Functional comparison of the effects of TARPs and cornichons on AMPA receptor trafficking and gating

Yun Shi[a,1], Young Ho Suh[b,1], Aaron D. Milstein[a,c,2], Kaname Isozaki[b], Sabine M. Schmid[a], Katherine W. Roche[b], and Roger A. Nicoll[a,c,3]

Departments of [a]Cellular and Molecular Pharmacology and [c]Physiology, University of California, San Francisco, CA 94143; and [b]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892.

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Glutamate receptors of the AMPA subtype (AMPARs) mediate fast synaptic transmission in the brain. These ionotropic receptors rely on auxiliary subunits known as transmembrane AMPAR regulatory proteins (TARPs) for both trafficking and gating. Recently, a second family of AMPAR binding proteins, referred to as cornichons, were identified and also proposed to function as auxiliary subunits. Cornichons are transmembrane proteins that modulate AMPAR function in expression systems much like TARPs. In the present study we compare the role of cornichons in controlling AMPA receptor function in neurons and HEK cells to that of TARPs. Cornichons mimic some, but not all, of the actions of TARPs in HEK cells; their role in neurons, however, is more limited. Although expressed cornichons can affect the trafficking of AMPARs, they were not detected on the surface of neurons and failed to alter the kinetics of endogenous AMPARs. This neuronal role is more consistent with that of an endoplasmic reticulum (ER) chaperone rather than a bona fide auxiliary subunit.

stargazin | synapse | auxiliary subunit | hippocampus

Fast excitatory synaptic transmission in the brain is mediated primarily by glutamate acting on AMPA receptors (AMPARs). AMPARs are heterotetramers composed of homologous subunits, GluA1–4, which are differentially expressed in distinct neuronal cell types, resulting in heterogeneity in the functional properties of AMPARs. In addition to their pore-forming subunits, AMPARs also assemble with transmembrane AMPAR regulatory proteins (TARPs) (1–4). The prototypical TARP γ-2 (stargazin) is mutated in the ataxic *stargazer* mouse. Cerebellar granule neurons (CGNs) in this mouse lack surface and synaptic AMPARs, demonstrating a necessary role for TARPs in AMPAR trafficking (5, 6). Further studies have shown that TARPs bind directly to AMPARs, promote their trafficking to the cell surface, and target the receptors to synapses via binding to the scaffolding protein PSD-95. In addition, TARPs dramatically alter the gating and pharmacological properties of AMPARs (4, 7–9).

Recently, a new family of AMPAR binding proteins, referred to as cornichons, was identified (10). Native AMPARs were affinity purified and interacting proteins were identified by mass spectrometry, including cornichon homolog 2 (CNIH2) and cornichon homolog 3 (CNIH3). Using antibody shift assays, it was concluded that AMPARs assemble primarily with CNIHs and that TARP-associated AMPARs represent a smaller and mostly nonoverlapping population of receptors. Coexpression of CNIH with GluA subunits in cultured cell lines and oocytes enhanced surface expression of AMPARs and dramatically slowed the time courses of AMPAR channel deactivation and desensitization in response to brief applications of glutamate to outside-out patches. These findings raised the intriguing possibility that AMPAR trafficking and gating are under the control of two distinct families of auxiliary subunits: TARPs and CNIHs. This would represent an evolutionarily recent gain of function for CNIHs, whose *Drosophila* and yeast homologs, Cni and Erv14p, serve as chaperones that aid in the forward trafficking of EGFR ligands from the ER to Golgi (11–13).

However, a number of issues remain unresolved (14, 15). Most importantly, do the profound effects of CNIHs on the biophysical properties of AMPARs in heterologous cells translate to native AMPARs in neurons? In this study, we carried out a series of biochemical, immunocytochemical, and electrophysiological experiments to define the roles of CNIHs in neurons.

Results

We first carried out electrophysiological recordings of AMPARs expressed in HEK cells to examine the functional effects of CNIHs. HEK cells were transfected with the AMPAR subunit GluA1 with or without the following proteins: CNIH1, CNIH2, CNIH3, or TARP γ-2 (stargazin). Both CNIH2 and CNIH3 greatly enhanced current amplitudes evoked by glutamate alone or in the presence of cyclothiazide (Fig. S1 *A* and *B*), indicating that the enhancement cannot be solely explained by an effect of CNIH2 on AMPAR channel desensitization. We next applied a voltage ramp in the presence of glutamate and, similar to TARPs (16), CNIH2 reduced inward rectification by relieving the voltage-dependent block of GluA2-lacking AMPARs by intracellular spermine (Fig. S1 *C* and *D*). These data establish that CNIH2 affects the biophysical properties of AMPARs and not simply the trafficking of receptors to the plasma membrane.

The most dramatic effects of CNIH2 on AMPARs in oocytes were a profound slowing of deactivation and reduction in desensitization (10). We used a fast perfusion system to compare the effects of CNIH2 and TARP γ-8 expressed in HEK cells, on AMPAR channel kinetics. Recording from outside-out patches, we found that CNIH2 was more effective at slowing deactivation (Fig. 1 *A* and *B*) and reducing desensitization (Fig. S2 *A* and *B*) than γ-8. Therefore, in HEK cells CNIH2 profoundly modulates the functional properties of AMPARs.

We next addressed the question of whether CNIH2 and TARPs can modulate a common pool of AMPARs. In a previous study, we were able to unequivocally express AMPARs containing a saturating stoichiometry of four TARPs per AMPAR channel by covalently linking GluA subunits to TARPs (17). Importantly, the tethered GluA1γ-8 construct functioned identically to GluA1 and γ-8 expressed separately. To determine whether saturating the TARP–AMPAR association occludes the ability of CNIH2 to exert its effect on AMPAR gating, we coexpressed the fusion construct GluA1γ-8 with CNIH2. CNIH2 further slowed the time course of AMPAR deactivation and decreased desensitization relative to GluA1γ-8 alone (Fig. 1 *A* and *B* and Fig. S2 *A* and *B*), demon-

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The authors declare no conflict of interest.

[1]These individuals contributed equally to this work.

[2]Present address: Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, VA 20147.

[3]To whom correspondence should be addressed. E-mail: nicoll@cmp.ucsf.edu.

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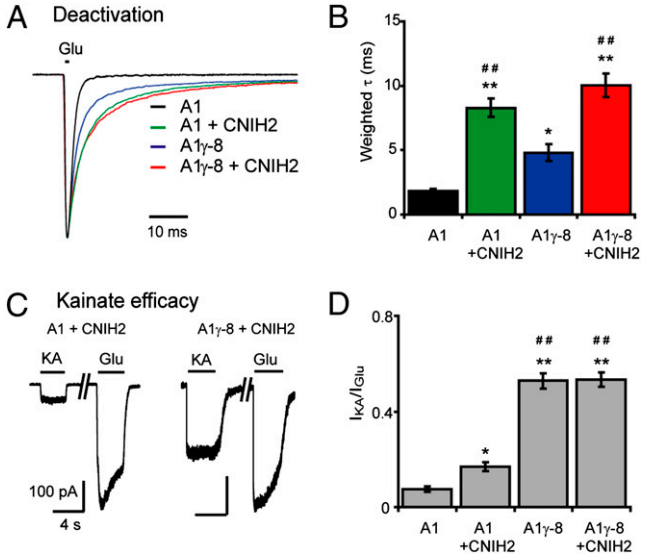

Fig. 1. CNIH2 modulates AMPAR channel properties in HEK cells. (*A*) Deactivation of GluA1 was slowed by coexpression of CNIH2 or linked TARP γ-8. (*B*) Summary of deactivation. $^{*}P < 0.05$; $^{**}P < 0.01$ relative to GluA1; $^{\#\#}P < 0.01$ relative to A1γ-8. (*C*) Examples of kainate and glutamate currents from patches excised from HEK cells in the constant presence of 100 μM cyclothiazide. Dashes indicate an 11.5-s break in the recordings. GluA1 coexpression with CNIH2 had a low KA efficacy whereas A1γ-8 coexpression with CNIH2 had a high KA efficacy. (*D*) Summary of kainate and glutamate ratio. $^{*}P < 0.05$; $^{**}P < 0.01$ relative to GluA1; $^{\#\#}P < 0.01$ relative to A1 + CNIH2. The KA efficacy of GluA1 and A1γ-8 reported here also appeared in ref. 17. Permission to reprint this data has been acquired from Cell Press. Data were shown in mean ± SEM.

strating that the actions of CNIHs and TARPs on AMPARs are not mutually exclusive and consistent with the presence of two distinct binding sites for these two families of transmembrane proteins.

An alternative explanation would be that CNIH2 displaces the linked γ-8 and disrupts the γ-8–dependent AMPAR modulation. We thus examined the efficacy of the partial agonist kainate, which is known to increase with TARP association (7, 9). Compared with γ-8, CNIH2 only modestly increased kainate efficacy (Fig. 1 *C* and *D*). If CNIH2 expression had disrupted the γ-8 modulation of AMPARs, then kainate efficacy would have decreased. However, kainate efficacy was not diminished when CNIH2 was coexpressed with the TARP fusion construct GluA1γ-8. Rather, coexpression of CNIH2 and GluA1γ-8 results in channels exhibiting both deactivation kinetics consistent with CNIH association and kainate efficacy consistent with TARP association, demonstrating that these proteins can modulate AMPARs simultaneously. We further tested whether CNIH2 shares with TARPs the ability to convert the competitive antagonist CNQX into a partial agonist (18) and found that CNIH2 has no effect on the action of CNQX (Fig. S2*C*).

TARPs also modulate the single channel conductance of AMPARs (7, 16). In agreement with previous findings for GluA1 coexpressed with γ-2 (16), nonstationary fluctuation analysis revealed that fusion of GluA1 to γ-8 caused a doubling of AMPAR single channel conductance (Fig. S3 *A*, *C*, and *E*). CNIH2 caused a similar increase in conductance (Fig. S3 *A*, *B*, and *E*), but coexpression with the fusion construct GluA1γ-8 did not result in a further increase (Fig. S3 *D* and *E*). Neither γ-8 nor CNIH2 affected the channel open probability (P_O, peak) (Fig. S3*F*).

We next directly compared AMPAR binding and surface expression of CNIH2 and TARPs using a variety of biochemical approaches. When CNIH2 is coexpressed with FLAG–GluA1 in HEK cells, we observed a robust interaction of GluA1 with CNIH2 by coimmunoprecipitation (Fig. 2 *A* and *B*). However, when compared with TARPs, substantially more γ-2 was associated with GluA1 than CNIH2 (Fig. 2 *A* and *B*). We also used biotinylation to determine whether CNIH2 is expressed on the surface of HEK cells. Although we detected considerable surface expression of CNIH2, it was significantly less than that of γ-2 (Fig. 2 *C* and *D*). The surface expression of CNIH2 and γ-2 was unaffected by coexpression with GluA1 (Fig. 2*D*).

CNIHs act as chaperones for other proteins. Our physiology data in HEK cells (Fig. S1) support a similar role of CNIHs for AMPARs. To directly compare the effect of CNIH2 and TARPs on AMPAR trafficking and surface expression, we coexpressed GluA1 with either CNIH2 or γ-2 and found that both increased GluA surface expression (Fig. 2 *C* and *E*). In addition, we evaluated the glycosylation state of GluA1 expressed alone or with

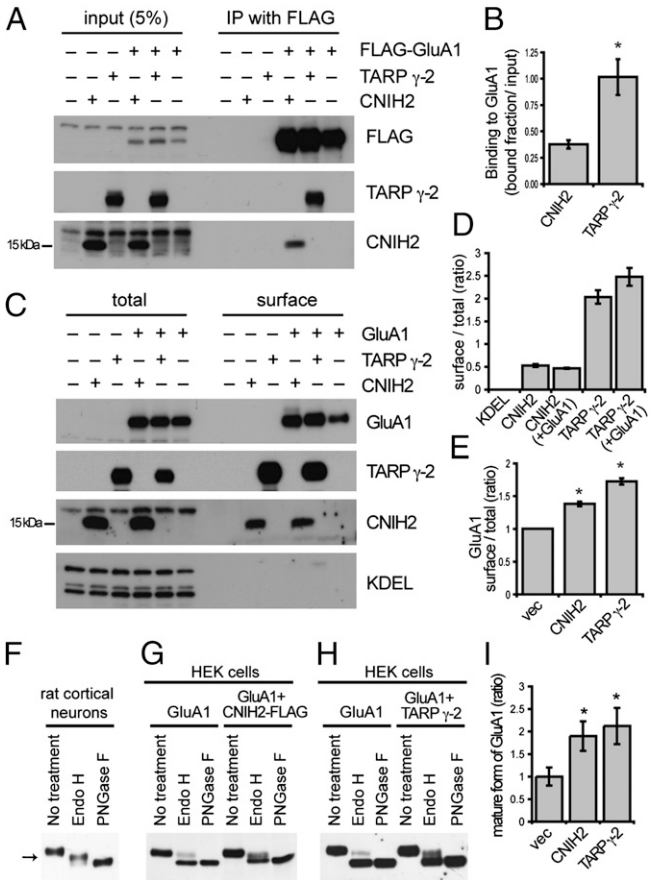

Fig. 2. CNIH2 binds to GluA1 and is expressed on the surface in HEK cells, but less than TARPs. (*A*) HEK cells were transfected with CNIH2 or TARP γ-2 or cotransfected with FLAG–GluA1, solubilized in 1% Triton X-100 lysis buffer, immunoprecipitated with FLAG M2-agarose, and immunoblotted with the indicated antibodies. (*B*) Quantitation of the bound fractions to GluA1 was performed by measuring the band intensity of the immunoprecipitated fraction compared with total input (5%) using ImageJ software, $^{*}P < 0.05$ ($n = 3$). (*C*) HEK cells were transfected with CNIH2 or TARP γ-2, or cotransfected with GluA1 and surface expression was evaluated using a surface biotinylation assay. (*D*) Quantitation of surface expression of CNIH2 or TARP from *C* was presented as a ratio of surface to total input (10%). (*E*) Quantitation of surface expression of GluA1 from *C*, $^{*}P < 0.001$ relative to GluA1 alone ($n = 3$). (*F*) The majority of GluA1 in rat cortical neurons is mature and displays little endo H sensitivity. GluA1 was immunoprecipitated from cultured cortical neurons, treated with endo H or PNGase F, and immunoblotted with GluA1. The mature form of GluA1 (arrow) is partially endo H sensitive. (*G* and *H*) Coexpression of GluA1 with CNIH2 (*G*) or TARP γ-2 (*H*) in HEK cells increases the ratio of mature GluA1. GluA1 expressed with or without CNIH2-FLAG (*G*) or TARP γ-2 (*H*) in HEK cells was immunoprecipitated and treated with endo H or PNGase F. The immunoblots were probed with GluA1 antibody. (*I*) The ratio of the mature versus immature form of GluA1 was quantified, $^{*}P < 0.05$ ($n = 4$). Bars represent mean ± SEM.

CNIH2 or γ-2. In rat cortical neurons the majority of GluA1 is endo H resistant (mature form; see arrow in Fig. 2*F*). In contrast, when GluA1 is expressed alone in HEK cells, the vast majority of the receptor is sensitive to endo H treatment or immature (Fig. 2 *G*, *H*, and *I*). However, when coexpressed with CNIH2 or γ-2, the amount of endo H resistant GluA1 increases dramatically (Fig. 2 *G*, *H*, and *I*), demonstrating that CNIH2 promotes the forward trafficking of GluA1.

To study the role of CNIHs in regulating native AMPAR trafficking and gating in neurons, we examined the effects of CNIH expression on AMPARs in CGNs. CGNs do not express CNIHs natively (10) and are known to depend on TARP γ-2 for the surface expression of AMPARs (5); therefore, we tested whether expression of CNIH2 in *stargazer* CGNs could rescue surface AMPAR expression. Twenty-four hours after transfection with CNIH2, CGNs exhibited a small but significant increase in whole-cell responses both to kainate and glutamate (Fig. 3 A_1, *B*, and *C*), an effect that was small relative to that observed with TARP γ-2. CNIH did not change the efficacy of kainate (Fig. 3*D*), consistent with what we had observed in HEK cells (Fig. 1*B*). These results indicate that CNIH can modestly enhance the surface expression of AMPARs in neurons, in the absence of TARPs.

We next analyzed the kinetics of AMPAR mEPSCs (miniature excitatory postsynaptic currents) (19). However, mEPSCs were not detected in either *stargazer* CGNs alone or when transfected with CNIH, in sharp contrast to the effect observed with the transfection of TARPs. We therefore repeated the above experiments in +/*stg* CGNs, which have a reduced expression level of TARP γ-2 and a reduced AMPAR/TARP stoichiometry resulting in slightly larger surface glutamate-evoked AMPAR currents, as well as frequent mEPSCs (17, 19). +/*stg* neurons expressing CNIH2 exhibited glutamate responses significantly larger than control (Fig. 3 A_2 and *C*), whereas kainate responses remained unchanged (Fig. 3 A_2 and *B*), resulting in a decrease in kainate efficacy (Fig. 3*D*). This suggests that most of the additional AMPARs trafficked to the surface by CNIH2 were not associated with γ-2. Perhaps in +/*stg* neurons, all available TARPs are already associated with surface AMPARs, leaving only intracellular AMPARs that are not bound to TARPs, and it is those receptors that are available to overexpressed CNIH2 for trafficking to the plasma membrane. If these "TARPless" AMPARs remain associated with CNIH2 on the cell surface, one would expect a low kainate efficacy and a slow mEPSC time course. However, AMPAR mEPSCs recorded from +/*stg* CGNs expressing CNIH2 exhibited no change in amplitude (Fig. 4 *A* and *B*), frequency (Fig. 4 *C*), or kinetics (Fig. 4 *D* and *E*) compared with untransfected cells. Furthermore, using nucleated patches we observed no change in AMPAR deactivation between untransfected CGNs and those expressing CNIH2 (Fig. 4 *F* and *G*). Therefore, CNIH2 can enhance surface expression of AMPARs in neurons, but those surface AMPARs do not display the slow kinetics characteristic of AMPARs coexpressed with CNIH2 in HEK cells.

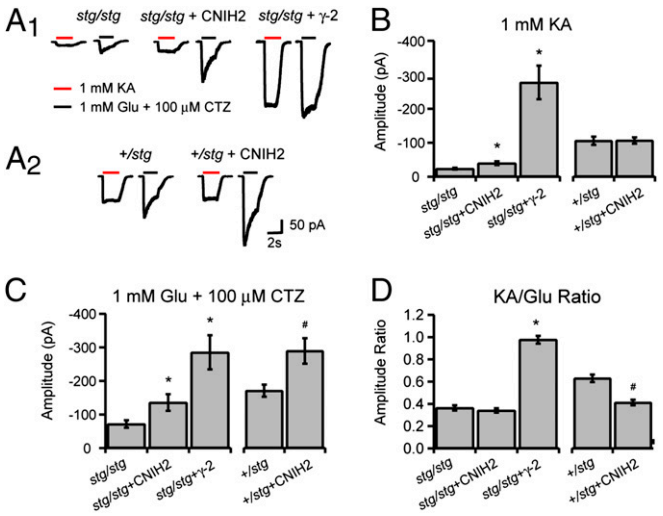

Fig. 3. The effect of CNIH2 on surface AMPAR expression in *stargazer* CGNs. (*A*) Sample records are shown of whole-cell recordings from CGNs on DIV5 during the application of either 1 mM glutamate + 100 μM CTZ, or 1 mM kainate. In *stg/stg* cultures, CNIH2 expression slightly increases responses to both agonists to a lesser extent than TARP γ-2. In +/*stg* cultures, CNIH2 expression increases responses to glutamate, but not kainate, resulting in an apparent decrease in kainate efficacy. (*B*) Whole-cell responses to kainate are quantified. $^{*}P < 0.02$ relative to untransfected *stg/stg*. (*C*) Whole-cell responses to glutamate are quantified. $^{*}P < 0.03$ relative to untransfected *stg/stg*; $^{\#}P < 0.03$ relative to untransfected +/*stg*. (*D*) Kainate efficacy (ratio of whole-cell responses to kainate and glutamate) is quantified. $^{*}P < 0.0001$ relative to untransfected *stg/stg*; $^{\#}P < 0.0001$ relative to untransfected +/*stg*.

If CNIH2 does not modulate AMPAR kinetics in neurons, where does CNIH2 reside in the cell and what might be its function? To address these questions, we performed biochemical characterization comparing CNIH to TARPs in neurons. To characterize CNIHs, we used several CNIH antibodies including one specific for CNIH1, CNIH2, and CNIH3 (Fig. S4). Each of these antibodies recognized the respective CNIH family member expressed in HEK cells (Fig. S4), as well as the FLAG-tagged CNIH2. However, surprisingly, endogenous CNIH expression was barely detectible. In fact, we detected no neuronal CNIH1 or CNIH3 and only very low levels of CNIH2 restricted primarily to hippocampus with a number of nonspecific bands of higher molecular weight (Fig. S4). Using immunoprecipitation, we can isolate and enrich CNIH2 expressed in HEK cells (Fig. S5*A*) as well as endogenous CNIH2 from mature hippocampus (Fig. S5*B*). Comparing CNIH2 immunoprecipitations from various brain regions, we again find CNIH2 expression is low and restricted primarily to hippocampus (Fig. S5*C*), consistent with other reports of CNIH2 distribution in the brain (http://biogps.gnf.org). It is important to note that even with the low expression levels of endogenous CNIH2, we were able to coimmunoprecipitate CNIH2 and GluA1 from hippocampus (Fig. S5 *D–F*) as reported previously (10). However, given the low levels of endogenous CNIH2 and the limited specificity of our antibodies, we focused our biochemical studies to exogenously expressed CNIH2.

We next turned to hippocampal pyramidal neurons, which are known to express CNIH2 (http://biogps.gnf.org) (10, 20, 21) (Fig. S4). Following 1–2 d of CNIH2 expression via biolistic transfection in hippocampal slice culture, we assayed AMPAR channel deactivation and desensitization using rapid glutamate application to outside-out somatic patches. We observed no change in the time course of either channel deactivation (Fig. 4 *H* and *I*), desensitization (Fig. 4 *J* and *K*), or in the size of the peak current (control, 198 ± 44 pA, $n = 9$; CNIH2, 250 ± 51 pA, $n = 11$; $P > 0.05$). If AMPARs were already saturated with CNIH2, one would expect much slower deactivation kinetics than that observed (see Fig. 1*A*) (10). Thus we conclude that CNIH2 does not modulate the kinetics of AMPARs even when CNIH2 is overexpressed substantially above endogenous levels in these neurons (see below).

We next expressed CNIH2 in cultured hippocampal neurons to evaluate its surface expression and binding to GluA1. Using lentivirus, we expressed exogenous CNIH2, TARP γ-2, or TARP γ-8 (Fig. 5 *A–D*) and performed coimmunoprecipitation assays with endogenous GluA1. We found that both CNIH2 and TARPs interact with GluA1 in neurons, but TARPs bind more efficiently (Fig. 5 *A* and *C* and Fig. S5 *D–F*). We next compared the surface expression of CNIH2 and TARPs. Using biotinylation, we found that surface expression of both γ-2 and γ-8 are robust in neurons. In sharp contrast surface expression of CNIH2 was barely detectable (Fig. 5 *B* and *D*). In these experiments KDEL, a protein that is restricted to intracellular membranes, was also negligible.

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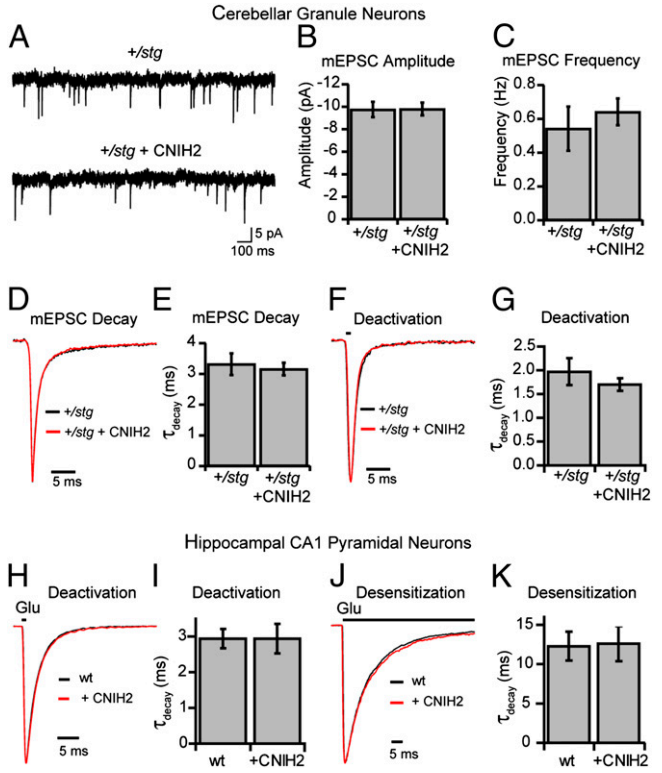

Fig. 4. CNIH2 does not affect AMPAR kinetics in neurons. (*A*) Samples of whole-cell recordings from *+/stg* CGNs on DIV8 demonstrate that AMPAR mEPSCs are readily detectable both in untransfected cells and those expressing CNIH2. mEPSC amplitude (*B*) and frequency (*C*) are unaffected by CNIH2. (*D*) Averaged traces of mEPSCs show no difference in the decay of events from a control neuron (black trace) and a CNIH2 expressing neuron (red trace). (*E*) Graph showing that mEPSC decay is not affected by the expression of CNIH2. (*F*) Averaged traces of deactivation of AMPARs from nucleated patches pulled from cultured CGNs are scaled to their peak amplitudes. (*G*) AMPAR deactivation time course from CGNs is quantified and shows no difference between untransfected *+/stg* neurons and those expressing CNIH2. (*H*) Averaged traces of deactivation of AMPARs from outside-out patches pulled from CA1 pyramidal cell somas in hippocampal slice culture are scaled to their peak amplitudes. (*I*) AMPAR deactivation time course from CA1 neurons is not different between untransfected WT neurons and those expressing CNIH2. (*J* and *K*) Same as in *H* and *I* but for the time course of AMPAR desensitization.

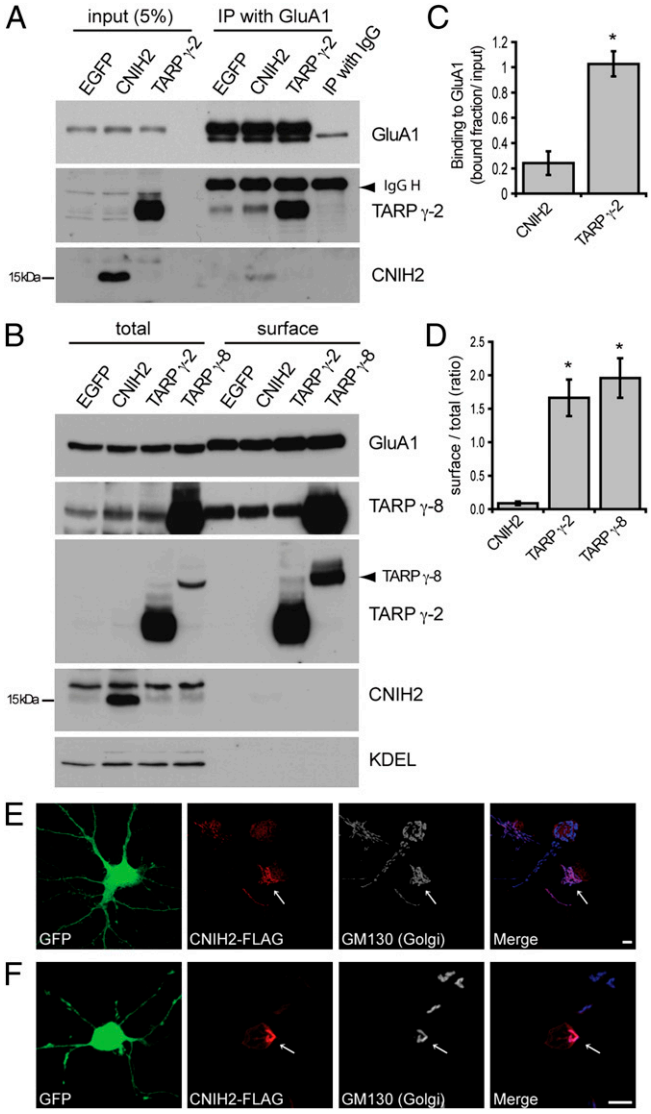

Fig. 5. CNIH2 expressed in neurons interacts with GluA1, but not as efficiently as TARPs and is almost undetectable on the cell surface. (*A* and *B*) CNIH2 or TARPs were expressed in primary hippocampal neurons using lentivirus as described in *SI Materials and Methods*. (*A*) Neurons were solubilized in 1% Triton X-100 lysis buffer, immunoprecipitated with GluA1, and immunoblotted with the indicated antibodies. The arrowhead in TARP γ-2 blot indicates Ig heavy chain (IgG H) bands. (*B*) Neurons were solubilized and surface expression of GluA1, CNIH2, or TARPs was evaluated using a surface biotinylation assay. The arrowhead in TARP γ-2 blot indicates TARP γ-8 due to the cross reactivity of this antibody. (*C*) Quantitation of the bound fractions to GluA1 from *A* was performed by measuring the band intensity of the immunoprecipitated fraction compared with total input (5%). $*P < 0.005$ ($n = 3$). (*D*) Quantitation of surface expression was presented as a ratio of surface to total input (20%), $*P < 0.005$ relative to CNIH2 ($n = 4$). Bars represent mean ± SEM. (*E* and *F*) CNIH2–FLAG was expressed in primary hippocampal neurons (*E*) or CGNs (*F*) and analyzed at DIV17 or DIV6, respectively. Neurons were fixed, permeabilized, and stained with rabbit FLAG (red) and mouse GM130 (white) antibodies as indicated. EGFP (green) was used to visualize the transfected neurons. CNIH2 is colocalized with the *cis*-Golgi marker, GM130 both in hippocampal neurons and CGNs (see arrows). (Scale bar, 5 μm.)

If CNIH2 is not primarily expressed on the cell surface, where is CNIH2 localized in neurons? To address this issue we expressed FLAG-tagged CNIH2 in hippocampal neurons (Fig. 5*E*) and CGNs (Fig. 5*F*) and found that CNIH2 staining overlapped with the *cis*-Golgi marker GM130. These results are in sharp contrast to the distribution of AMPARs and are inconsistent with the majority of CNIH being coassembled with AMPARs in neurons.

Discussion

The goal of this study was to determine whether cornichon proteins are the principle auxiliary subunits of native AMPARs in neurons, rather than TARPs, as was recently proposed (10). We have systematically compared the properties of CNIHs and TARPs both in expression systems and in two neuronal preparations. In HEK cells we find that the prototypical neuronal cornichon CNIH2 binds to AMPARs, is expressed on the cell surface, and enhances AMPAR surface expression. However, TARPs bind to AMPARs more efficiently, are expressed on the cell surface in greater abundance, and enhance the surface expression of AMPAR to a greater extent. Both CNIH2 and TARPs modulate kinetics of AMPARs in HEK cells, but, notably, CNIH2 profoundly slows the deactivation and desensitization of AMPARs, compared with the modest slowing caused by TARPs. Thus, from the studies in heterologous cells, it appears that the most striking role for CNIH2 is functional regulation of AMPARs.

Western blotting failed to detect CNIH1 and CNIH3 in brain, and CNIH2 was detected primarily in the hippocampus, but only at very low levels, consistent with the regional distribution of CNIH2

mRNA in mouse (http://biogps.gnf.org). Thus, in contrast to the wide distribution of TARPs throughout the brain, CNIHs display a restricted distribution and are much less abundant than TARPs. In CGNs, but not in hippocampal neurons, we show that overexpression of CNIH2 results in a modest increase in the surface expression of AMPARs. However, the biophysical properties of those receptors show no sign of their being functionally associated with CNIH2. Furthermore, in contrast to HEK cells, CNIH2 is essentially undetectable on the surface of neurons. Given the known function of the *Drosophila* and yeast homologs of CNIHs as ER chaperones (12, 13), it is possible that CNIH2 could facilitate the exit of AMPARs from the ER, but does not remain associated with AMPARs delivered to the neuronal plasma membrane. Regardless of what the actual role of CNIHs in neurons is, our data indicate that TARPs, not CNIHs, are the primary auxiliary AMPAR subunits in the brain.

Our results in HEK cells demonstrate that both CNIHs and TARPs can interact functionally with the same AMPAR channel. We also establish that CNIHs share with TARPs the ability to reduce block of GluA2-lacking AMPARs by spermine. CNIH2 also increases AMPAR single channel conductance similarly to TARPs. However, CNIHs differ from TARPs in that they only minimally increase the efficacy of the partial agonist kainate (7, 9) and fail to convert the competitive antagonist CNQX into a partial agonist (18, 22).

In contrast to the study of CNIHs expressed in HEK cells, our neuronal studies indicate that CNIHs do not play a major role in functionally regulating endogenous AMPARs. Despite the claim by Schwenk et al. (10) that the majority of AMPARs in the brain are associated with CNIHs and not TARPs, none of our findings support this claim. First, in contrast to TARPs, the expression of CNIHs in brain is extremely limited. Second, using a variety of approaches and neuronal cell types and preparations, we find no effect of CNIHs on AMPAR kinetics. This is in contrast to the consistent and profound effect that TARPs have on AMPAR trafficking, pharmacology, and kinetics in neurons. For example, we have previously demonstrated that diverse neuronal cell types in the brain exhibit high kainate efficacy and depolarize in response to CNQX application (18), suggesting that the vast majority of surface-expressed AMPARs in the brain are associated with TARPs. In summary, the longstanding enigma of the inability of AMPARs expressed in heterologous cell systems to mimic the properties of neuronal AMPARs was resolved by the discovery of TARPs. In striking contrast, the profound slowing of AMPAR deactivation by CNIH2 has no known counterpart with the neuronal AMPARs examined in this study.

The findings in this study raise two related questions. First, why does the expression of CNIHs modify the kinetics of surface AMPARs in HEK cells but not neurons? Second, what might be the benefit of CNIHs modifying AMPAR kinetics in neurons if the interaction only occurs in the ER/*cis*-Golgi? The difference between neurons and HEK cells with respect to trafficking CNIHs to the cell surface is not clear and presumably reflects differences in the mechanisms that regulate CNIH trafficking from ER to Golgi. Recent work on the biosynthesis of AMPARs may provide some insight into the second question. It has been proposed that during AMPAR biosynthesis, glutamate-induced conformational changes in the ER serve as early trafficking checkpoints to ensure that only functional receptors are exported to the cell surface (23–27). It may be a common function of CNIHs and TARPs to participate in glutamate-induced conformational changes by immature AMPARs, which then disengage from CNIHs but remain bound to TARPs as they are delivered to the plasma membrane and then targeted to synapses. However, even if CNIHs were to have a role in AMPAR trafficking, this role is modest and variable among neuronal type compared with the role of TARPs (28).

Materials and Methods

All of the CNIH cDNAs were subcloned in pIRES2–EGFP vector (Clontech). They were cotransfected in HEK cells with GluA1 to test their effects in changing AMPAR physiological properties. Their functional effects on endogenous AMPAR in neurons were tested by transfecting them in cultured cerebellar granule neurons and CA1 pyramidal neurons in cultured hippocampal slices. Immunoblotting and immunoprecipitation were used to analyze the coassembly of AMPARs and CNIH2 or TARPs. Surface expression of transfected HEK cells and neurons was analyzed using a surface biotinylation assay (29). To effectively transfect TARPs and CNIH2 in neurons for biochemical purpose, we used FUGW lentivirus vector (29). The localization of expressed CNIH2 was examined in cultured cerebellar granule neurons and hippocampal neurons using a FLAG-tagged CNIH2 and immunostaining. Detailed protocols for electrophysiological recording and biochemical experiments are provided in *SI Materials and Methods*.

1. Nicoll RA, Tomita S, Bredt DS (2006) Auxiliary subunits assist AMPA-type glutamate receptors. *Science* 311:1253–1256.
2. Osten P, Stern-Bach Y (2006) Learning from stargazin: The mouse, the phenotype and the unexpected. *Curr Opin Neurobiol* 16:275–280.
3. Ziff EB (2007) TARPs and the AMPA receptor trafficking paradox. *Neuron* 53:627–633.
4. Milstein AD, Nicoll RA (2008) Regulation of AMPA receptor gating and pharmacology by TARP auxiliary subunits. *Trends Pharmacol Sci* 29:333–339.
5. Chen L, et al. (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408:936–943.
6. Hashimoto K, et al. (1999) Impairment of AMPA receptor function in cerebellar granule cells of ataxic mutant mouse stargazer. *J Neurosci* 19:6027–6036.
7. Tomita S, et al. (2005) Stargazin modulates AMPA receptor gating and trafficking by distinct domains. *Nature* 435:1052–1058.
8. Priel A, et al. (2005) Stargazin reduces desensitization and slows deactivation of the AMPA-type glutamate receptors. *J Neurosci* 25:2682–2686.
9. Turetsky D, Garringer E, Patneau DK (2005) Stargazin modulates native AMPA receptor functional properties by two distinct mechanisms. *J Neurosci* 25:7438–7448.
10. Schwenk J, et al. (2009) Functional proteomics identify cornichon proteins as auxiliary subunits of AMPA receptors. *Science* 323:1313–1319.
11. Roth S, Neuman-Silberberg FS, Barcelo G, Schüpbach T (1995) Cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. *Cell* 81:967–978.
12. Bökel C, Dass S, Wilsch-Bräuninger M, Roth S (2006) Drosophila Cornichon acts as cargo receptor for ER export of the TGFalpha-like growth factor Gurken. *Development* 133:459–470.
13. Castillon GA, Watanabe R, Taylor M, Schwabe TM, Riezman H (2009) Concentration of GPI-anchored proteins upon ER exit in yeast. *Traffic* 10:186–200.
14. Jackson AC, Nicoll RA (2009) Neuroscience: AMPA receptors get 'pickled'. *Nature* 458: 585–586.
15. Tigaret C, Choquet D (2009) Neuroscience. More AMPAR garnish. *Science* 323:1295–1296.
16. Soto D, Coombs ID, Kelly L, Farrant M, Cull-Candy SG (2007) Stargazin attenuates intracellular polyamine block of calcium-permeable AMPA receptors. *Nat Neurosci* 10: 1260–1267.
17. Shi Y, Lu W, Milstein AD, Nicoll RA (2009) The stoichiometry of AMPA receptors and TARPs varies by neuronal cell type. *Neuron* 62:633–640.
18. Menuz K, Stroud RM, Nicoll RA, Hays FA (2007) TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists. *Science* 318:815–817.
19. Milstein AD, Zhou W, Karimzadegan S, Bredt DS, Nicoll RA (2007) TARP subtypes differentially and dose-dependently control synaptic AMPA receptor gating. *Neuron* 55:905–918.
20. Lein ES, et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445:168–176.
21. Wu C, et al. (2009) BioGPS: An extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 10:R130.
22. Kott S, Sager C, Tapken D, Werner M, Hollmann M (2009) Comparative analysis of the pharmacology of GluR1 in complex with transmembrane AMPA receptor regulatory proteins gamma2, gamma3, gamma4, and gamma8. *Neuroscience* 158:78–88.
23. Priel A, Selak S, Lerma J, Stern-Bach Y (2006) Block of kainate receptor desensitization uncovers a key trafficking checkpoint. *Neuron* 52:1037–1046.
24. Greger IH, Akamine P, Khatri L, Ziff EB (2006) Developmentally regulated, combinatorial RNA processing modulates AMPA receptor biogenesis. *Neuron* 51:85–97.
25. Penn AC, Williams SR, Greger IH (2008) Gating motions underlie AMPA receptor secretion from the endoplasmic reticulum. *EMBO J* 27:3056–3068.
26. Fleck MW (2006) Glutamate receptors and endoplasmic reticulum quality control: Looking beneath the surface. *Neuroscientist* 12:232–244.
27. Coleman SK, et al. (2009) Agonist occupancy is essential for forward trafficking of AMPA receptors. *J Neurosci* 29:303–312.
28. Vandenberghe W, Nicoll RA, Bredt DS (2005) Interaction with the unfolded protein response reveals a role for stargazin in biosynthetic AMPA receptor transport. *J Neurosci* 25:1095–1102.
29. Suh YH, et al. (2010) A neuronal role for SNAP-23 in postsynaptic glutamate receptor trafficking. *Nat Neurosci* 13:338–343.