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ORIGINAL ARTICLE

Synaptic Targeting of Kainate Receptors

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Abstract

When native and recombinant kainate receptors (KARs) are compared, there is a mismatch in several of their functional properties. While both generate currents, synaptic responses mediated by KARs have rarely observed in cultured hippocampal neurons. The recent discovery of auxiliary proteins for KARs, such as Netos, offers an explanation for these discrepancies. We found that the GluK5 KAR subunit and the ancillary proteins, Neto1 and Neto2, are not expressed by hippocampal neurons in culture. Therefore, we used this model to directly test whether these proteins are required for the synaptic localization of KARs. Transfection of GluK4, GluK5, Neto1, or Neto2 into hippocampal neurons was associated with the appearance of synaptic KAR-mediated EPSCs. However, GluK4 or GluK5 alone produced synaptic activity in a significant proportion of cells and with reliable event frequency. While neurons expressing GluK4 or GluK5 subunits displayed synaptic responses with rapid kinetics, the expression of Neto proteins conferred these synaptic responses with their characteristic slow onset and decay rates. These data reveal some requirements for KAR targeting to the synapse, indicating a fundamental role of high affinity KAR subunits in this process.

Key words: cultures, GluK4, GluK5, hippocampus, Neto1, Neto2, synaptic transmission, trafficking

Introduction

It is becoming evident that the formation of heteromeric complexes alters the biophysical and pharmacological properties of neurotransmitter receptors and, indeed, interactions with ancillary proteins may guide their subcellular distribution. While this is true for most synaptic receptors, it is becoming particularly apparent for ionotropic glutamate receptors. Auxiliary subunits of AMPA receptors, such as stargazin and others transmembrane AMPA receptor-associated proteins (see Jackson and Nicoll 2011 for a review), CKAMP44 (Cysteine-knot AMPA receptor-modulating protein of 44 kDa; von Engelhardt et al. 2010), and cornichons (Schwenk et al. 2009) are determinants for the trafficking and for many biophysical characteristics of AMPA receptors. Although proteins that associate functionally with Kainate receptors (KARs) have remained elusive for a long time, a number of such proteins are now being identified. Indeed, the interactions with these proteins might account for the mismatch traditionally observed between recombinant and synaptic KARs (reviewed by Lerma and Marques 2013).

KARs are formed by 5 different subunits, GluK1-5, of which GluK1-3 can co-assemble as homomers or heteromers and give rise to functional receptors (Cui and Mayer 1999; Paternain et al. 2000). In contrast, GluK4 and GluK5 must co-assemble with GluK1-3 subunits to form functional receptor channels. In CA3 pyramidal neurons, heteromeric assembly seems to be necessary for KARs to be targeted to the synapse (Fernandes et al. 2009), although this issue is still not absolutely clear. Two auxiliary KAR proteins have recently been identified, Neto1 and Neto2 (NEuropilin TOlloid-like 1 and 2; Zhang et al. 2009; Straub, Hunt, et al. 2011), single transmembrane proteins expressed strongly in the cortex and hippocampus, that are very similar to one another.

While the effect of Neto proteins on KAR gating and pharmacology has been defined to some extent (Zhang et al. 2009; Copits et al. 2011; Straub, Hunt, et al. 2011; Fisher and Mott 2012), their effect on KAR targeting is less clear. Certainly, genetic ablation of Neto1 and Neto2 in the hippocampus reduces the affinity of CA3 neurons for kainate but does not abolish KAR-mediated EPSCs ($EPSC_{KAR}$; Straub, Hunt, et al. 2011; Tang et al. 2011). Indeed, it was recently shown that transfection of Neto2 promotes the synaptic localization of exogenous GluK1 receptors in rat hippocampal neurons, although the number of recorded synaptic events was extremely low (Copits et al. 2011). Although native

KARs are abundantly expressed in cultured hippocampal neurons, by unknown reasons synaptic KARs are not found (Lerma et al. 1993, 1997). Moreover, single-cell PCR analysis of cultured hippocampal cells did not reveal any expression of GluK4 or GluK5 (Ruano et al. 1995) and surprisingly, cultured hippocampal neurons lack Neto proteins. Therefore, these neurons seem to be ideal to test the influence of these proteins on the synaptic targeting of KARs.

In the present work, we have characterized the impact of Neto1 and Neto2 on different types of KARs as well as the effect on their synaptic localization, validating previously published data. However, we found that GluK4 and GluK5 subunits significantly contribute for targeting KARs to the synapse, evidence that most if not all synaptic KARs must be heteromeric. Indeed, these data reveal a fundamental role of high-affinity KAR subunits in the targeting of synaptic KARs.

Materials and Methods

cDNA Constructs and Transfection

The Neto1 plasmid was purchased from Thermo Scientific, subcloned by PCR into pCDNA3 and tagged with GFP after the signal peptide. The GFP-Neto2 plasmid was a generous gift from Dr Y. Stern-Bach (The Hebrew University-Hadassah Dental School, Jerusalem). HEK293 cells were co-transfected with KAR subunits and Neto proteins by electroporation (Gene pulser; Bio-Rad, Hercules, CA, USA) at a ratio 2:1 (8 µg of cDNA total). Afterward, the cells were seeded in Petri dishes coated with poly-D-lysine (25 mg/mL), cultured in a humidified incubator at 37 °C (5% CO_2) in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, and they were used the next day for electrophysiological experiments.

PCR Amplification

Total RNA from the P0 hippocampus and from cultured hippocampal neurons (DIV 19) was isolated using the RNeasy mini kit (Qiagen). Reverse transcription was performed with 500 ng of total RNA using a SuperScript Double-stranded cDNA Synthesis Kit (Invitrogen). PCR was performed using the following primers: Neto1 reverse: 5′GTTGTATTCAGATTCATGTTTG3′; Neto1 forward: 5′ CATCACTGTGGATCCCAACTGTC3′, giving a band of 310 bp. Neto2 forward: 5′ GAAGCAGTGCTATCGAAAATCT3′; Neto2 reverse: 5′ CTTAGTGAAAATAGTTCATAATG3′, giving a band of 500 bp; GADPH forward: 5′ TGCTGAGTATGTCGTGGAGTCT3′; GADPH reverse: 5′GGTCCAGGGTTTCTTACTCCTT3′ giving a band of 730 bp.

Determination of the Surface Fraction of KARs

Transfected HEK293 cells were grown until a full monolayer was obtained, when they were washed with PBS containing 1.8 mM Ca^{2+} and 1 mM Mg^{2+} (PBS/Ca^{2+}/Mg^{2+}), and then incubated for 15 min with biotinylation reagent (1.5 mg/mL sulfo-NHS-SS-biotin in PBS/Ca^{2+}/Mg^{2+}; Pierce) at 4 °C with gentle agitation. The cells were washed again 3 times in PBS/Ca^{2+}/Mg^{2+} and incubated for 10 min in quenching buffer (192 mM glycine, 25 mM Tris in PBS/Ca^{2+}/Mg^{2+}). The cell extracts were then collected in 500 µL lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 1% Triton-X, 0.1% SDS and protease inhibitor cocktail tablet [pH 7.4]), incubated for 1 h with rotation at 4 °C and then centrifuged at 10 000 g. The pellet recovered was discarded and an aliquot of the supernatant was used as the input (25 µL, 5%), while the remainder was incubated with 50 µL of a 1:1 slurry streptavidin beads (Pierce) overnight at 4 °C with rotation. Subsequently, the beads were washed 3 times with lysis buffer, and the proteins were recovered by adding 35 µL of loading buffer containing 1% SDS and boiling for 5 min (95 °C). The input and biotinylated samples were then resolved by SDS–PAGE and transferred to nitrocellulose membranes (Amersham), which were then probed with polyclonal c-myc, GluK2/3, and GAPDH antisera. The proteins recognized were then visualized by ECL (ECL plus, Pierce) and quantified using Quantity one (Bio-Rad) to measure the band densities of the input and biotinylated samples.

Primary Hippocampal Cell Cultures

Cells were mechanically dissociated from the hippocampus of P0 mice after trypsin digestion (Type I 0.12 mg/mL, 30 min at 37 °C; Sigma). The cells were then plated on coverslips coated with laminin (4 mg/mL; Sigma) and poly-D-lysine (5 mg/mL; Sigma) in DMEN plus 10% FBS and 1% Penicillin/streptomycin. Five hours after seeding, the coverslips were transferred to M24 plates containing an astrocyte monolayer growing in Neurobasal medium (containing 1% Penicilin/streptomycin, 1% Glutamax, and 2% NS-21 medium) and incubated at 37 °C in an atmosphere of 95%/5% O_2/CO_2 until they were used 17–20 days later. The medium was partially (40%) replaced on days 3, 7, 11, and 15.

Electrophysiology Recordings and Perfusion

Membrane currents were recorded using the patch-clamp technique under whole-cell configuration. Patch pipettes were fabricated from borosilicate glass and they had resistances of 3–7 MΩ. Currents were filtered at 10 kHz (two-pole Butterworth filter, −12 db/octave) and transferred to a personal computer for analysis at a sampling rate of 5–10 kHz, and they were displayed using pClamp software (Axon Instruments, Foster City, CA, USA). All experiments were performed at room temperature (22–25 °C) and the cells were rapidly perfused using a linear array of 7 glass tubes placed 200–300 µm from cell body, as described previously (Lerma 1992). Measurement of open tip potential drop revealed that this perfusion system completed solution exchange in <2 ms.

The external solution was (in mM): NaCl 160, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 2, glucose 10, and HEPES 10 [pH 7.4]. When necessary, the external sodium was substituted by equimolar cesium and the patch pipettes were filled with (in mM): cesium methanesulfonate 135, NaCl 8, HEPES 10, EGTA 1 [pH 7.3]. When required the osmolarity of the external and internal solutions was also adjusted with sucrose to 330 and 305 mOsm, respectively. Agonists and antagonists were diluted from stock solutions to the desired concentration in external solution: 1 M for glutamate, 50 mM for kainate or APV in distilled water; 50 mM for picrotoxin in ethanol; 100 mM for (-)GYKI 53655 in DMSO.

Statistical Evaluation

For statistical assessment, we used Student's *t*-test, whenever possible. For multiple comparisons, one-way ANOVA was used after evaluating Gaussian approximation data distribution by the Kruskal–Wallis test. Significances were obtained by Dun's multiple comparison post hoc test, using GraphPad Prism or SigmaPlot software.

Results

Neto1 and Neto2 Differentially Modulate KAR Gating and Pharmacology

A number of studies have indicated that Neto proteins affect the gating properties of different KARs (Copits et al. 2011; Straub,

Hunt, et al. 2011; Fisher and Mott 2013). We confirmed these results by studying the effect of Neto proteins on the properties of several types of recombinant homomeric KARs expressed in HEK cells. Neto 1 or Neto2 was co-expressed with one of the KAR subunits and the electrophysiological responses to the rapid application of a saturating concentration of glutamate (10 mM) were studied. The effects of these ancillary proteins on amplitude, desensitization and affinity of KARs can be found in Supplementary Table 1. Briefly, Neto proteins had differential effects on the kinetics of GluK1 and GluK3 desensitization. While Neto1 accelerated the onset of desensitization, Neto2 slowed down onset kinetics and reduced the steady-state desensitization of both receptors. In contrast, both Neto 1 and Neto 2 decelerated the desensitization of GluK2. Conversely, Neto1 drastically accelerated the recovery from desensitization of GluK1, GluK2, and GluK3, while Neto2 only influenced the recovery of GluK2.

Co-expression with Neto proteins also affected the affinity of KARs for glutamate. GluK1 receptors increased their glutamate affinity 7.5-fold when associated to Neto2 and 34-fold when associated with Neto1. Similarly, Neto1 and Neto2 increased the glutamate affinity of GluK2 receptors, although more modestly (3- to 4-fold; Supplementary Table 1). In the presence of Neto proteins, the dose–response curve for GluK3 was also shifted to the left, although the extremely low affinity of this subunit for glutamate precluded the accurate estimation of the EC_{50} in either case.

Neto1, and especially Neto2, consistently increased the amplitude of glutamate-induced responses (Fig. 1), although it remains unclear whether this increase reflected a change in gating properties (e.g., desensitization, on and off rates, mean open times, etc.; Straub, Zhang, et al. 2011) or an increase in the functional receptors at the membrane. To clarify this issue, we measured the density of membrane receptors in the presence and absence of Neto protein. Streptavidin recovery of biotinylated surface protein indicated a substantial increase in the KARs at the membrane when they are co-expressed with either Neto protein (Fig. 1).

In summary, these results confirm and extend previous observations on the capacity of Neto proteins to modulate the main KAR subunits, and they indicate that, in addition to modifying their gating properties, Neto proteins promote the presence of KARs in the membrane. Whether this is a forward trafficking effect or a decrease in receptor endocytosis, as it has been recently suggested (Witehead et al. 2014, 9th FENS Forum Abstr. 1715), remains to be established.

Primary Hippocampal Cultures Do Not Express Auxiliary Neto Proteins or KARs Containing High-Affinity Subunits

When Neto mRNA expression was assessed by semiquantitative PCR, it appeared that hippocampal cells in primary culture do not express Neto1 or Neto2 (Fig. 2A,B). Indeed, when Neto mRNA expression was assessed at P0 hippocampi and in primary hippocampal cells after 19 days in culture, we could not amplify the Neto1 or Neto2 genes in these cultures, although they were amplified from hippocampal tissue. We were also able to amplify Neto1 and Neto2 mRNA from transfected cultures, and these experiments demonstrated the high specificity for used primers despite the strong expression of both mRNAs (Fig. 2A,B).

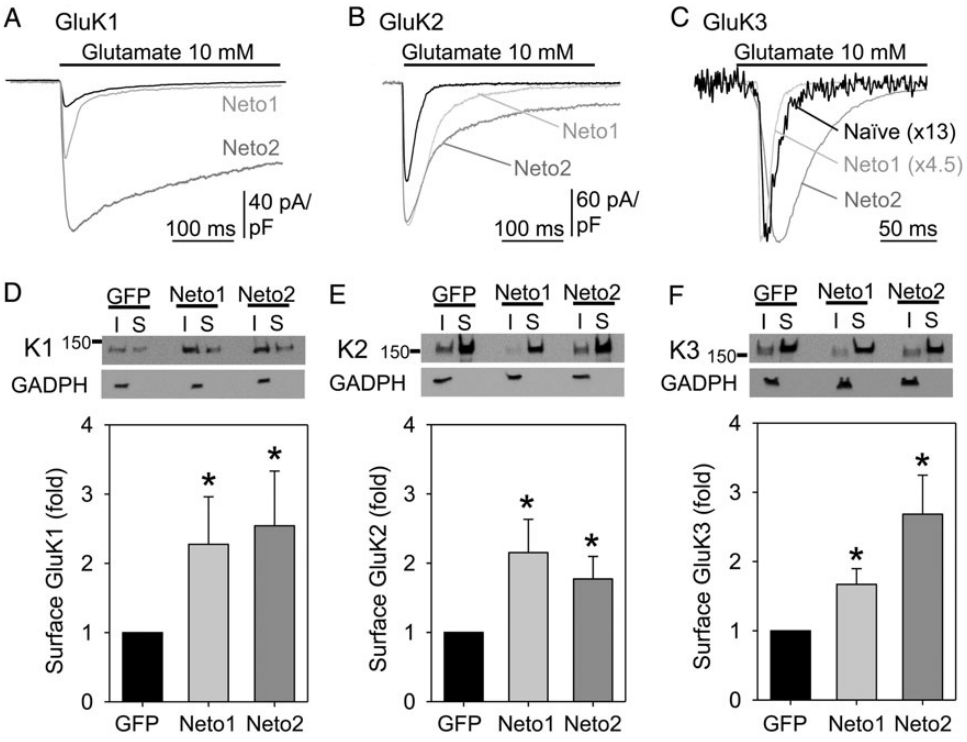

Figure 1. Neto1 and Neto2 promote the surface expression of KARs, studied in HEK cells. (*A–C*) responses elicited by rapid application of glutamate (500 ms) when GluK1 (*A*), GluK2 (*B*), and GluK3 (*C*) are expressed alone (black traces), or along with Neto1 (gray) or Neto2 (dark gray). In (*C*), responses are normalized to the amplitude obtained with Neto2. (*D–F*) HEK293 cells were transfected with myc-tagged GluK1, GluK2, or GluK3 subunits, with or without Neto1 or Neto2. Membrane fractions were separated, immunoprecipitated with streptavidin, immunoblotted and probed with anti-myc (for GluK1) or anti-GluK2/3 (for GluK2 and GluK3) antibodies. In all cases, 5% of the total sample (input) was loaded for quantification. The density of the biotinylated band was calculated as the fraction of the total input to estimate surface expression. Immunoblots for GAPDH served as a control of loading and to demonstrate the absence of contamination by the soluble fraction. Histograms show the quantification of several experiments ($n \geq 4$) and the data represent the mean ± SEM; *$P < 0.05$, Student's *t*-test.

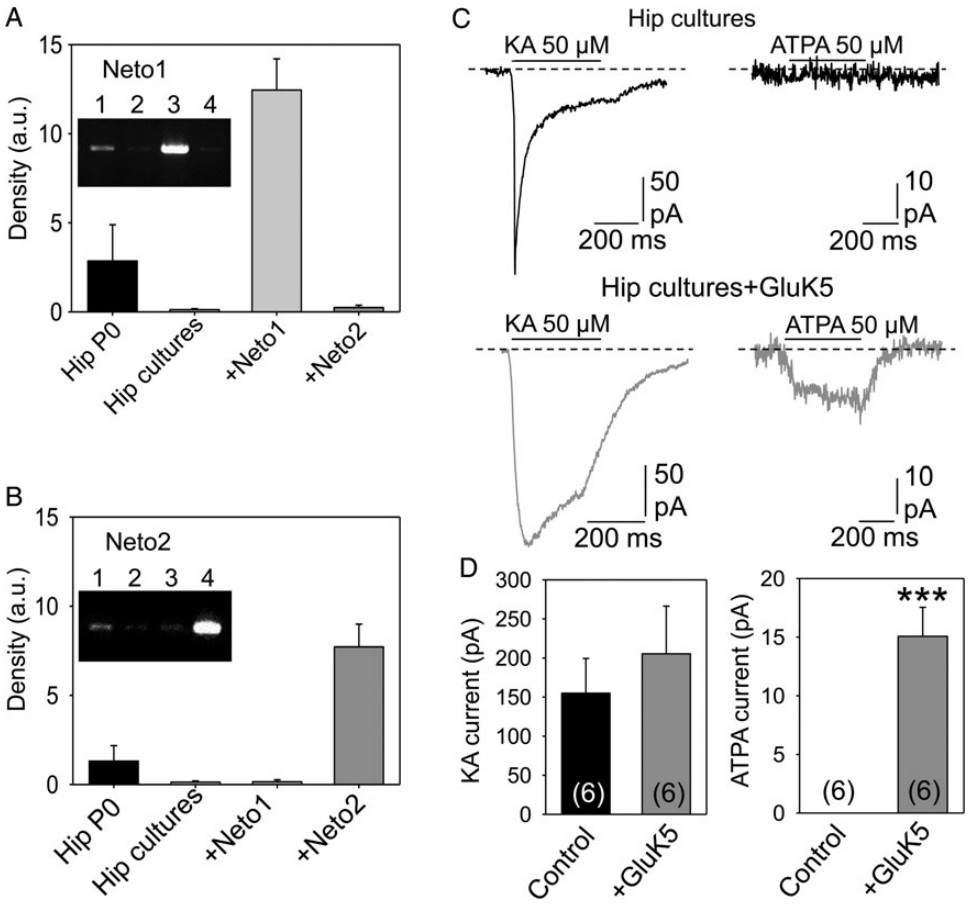

Figure 2. Hippocampal cultures do not express Neto proteins or GluK5 subunits. Neto1 and Neto2 mRNA was amplified by RT-PCR from either the hippocampus of P0 mice or 17–20 DIV hippocampal neuronal cultures that had been transfected with Neto1 (*A*) or Neto2 (*B*), or not. In each case, 4 different animals and cultures were analyzed. (*C*) Control (black) and GluK5-transfected (green) cultures were exposed to Kainate (KA) to activate the whole population of KARs, or to ATPA to test the presence of heteromeric GluK5-containing receptors. Only cells in cultures transfected with GluK5 responded to ATPA (lower row). (*D*) Quantification of these data shown as the mean ± SEM, where the numbers in parenthesis indicate the number of neurons studied; ***$P < 0.001$, Student's *t*-test.

Previous data from single-cell PCR experiments carried out in our laboratory indicated that cultured hippocampal neurons with functional KARs consistently expressed GluK2 (Ruano et al. 1995). However, in these experiments, we were unable to detect GluK4 or GluK5. Here, we have further confirmed the lack of GluK4/5 expression by pharmacology. In conditions that isolate KARs (i.e., in the presence of APV, GYKI, and picrotoxin to prevent activation of NMDARs, AMPARs, and $GABA_A$ receptors, respectively), kainate elicited currents mediated by KARs (155.0 ± 44.5 pA, $n = 6$). However, ATPA (50 μM), a KAR agonist that acts on GluK2 receptors whenever this heteromerizes with GluK1, GluK4, or GluK5 (see Paternain et al. 2000) did not elicit any response, further indicating that heteromeric receptors containing either GluK4 or GluK5 subunits were not present in these cells. In contrast, ATPA consistently induced nondesensitizing responses in neurons transfected with GluK5 (15.07 ± 2.47 pA, $n = 6$; $P < 0.01$; Fig. 2*C*,*D*). Kainate induced responses with similar amplitudes in both GluK5-expressing cells and untransfected neurons (205.4 ± 61.0 pA, $n = 6$), and the kinetic properties of these responses were consistent with the activation of heteromeric GluK2/GluK5 receptors (Fig. 2*C*,*D*). These data demonstrate that under normal conditions, cultured hippocampal neurons express native KARs, probably formed by homomeric GluK2 receptors, and that heteromeric receptors are assembled and functional upon transfection of GluK5.

Neto1 and Neto2 Barely Favor the Insertion of KARs at Synaptic Sites

Despite the presence of native KARs in the membrane of cultured hippocampal neurons, synaptic responses are not readily induced (Lerma et al. 1997). This was confirmed by the total absence of synaptic activity after NMDAR and AMPAR blockade in cultures that displayed considerable spontaneous EPSCs before blocking glutamate receptors (Fig. 3A, top). The same conclusion was reached when a barrage of firing was forced by applying brief depolarizing pulses of K^+ (50 mM) to neurons in the neighborhood of the recorded cell (not shown). However, in cells transfected with Neto1 or Neto2, exposure to APV and GYKI unmasked the presence of small and slow EPSCs (Fig. 3A, gray and dark-gray traces) that were abolished by exposure to CNQX, an AMPAR and KAR mixed antagonist (not shown). The association of endogenous KARs with exogenous Neto proteins in these experiments was demonstrated by looking at the steady state to peak ratio of kainate-induced responses (300 μM; Fig. 3A, left hand recordings), a parameter that was increased significantly in Neto-transfected cells as it was the amplitude of these responses (Fig. 3B). Examination of synaptic activity after AMPAR blockade revealed the presence of $EPSC_{KAR}$ with slow activation and deactivation kinetics, with similar values to those $EPSC_{KAR}$ found in hippocampal slices (Fig. 3C) (e.g., Straub, Hunt, et al. 2011). They were found,

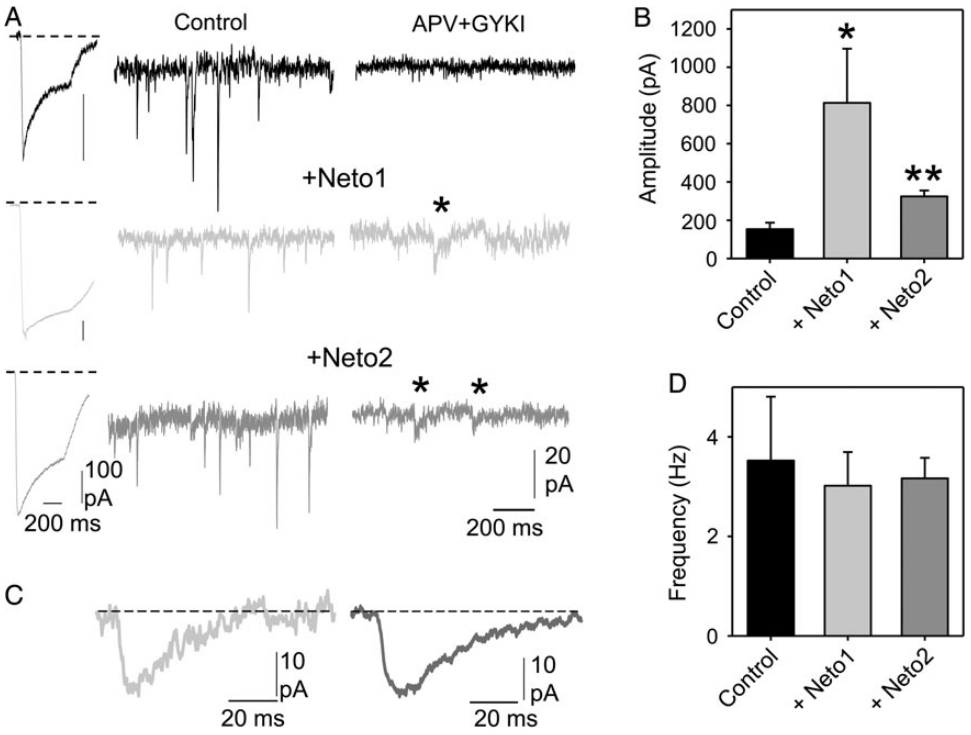

Figure 3. Auxiliary Neto proteins promote KAR synaptic responses. Hippocampal cultured neurons were recorded after 16–20 DIV. (*A*) The presence of functional KARs was probed by rapid application of 300 µM KA (inserts on the left) in control (black traces), Neto1 (gray traces)- and Neto2 (dark-gray traces)-transfected neurons in which current amplitude was quantified (*B*) showing an increase in the KA-evoked current in Neto-expressing neurons (mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.005$, Student's *t*-test). Excitatory synaptic transmission was recorded after blocking inhibitory transmission (Picrotoxin 50 µM; Control traces) and after additional blockade of NMDA and AMPA receptors (APV 50 µM and GYKI 50 µM). Only in Neto1- or Neto2-transfected neurons could some synaptic activity be detected (asterisks) after AMPA and NMDA blockade. (*C*) Amplification of these small synaptic responses from Neto1- to 2-transfected cells. (*D*) Transfection of Neto1 or Neto2 did not affect the frequency of appearance of AMPAR-mediated EPSC.

however, in a small proportion of neurons (23–26%), and the frequency of events was extremely low: 0.58 ± 0.1 and 0.75 ± 0.2 events/min for Neto1 and Neto2, respectively. The frequency of AMPAR-mediated EPSCs was unchanged in Neto1- or Neto2-transfected neurons (Fig. 3*D*). Altogether these data indicate that Neto1 and Neto2 favor the trafficking of KARs to the membrane and to some extent to the synapse.

GluK4 and GluK5 Traffic KARs to the Synapse

Since all the KARs endogenously expressed by hippocampal cells in culture are homomeric receptors that are not trafficked to the synapse, we wondered whether assembly into heteromeric high-affinity KARs would be sufficient to target these receptors to synapses, forming functional receptors. Therefore, we transfected GluK4 or GluK5 into hippocampal neurons in culture and assessed the appearance of an $EPSC_{KAR}$. There were no differences in spontaneous synaptic activity in control or GluK4- or GluK5-transfected neurons when NMDAR and AMPAR were not blocked (Fig. 4*A*,*B*). However, $EPSC_{KAR}$ were clearly observed in GluK4- or GluK5-transfected neurons [Fig. 4*A*,*B* (gray traces), and *C*]. Interestingly, these $EPSC_{KARs}$ did not show slow activation–deactivation kinetics but rather, they presented a nearly identical shape to AMPAR-mediated EPSCs (Fig. 4*D*,*F*). Indeed, the time courses of synaptic events were very different from those in the presence of Neto proteins, in that both the decay and rise times were much slower (Fig. 5*A*). This could be seen readily when the averaged rise times recorded in a particular neuron were plotted against their respective decay times (Fig. 5*B*). $EPSC_{KARs}$ fell within the range of $EPSC_{AMPARs}$, far from the cloud formed by responses recorded in neurons expressing either Neto protein.

According to the amplitude of the events, we did not observe any significant difference between the 3 types of $sEPSC_{KARs}$ (8.4 ± 0.65 pA, $n = 6$; 8.13 ± 2.01 pA, $n = 5$; 8.08 ± 1.72 pA, $n = 5$; and 14.2 ± 2.13 pA, $n = 5$ for GluK4, GluK5, Neto1, and Neto2, respectively; Fig. 5*C*). However, to our surprise, the synaptic activity mediated by KARs was observed in 50% and 42% of the neurons studied (6/12 and 5/12) in GluK4- or GluK5-expressing neurons, respectively, and the frequency of synaptic events was virtually 10-fold higher (Fig. 5*D*). These results indicate that GluK5 or GluK4 targets KARs to synaptic sites much more efficiently than Neto1 or Neto2 does, and that the association of KAR receptors with Neto1 or Neto2 is not required to target synaptic KARs. We further studied the effect of Neto proteins and GluK5 co-expression and found a larger incidence of $EPSC_{KARs}$ in those cultures (66% of cells presented $EPSC_{KARs}$ when expressing GluK5 plus either Neto; $n = 9$). The frequency of these events was also slightly larger in these cells, although the values did not reach statistical significance (ANOVA test, $P > 0.05$) when compared with cultures transfected with GluK5 alone (Fig. 5*C*). With the exception of 2 cells that were not considered for quantification, the kinetic values observed for $EPSC_{KARs}$ fell well in the range of kinetics found for receptors incorporating Neto proteins (Fig. 5*E*,*F*).

We wanted to assess the action of Neto proteins on the appearance of synaptic GluK1 containing KARs, since this subunit is not readily expressed in cultured neurons (Ruano et al. 1995). For that reason, we transfected GluK1 and Neto2 in hippocampal neurons. After blocking AMPARs and NMDARs, $EPSC_{KAR}$ were found in 50% of the cells studied ($n = 10$) (Fig. 6*A*–*C*), but

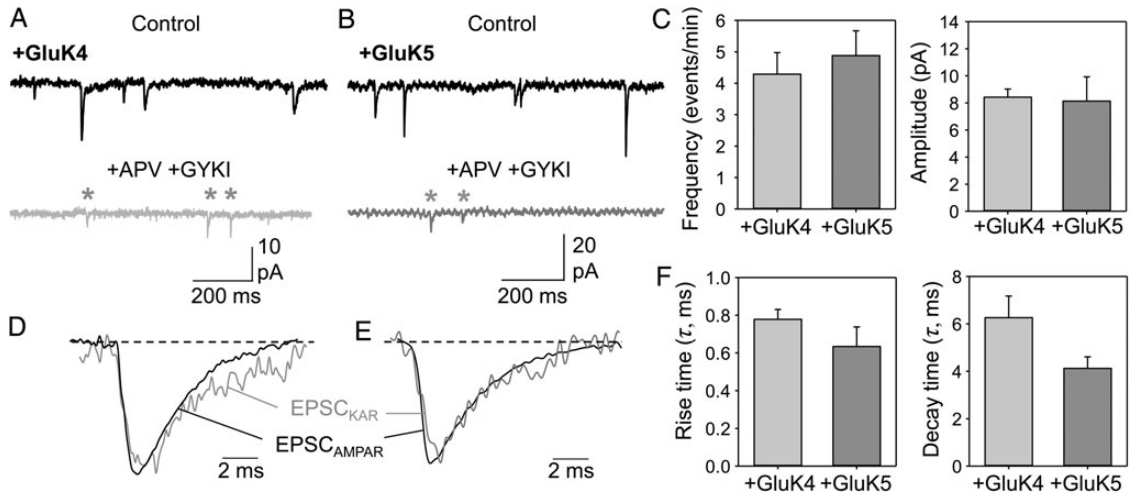

Figure 4. GluK4 and GluK5 subunits target KARs to synapses. Spontaneous excitatory synaptic transmission was recorded in 16–20 DIV hippocampal neurons before (dark traces) and after (gray traces) blocking AMPA and NMDA receptors. KAR-mediated synaptic EPSCs (asterisks) only appeared in (*A*) GluK4-or (*B*) GluK5-transfected neurons. (*C*) Frequency and amplitude of events observed in neurons transfected with GluK4 ($n = 6$ neurons) or GluK5 ($n = 5$ neurons). (*D* and *E*) Examples of ESPCs mediated by AMPARs (black) and KARs (gray), superimposed after amplitude normalization. (*F*) Quantification of rise and decay times of KAR-mediated EPSCs in neurons transfected with GluK4 or GluK5.

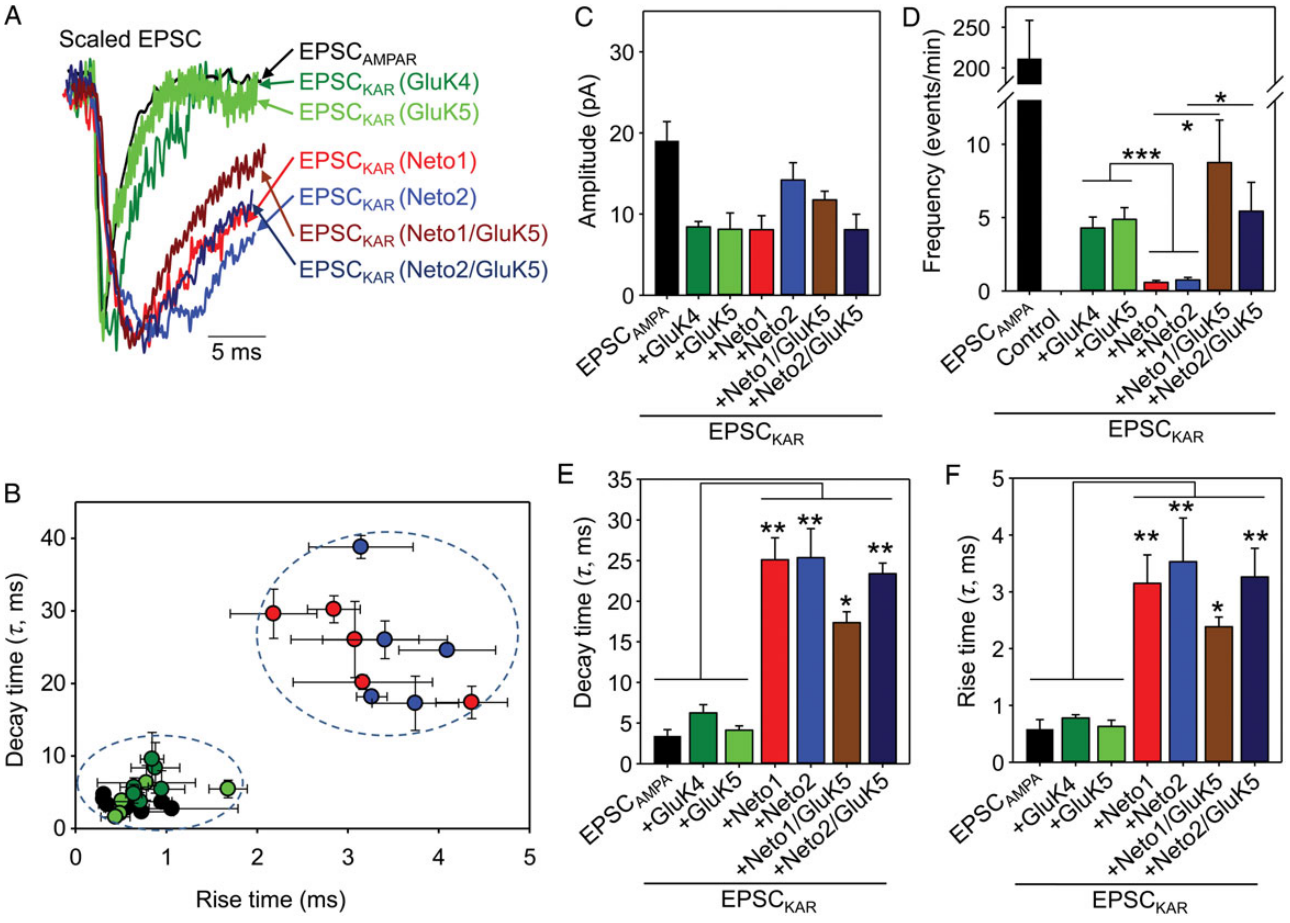

Figure 5. The properties of KAR-mediated EPSCs. (*A*) KAR-mediated EPSCs recorded under each of the indicated conditions were averaged and superimposed to AMPAR-mediated EPSC after normalizing the amplitude in order to show the different time courses in one or other experimental situation. (*B*) The averaged rise time was plotted against the averaged decay time for all EPSCs recorded in each cell. KAR-mediated EPSCs incorporating GluK4 or GluK5 present similar kinetics to AMPARs, while those incorporating Neto1 or Neto2 have considerably slower kinetics. Comparison of the amplitude (*C*), the frequency (*D*), and the decay and rise times (*E* and *F*, respectively) of synaptic events from different experimental conditions. The data are the mean ± SEM; One-way ANOVA followed by Dun's multiple comparison text, $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.005$. ($n = 5$–7 neurons per condition) .

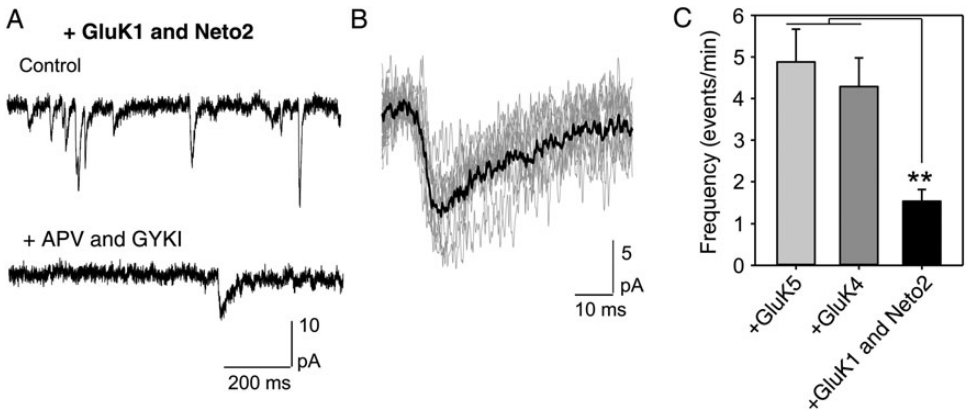

Figure 6. Synaptic KARs in GluK1- to Neto2-transfected neurons. (*A*) AMPAR- (control) and KAR-mediated EPSCs (lower trace) in neurons transfected with GluK1 subunits and Neto2. (*B*) KAR-mediated EPSCs are shown superimposed and averaged (dark trace), showing the typical slow kinetics as when Neto proteins form part of the receptor complex. (*C*) Comparison of frequency of KAR-mediated synaptic events recorded under different transfection conditions (data for GluK5 and GluK4 are the same as shown in Figs 4 and 5; $n = 5$ neurons for GluK1–Neto2). One-way ANOVA followed by Dun's multiple comparison text, $^{**}P < 0.01$.

the frequency of events was still low (1.5 ± 0.3 events/min; $n = 5$). The amplitude was similar to other $EPSC_{KAR}$ (8.4 ± 0.9 pA) and, interestingly, all events had slow activation and deactivation kinetics (Fig. 6*B*), indicating the incorporation of Neto2 to these synaptic receptors. These data are almost identical to data reported by Copits et al. (2011) and further stressed the key role of high-affinity receptor subunits in targeting KARs to the synapse.

Discussion

We have found that 2 auxiliary proteins that associate with KARs, Neto1, and Neto2, strongly modify the gating and affinity of homomeric and heteromeric KARs, confirming previous conclusions (Copits et al. 2011; Straub, Zhang, et al. 2011; Fisher and Mott 2013). Moreover, we observed that Neto proteins are not entirely required for the trafficking of KARs to synaptic sites, whereas the GluK4 and GluK5 subunits were sufficient to ensure the presence of KARs at synaptic contacts where they are exposed to the synaptically released glutamate. Interestingly, synaptic KARs lacking Neto proteins displayed rapid onset and decay kinetics, indistinguishable from AMPAR-mediated EPSCs, while the presence of Neto proteins conferred the slow kinetics on KAR-mediated synaptic events characteristic of these responses in the hippocampus.

We have studied hippocampal neurons in culture, cells in which Neto protein expression is suppressed for some unknown reason, or in which it is not activated during in vitro cellular differentiation and maturation. Indeed, Neto proteins are ubiquitously expressed in hippocampal neurons (e.g., Tang et al. 2011). It was previously proposed that Neto2 promotes the synaptic localization of exogenous GluK1 in hippocampal neurons (Copits et al. 2011; Straub, Zhang, et al. 2011), as assessed by the localization of KARs in dendritic spines promoted by Neto2. Elsewhere, a marked decrease in the levels of GluK2, GluK3, and GluK5 from PSD fractions were described in $Neto1^{-/-}$ mice (Tang et al. 2011), and a lack of spontaneous KAR-mediated synaptic currents in CA3 neurons has been recently reported in $Neto1^{-/-}$ but not $Neto2^{-/-}$ mice (Wyeth et al. 2014). Together, these data suggest that the association of KARs with Neto proteins may be required for their trafficking to the synapse. However, in cultured cells transfected with GluK1 and Neto2, there was a very low frequency of KAR-mediated EPSCs (<2 events/min), even though these recordings were obtained in the presence of $BaCl_2$ to increase the release probability (Copits et al. 2011), a result that we were able to replicate. Similar low frequencies (1 event/min) were found in cerebellar granule cells transfected with GluK2 and Neto2 (Zhang et al. 2009). Since hippocampal neurons in culture express a wealth of extrasynaptic GluK2 receptors but not other KAR subunits (Ruano et al. 1995; see Fig. 3), we were in a position to examine the requirement of one or another interacting protein to reconstitute synaptic functioning in the system (i.e., cultured hippocampal neurons). We observed that Neto1 or Neto2 might promote the synaptic localization of endogenous GluK2 or exogenous GluK1 in cultured hippocampal neurons, a result essentially consistent with previously reported data (Copits et al. 2011; Straub, Zhang, et al. 2011, 2009). However, the observed frequency of synaptic events was so low (~1 events every 2 min), that the association with Neto proteins is not likely to underlie KAR trafficking to the synapse, contrary to general believes. These rare synaptic events carried, however, the signature of KARs associated to Neto proteins, since they had the characteristic slow kinetics of $EPSC_{KARs}$ repeatedly seen in hippocampal or cortical slices.

The GluK4 and GluK5 subunits are also not expressed by these cells in culture (Ruano et al. 1995), 2 related KAR subunits that cannot form receptors by themselves but that heteromerize with GluK1, K2, or K3 subunits. We confirmed that our cultures did not express heteromeric GluK2/GluK5 or GluK2/GluK4 receptors, since neuronal KAR currents were not activated by ATPA, a GluK1 selective agonist that also activates heteromeric GluK2 receptors (Paternain et al. 2000). To our surprise, transfection of GluK4 or GluK5 produced $EPSC_{KARs}$ in our recordings with a frequency much higher than that observed with Neto proteins (from <1 to ~5 events/min). In addition, synaptic activity mediated by KARs was observed in >40% of the neurons transfected with GluK4 or GluK5, a larger proportion than when Netos were expressed. Since GluK4 or GluK5 cannot form functional receptors alone, we must conclude that the synaptic responses observed were mediated by either heteromeric GluK2/GluK4 or GluK2/GluK5 receptors. Importantly, these events were characterized by rapid onset and fast decay kinetics, resembling AMPAR-mediated synaptic responses, again indicating that association to Neto proteins is not required for KARs to be targeted to the synapse. Of course, this does not mean that under normal circumstances most of KARs could be associated with Neto proteins. Co-expression of GluK5 and Neto proteins slightly

augmented the proportion of cells presenting $EPSC_{KARs}$, although the increased frequency did not reach statistical significance. However, the presence of Neto proteins imposed the typical signature of slow kinetics to $EPSC_{KAR}$. Similarly, expression of GluK1 with Neto2 produced slightly more frequent synaptic events than Neto2 alone, also with slow kinetics. Indeed, the genetic ablation of Neto1 provokes much faster mossy fiber $EPSC_{KAR}$ decay kinetics, closely approaching AMPAR kinetics (Straub, Hunt, et al. 2011; see Lerma and Marques 2013 for a review). All together, these data support the idea that Neto proteins, although may help, are not an essential requirement for the targeting of KARs to the synapse.

One further issue to be considered is why the frequency of KAR-mediated synaptic events was so much lower in neurons transfected with GluK5 that expressed synaptic and nonsynaptic KARs, than the AMPAR-mediated EPSCs in these same neurons. One might think that AMPARs and KARs would co-localize at synapses and therefore, that the frequency of synaptic events would not be so different. However, 2 scenarios might arise in our experimental paradigm that would influence the development of EPSCs. First, blocking AMPARs might decrease general excitability and hence, the spontaneous activity of the in vitro network, probably resulting in a reduction in spontaneous firing and therefore in the synaptic events recorded. While this could indeed exert an influence, we still observed significant differences in the number of induced events provoked by directly applying K^+. The alternative scenario is that not all synapses are competent to host KARs and/or KARs are only targeted to a few synapses, as also seems to occur in vivo. For instance, synaptic KARs are detected in the synapses mossy fibers make on CA3 pyramidal cells but not in those made by Schaffer collaterals with CA1 pyramidal cells (Castillo et al. 1997). Similarly, $EPSC_{KAR}$ were not detected in all hippocampal interneurons (Frerking et al. 1998; Christensen et al. 2004) and in the barrel cortex, KARs and AMPARs segregate to different synapses during development (Kidd and Isaac 1999). These evidences suggest the existence of a as yet unknown mechanism to sort KARs to synaptic contacts.

Our data indicate that high-affinity subunits, GluK4 and GluK5, are key subunits in directing KARs to synapses. Although very few is known on GluK4 and GluK5, recent data drew attention to the role of GluK5 in synaptic plasticity and indeed, the regulation of GluK5 by phosphorylation emerged as a mechanism of long-term depression of $EPSC_{KARs}$ in mossy fiber to CA3 synapses (Selak et al. 2009; Carta et al. 2013). It also seems that GluK5 plays an important role in coupling KAR activity to developmental processes such as maturation and neurite extension (Marques et al. 2013). Therefore, although these subunits are unable to form functional KARs by itself, at least the GluK5 subunit appears to have a fundamental influence on the biology of KARs.

Altered GluK5 expression has been reported in schizophrenia (e.g., Meador-Woodruff and Healy 2000) and although association studies implicated polymorphisms in *GRIK5* in this disease (see Lerma and Marques 2013 for a review), the validity of this association remains unclear (Shibata et al. 2006), as does the role played by this subunit in this disease. It may be that the influence of GluK5 malfunction is subtle, as GluK4 could fulfill some of the roles played by this subunit (e.g., Fernandes et al. 2009). However, more attention must be paid to these subunits given the fundamental role they play in synaptic targeting of KARs revealed here.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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