-induced conformational changes in the intact GluR6 kainate receptor. *J Physiol* **572**, 201-213

36. Straub, C., Zhang, W., and Howe, J. R. (2011) Neto2 modulation of kainate receptors with different subunit compositions. *J Neurosci* **31**, 8078-8082

37. Sobolevsky, A. I. (2015) Structure and gating of tetrameric glutamate receptors. *J Physiol* **593**, 29-38

38. Lu, H. W., Balmer, T. S., Romero, G. E., and Trussell, L. O. (2017) Slow AMPAR Synaptic Transmission Is Determined by Stargazin and Glutamate Transporters. *Neuron* **96**, 73-80 e74

39. Twomey, E. C., Yelshanskaya, M. V., Grassucci, R. A., Frank, J., and Sobolevsky, A. I. (2017) Structural Bases of Desensitization in AMPA Receptor-Auxiliary Subunit Complexes. *Neuron* **94**, 569-580 e565

40. Meyerson, J. R., Kumar, J., Chittori, S., Rao, P., Pierson, J., Bartesaghi, A., Mayer, M. L., and Subramaniam, S. (2014) Structural mechanism of glutamate receptor activation and desensitization. *Nature* **514**, 328-334

41. Carbone, A. L., and Pleston, A. J. (2012) Coupled control of desensitization and gating by the ligand binding domain of glutamate receptors. *Neuron* **74**, 845-857

42. Schauder, D. M., Kuybeda, O., Zhang, J., Klymko, K., Bartesaghi, A., Borgnia, M. J., Mayer, M. L., and Subramaniam, S. (2013) Glutamate receptor desensitization is mediated by changes in quaternary structure of the ligand binding domain. *Proc Natl Acad Sci U S A* **110**, 5921-5926

43. Griffith, T. N., and Swanson, G. T. (2015) Identification of critical functional determinants of kainate receptor modulation by auxiliary protein Neto2. *J Physiol* **593**, 4815-4833

44. He, X. Y., Li, Y. J., Kalyanaraman, C., Qiu, L. L., Chen, C., Xiao, Q., Liu, W. X., Zhang, W.,
Yang, J. J., Chen, G., Jacobson, M. P., and Shi, Y. S. (2016) GluA1 signal peptide determines the spatial assembly of heteromeric AMPA receptors. *Proc Natl Acad Sci U S A* **113**, E5645-E5654

**Figure 1. Neto1/2 have two separate interaction sites with GluK2.**

*A and B*, Immunoblot of immunoprecipitates from transfected HEK293T cells. The identities of the transfected constructs were indicated above each lane. Full-length
GluK2, GluK2NTD and GluK2ΔNTD were co-immunoprecipitated with Neto1 and Neto2.

C, The GluK2NTD were co-immunoprecipitated with CUB1 domains of Neto1/2.

D, Co-immunoprecipitation of GluK2ΔNTD and Neto proteins without CUB1 domains.

E and F, Co-immunoprecipitation of GluK2NTD and Neto proteins with or without CUB1 domains. Deletion of CUB1 domains significantly suppressed the interaction between GluK2 and Neto proteins (arrows).

G, Quantification of immunoprecipitation in (E) and (F). The GluK2NTD pulled-down was normalized by the FLAG signal pulled-down. Compared to full-length Neto1 (1.00 ± 0.32), the GluK2NTD precipitated by Neto1ΔCUB1 was significantly reduced to 0.38 ± 0.08 (n = 4 pairs, **p < 0.01, paired t-test). Compared to full-length Neto2 (1.00 ± 0.22), the GluK2NTD pulled down by Neto2ΔCUB1 was significantly reduced to 0.24 ± 0.03 (n = 4 pairs, ***p < 0.001, paired t-test).

H, A schematic model shows the two interaction sites between GluK2 and Neto proteins.
Figure 2. Neto regulation on GluK2 desensitization

A, Deletion of NTD has no effects on GluK2 desensitization. Up panel, superimposed average desensitization traces of GluK2 and GluK2ΔNTD. Left low panel, statistical comparison between the $\tau_{\text{des}}$ of GluK2 and GluK2ΔNTD. Right low panel, a schematic model depicts deletion of NTD.

B, The modulatory effects of Neto1 and Neto2 on GluK2 desensitization. Up panel shows superimposed average desensitization traces. Left low panel, statistical comparison among the $\tau_{\text{des}}$ of GluK2 with and without Netos. While Neto1 has no effects on GluK2 desensitization, Neto2 slows GluK2 desensitization by about 4 times (**p < 0.001). Neto1 and Neto2 exhibited deferential modulation on GluK2 desensitization (GluK2+Neto1 vs. GluK2+Neto2, ***p < 0.001). Right low panel, a schematic model depicts the NTD-CUB1 and core-Neto interactions.

C, The modulatory effects of Neto1 and Neto2 on GluK2ΔNTD desensitization. Up panel shows superimposed average desensitization traces. Left low panel, statistical
comparison among the $\tau_{\text{des}}$ of GluK2ΔNTD with and without Netos. Both Neto1 and Neto2 has modest slowing effects on GluK2ΔNTD (*$p < 0.05$), no difference was found between GluK2ΔNTD+Neto1 and GluK2ΔNTD+Neto2. Right low panel, a schematic model depicts the core-Neto interaction under these conditions. 

D, CUB1-deleted Netos have no apparent effects on GluK2 desensitization.

E, The modulatory effects of CUB1-deleted Netos on GluK2ΔNTD. Neto1ΔCUB1 slowed the desensitization of GluK2ΔNTD (**$p < 0.01$). Neto2ΔCUB1 slowed the desensitization of GluK2ΔNTD (*$p < 0.05$). No significant difference was found between GluK2ΔNTD+Neto1ΔCUB1 and GluK2ΔNTD+Neto2ΔCUB1.

The raw desensitization traces were depicted and the average desensitization traces calculated in supporting Figure S2A. The data were analyzed using Two-way ANOVA with post hoc Tukey’s multiple comparisons tests (Netos or mutants, $F(4, 151) = 22.80$, $p < 0.001$; NTD, $F(1, 151) = 0.16$, $p = 0.69$; interaction, $F(4, 151) = 25.69$, $p < 0.001$). Statistical significance was denoted as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant.
Figure 3. Neto regulation on GluK2 deactivation.
A, Deletion of NTD has no effects on GluK2 deactivation.
B, The modulatory effects of Neto1 and Neto2 on GluK2 deactivation. Up panel shows superimposed average deactivation traces. Low panel, while Neto1 has no effects on GluK2 desensitization, Neto2 slows GluK2 deactivation (*p < 0.05). Neto1 and Neto2 exhibited deferential modulation on GluK2 deactivation (GluK2+Neto1 vs. GluK2+Neto2, ***p < 0.001).
C, The modulatory effects of Neto1 and Neto2 on GluK2ΔNTD deactivation. Up panel shows superimposed average desensitization traces. Low panel, both Neto1 and Neto2 has modest slowing effects on GluK2ΔNTD (*p < 0.05). No difference was found
D, Neto1ΔCUB1 slightly slowed (*p < 0.05) while Neto2ΔCUB1 has no effects on GluK2 deactivation. However, the effects were not significantly different between Neto1ΔCUB1 and Neto2ΔCUB1.

E, The modulatory effects of CUB1-deleted Netos on GluK2ΔNTD deactivation. Both Neto1ΔCUB1 and Neto2ΔCUB1 slowed the deactivation of GluK2ΔNTD (***p < 0.001). No significant difference was found between GluK2ΔNTD+Neto1ΔCUB1 and GluK2ΔNTD+Neto2ΔCUB1.

The raw deactivation traces were depicted and the average deactivation traces calculated in supporting Figure S2B. The data were analyzed using Two-way ANOVA with post hoc Tukey's multiple comparisons tests (Netos or mutants, F (4, 129) = 14.68, p < 0.001; NTD, F (1, 129) = 47.39, p < 0.001; interaction, F (4, 129) = 10.00, p < 0.001). Statistical significance was denoted as: *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.
**Figure 4. Neto regulation on GluK2 recovery from desensitization**

A, Representative recording traces of GluK2 and GluK2ΔNTD evoked by pairs of 50 ms applications of 10 mM glutamate. The interval time between two applications ranged from 5 ms to 8000 ms. The amplitude was normalized to the first peak.

B, Analysis of the recovery rates of GluK2 and GluK2ΔNTD. Left, the recovery was calculated by the peak amplitude of the 2nd response divided by that of the 1st response. Data points represented mean ± SD. The average data was fitted with a single exponential equation (GluK2, black; GluK2ΔNTD, red). Right, the time constants ($\tau_{rec}$) were compared between GluK2 and GluK2ΔNTD. Deletion of NTD significantly speeded the receptor recovery from desensitization (**p < 0.01).

C, Netos regulate GluK2 recovery from desensitization. Both Neto1 and Neto2 speeded GluK2 recovery from desensitization (*p < 0.05 and **p < 0.01 respectively).

D, Netos differentially regulate GluK2ΔNTD recovery from desensitization. Neto1 has no effects on GluK2ΔNTD recovery from desensitization. Neto2 slowed GluK2ΔNTD recovery from desensitization (*p < 0.05).

E, CUB1 deleted Netos differentially regulate GluK2 recovery from desensitization. Neto1ΔCUB1 has no effects on GluK2 recovery from desensitization. Neto2ΔCUB1 slowed GluK2 recovery from desensitization (**p < 0.01).

F, CUB1 deleted Netos differentially regulate GluK2ΔNTD recovery from desensitization. Neto1ΔCUB1 has no effects on GluK2ΔNTD recovery from desensitization.
desensitization. Neto2ΔCUB1 slowed GluK2ΔNTD recovery from desensitization (*p < 0.05)
The representative traces for recovery from desensitization were depicted in supporting Figure S3. The data were analyzed using Two-way ANOVA with post hoc Tukey’s multiple comparisons tests (Netos or mutants, F (4, 93) = 29.92, p < 0.001; NTD, F (1, 93) = 109.80, p < 0.001; interaction, F (4, 93) = 20.22, p < 0.001). Statistical significance was denoted as: *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.
Figure 5. Critical residues on CUB1 domains for NTD-CUB1 interactions.

A, A homology model for Neto2CUB1 domain. Left panel, distribution of the charges on Neto2CUB1 surface. Negatively charged residues are shown in red, and positively charged residues are shown in blue. The molecule is polarized according to its charge distribution. Middle panel, the model is rotated for better view of the negatively charged pole. Right panel, sequence alignment around the negatively charged residues between Neto1 and Neto2.

B, Co-immunoprecipitation of Neto2CUB1 mutants with GluK2NTD. The interaction between GluK2NTD and Neto2CUB1 was significantly diminished when 4 negatively charged residues were mutated to alanine residues (arrow). The mutation on 5 residues on the positively charged pole did not affect CUB1 interaction with GluK2NTD. Right panel, bar graph shows the relative pull-down efficiency from 3 experiments. The GluK2NTD pulled-down were normalized by the FLAG signal pulled-down.

C, Mutation on the 3 negatively charged residues in Neto1CUB1 domain diminished its binding to GluK2NTD (arrow). Right panel, bar graph shows the relative pull-down
efficiency from 3 experiments. The GluK2NTD pulled-down were normalized by the FLAG signal pulled-down.

D, Neutralization on the negative charges affect Neto regulation on GluK2 desensitization. Left, superimposed average desensitization traces of GluK2 with or without mutated Netos recorded in a parallel experiment. Right panel, bar graph shows the weighted $\tau_{\text{des}}$. GluK2, 2.69 ± 0.35 ms, n = 8; GluK2 + Neto1(DE3A), 4.10 ± 0.91 ms, n = 10; GluK2 + Neto2(DE4A), 3.75 ± 0.83 ms, n = 8. One-way ANOVA with post hoc Tukey's multiple comparisons tests, F (2, 23) = 7.24, p < 0.01. *p < 0.05; **p < 0.01; n.s., not significant.

E, Neutralization on the negative charges affect Neto regulation on GluK2 recovery from desensitization. Left, the recovery of GluK2 with or without mutant Netos recorded in a parallel experiment. Right, analysis of the recovery rates. GluK2+Neto1(DE3A) (2.36 ± 0.77 s, n = 13) was not different from GluK2 (2.45 ± 0.92 s, n = 12). Neto2(DE4A) slowed GluK2 recovery (4.24 ± 1.40 s, n = 8, **p < 0.01, one-way ANOVA with post hoc Tukey's multiple comparisons, F (2, 30) = 9.17, p < 0.001).
Figure 6. Critical residues in GluK2NTD for NTD-CUB1 interaction.

A, The NTD dimer of GluK2 is adapted from Cryo-EM structure of GluK2 (PDB ID: 5kuf). Highly positive patches on the surface of subunit A containing at least two positively charged residues were identified (in blue). Positively charged residues scattered on NTD surface were shown in pink. Right, the residues composed of the 6 highly positive patches on NTD surface.

B, Co-immunoprecipitation of Neto2CUB1 domain coexpressed with GluK2NTD with...
or without mutations. Lanes 1-6 are GluK2NTD mutations carry alanine replacement of positively charged residue identified in (A). The interaction between GluK2NTD and CUB1 domain was significantly disrupted when the 4 positively charged residues in group 1 were mutated to alanine residues (arrow). Low panel, bar graph shows the pull-down efficiency from 3 experiments.

C, Co-immunoprecipitation of Neto2CUB1 domain with GluK2NTD mutations. 4A, the same mutant as lane 1 in (B). 2A, R58A_K82A. The interaction between GluK2NTD and CUB1 domain was significantly disrupted in 4A mutation of the GluK2NTD but not in the series of single mutation or double mutation. Low panel, bar graph quantified the pull-down efficiency from 3 experiments.

D, Neto1 and Neto2 regulation on the desensitization of GluK2(RK4A). Both Neto1 (5.89 ± 3.01 ms, n = 19) and Neto2 (5.78 ± 1.72, n = 12) slowed the desensitization of GluK2(RK4A) (2.47 ± 0.23 ms, n = 10). **p < 0.01, one-way ANOVA with post hoc Tukey's multiple comparisons, F (2, 30) = 9.17, p < 0.001.

E, GluK2(RK4A) recovery from desensitization with or without Netos. Neto1 (1.27 ± 0.22 s, n = 13) had no effects on GluK2(RK4A) recovery (1.19 ± 0.38 s, n = 18). Neto2 (1.95 ± 0.69 s, n = 15) slowed GluK2(RK4A) recovery. ***p < 0.001, one-way ANOVA with post hoc Tukey's multiple comparisons, F (2, 43) = 11.08, p < 0.001.
Figure 7. Schematic models summarizing NTD and Neto modulation of GluK2 gating.

A, The effects of the three factors, NTD, core-Neto interaction and NTD-CUB1 interaction on GluK2 desensitization. The model starts from the smallest functional receptor GluK2ΔNTD. NTD has no effects on desensitization (comparison 1). Core-Neto interaction with either Neto1 or Neto2 slows desensitization (comparison 2). NTD-Neto1 speeds up desensitization while NTD-Neto2 slows desensitization (comparison 3). These models can be applied for GluK2 deactivation.

B, The three factors on the recovery from desensitization. Among the three factors, NTD appear to have most dramatic effects, which stabilizes the receptor in desensitization state (comparison 1). Core-Neto1 has little effects on while core-Neto2 slows the recovery speed either on NTD truncated (comparison 2) or full-length GluK2 (comparison 2'). The NTD-CUB1 interaction speeds GluK2 recovery (comparison 3).
Neto proteins regulate gating of the kainate-type glutamate receptor GluK2 through two binding sites
Yan-Jun Li, Gui-Fang Duan, Jia-Hui Sun, Dan Wu, Chang Ye, Yan-Yu Zang, Gui-Quan Chen, Yong-Yun Shi, Jun Wang, Wei Zhang and Yun Stone Shi

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