Channel properties reveal differential expression of TARPed and TARPl-less AMPARs in stargazer neurons

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Dynamic regulation of calcium-permeable AMPA receptors (CP-AMPARs) is important for normal synaptic transmission, plasticity and pathological changes. Although the involvement of transmembrane AMPAR regulatory proteins (TARPs) in trafficking of calcium-impermeable AMPARs (CI-AMPARs) has been extensively studied, their role in the surface expression and function of CP-AMPARs remains unclear. We examined AMPAR-mediated currents in cerebellar stellate cells from stargazer mice, which lack the prototypical TARP stargazin (γ-2). We found a marked increase in the contribution of CP-AMPARs to synaptic responses, indicating that, unlike CI-AMPARs, these can localize at synapses in the absence of γ-2. In contrast with CP-AMPARs in extrasynaptic regions, synaptic CP-AMPARs displayed an unexpectedly low channel conductance and strong block by intracellular spermine, suggesting that they were 'TARPless'. As a proof of principle that TARP association is not an absolute requirement for AMPAR clustering at synapses, miniature excitatory postsynaptic currents mediated by TARPl-less AMPARs were readily detected in stargazer granule cells following knockdown of their only other TARP, γ-7.

A majority of fast excitatory synaptic transmission in the CNS is mediated by AMPARs. The subunits forming these receptors (GluA1–4) assemble as homo- or hetero-tetramers, the functional properties of which depend on their precise composition. In addition to these core subunits, the native receptors contain TARPs (γ-2, γ-3, γ-4, γ-5, γ-7 and γ-8). These accessory subunits have pivotal roles in the neuronal trafficking of AMPARs, promoting their maturation, delivery to the cell surface and accumulation at synapses. In particular, TARPs can interact, via their C-tail, with PDZ-containing proteins of the postsynaptic scaffold, such as PSD-95. This interaction is generally thought to be crucial for AMPAR synaptic clustering. TARPs also regulate many functional properties of AMPARs, slowing deactivation and desensitization and increasing single-channel conductance. The various TARP isoforms differ in their influence on AMPAR properties and display distinct, yet partially overlapping, patterns of expression in the brain. This large family of auxiliary subunits adds greatly to the functional diversity of AMPARs in neurons and glia. Most fast excitatory transmission in the brain is mediated by GluA2-containing CI-AMPARs. However, it has become apparent that CP-AMPARs are more widespread than was originally thought, contributing to normal transmission at numerous synapses and having a key role in several important forms of plasticity. Furthermore, the disordered regulation of CP-AMPARs is linked to a wide variety of neurological conditions. Despite their importance in normal and pathological states, the molecular mechanisms that regulate CP-AMPAR trafficking to the neuronal membrane and control synaptic clustering remain unclear.

The prototypical TARP, stargazin (γ-2), is intensely expressed in the cerebellum. In the stargazer (stg/stg) mouse, which has a mutation affecting the Caeng2 gene encoding stargazin and lacks functional protein, cerebellar granule cells display a characteristic loss of surface AMPARs. We investigated the effect of the stargazer mutation on the functional expression of AMPARs in cerebellar stellate cells. Similar to granule cells, these neurons contain both γ-2 and the atypical TARP γ-7. In contrast with CP-AMPARs in extrasynaptic regions, synaptic CP-AMPARs displayed an unexpectedly low channel conductance and strong block by intracellular spermine, suggesting that they were 'TARPless'. As a proof of principle that TARP association is not an absolute requirement for AMPAR clustering at synapses, miniature excitatory postsynaptic currents mediated by TARPl-less AMPARs were readily detected in stargazer granule cells following knockdown of their only other TARP, γ-7.

RESULTS

Increased EPSC rectification in stg/stg stellate cells

To investigate the role of γ-2 in the expression of CP-AMPARs at parallel fiber–stellate cell synapses, we first recorded evoked excitatory postsynaptic currents (eEPSCs) from stg/stg mice. Parallel fiber stimulation reliably evoked currents (Fig. 1a), but usually required stimulus

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Figure 1 Loss of γ-2 increases EPSC rectification in stellate cells. (a) AMPAR-mediated eEPSCs are strongly rectifying in stg/stg stellate cells. Representative parallel fiber-evoked synaptic currents in two stellate cells in cerebellar slices from a control mouse (left) and a stg/stg mouse (right). Currents are averaged responses at −80, −60, −40, 0 and +40 mV. (b) Corresponding I-V relationships normalized to −80 mV. The fitted curves are fifth-order polynomials. (c) Pooled data showing rectification index values determined as the ratio of synaptic conductance at +40 and −60 mV. Box-and-whisker plots indicate the median value (red line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); open circles represent individual values. The rectification index was significantly less in stg/stg than in control stellate cells (n = 8 cells from 5 animals and 9 cells from 4 animals, respectively, **P < 0.01). (d) Rectification of stg/stg mEPSCs persists in presence of wild-type innervation. Representative recordings of mEPSCs from cultured stellate cells at −80 and +60 mV are shown. Traces are from a control cell (top) and a stg/stg cell (bottom). Traces to the right show summed mEPSCs from equivalent time periods at the two voltages. (e) Global averages of normalized summed mEPSCs (five control cells and nine stg/stg cells from two cultures). Shaded areas denote s.e.m. (f) Pooled data showing rectification index values determined as the ratio of summed peak conductance at +60 and −80 mV. Open circles represent individual values. The rectification index was significantly less in stg/stg compared with control cells (**P < 0.001).

Intensities higher than those used in slices from control littersmates. Thus, even in the absence of γ-2, AMPARs were capable of clustering at parallel fiber–stellate cell synapses. In the presence of intracellular spermine (100 µM), which produces a voltage-dependent block of CP-AMPARs, eEPSCs displayed I-V relationships that were strongly rectifying (Fig. 1b,c). On average, the rectification index (+40/−60 mV; see Online Methods) was less than half that found in control cells (Supplementary Table 1). Although increased EPSC rectification is likely to reflect an increased AMPAR/CI-AMPAR ratio, it could have another possible origin. We have previously shown that polyamine block of CP-AMPARs is attenuated by TARP association and that the magnitude of the relief may differ between TARP subtypes. TARP γ-7 is normally expressed along with γ-2 in stellate cells. In recordings from recombinant receptors expressed in tsA201 cells, we found that γ-7 was ~50% less effective than γ-2 at relieving polyamine block of homomeric GluA3 CP-AMPARs (Supplementary Fig. 1a). Thus, an increase in rectification in stg/stg would be expected if γ-2-containing CP-AMPARs were replaced by γ-7-containing assemblies. However, given the rectification changes that we observed in cultured stellate cells and the blocking action of philanthotoxin (PHTx-433, see below), we feel that this possibility is excluded.

It has been shown that the relative expression of CI-AMPARs and CP-AMPARs at stellate cell synapses can be regulated by parallel fiber activity. As cerebellar granule cells show a near-complete loss of surface AMPA receptors in stg/stg mice, the activity-dependent release of glutamate from parallel fibers (granule cell axons) may be decreased in vivo. It could be argued that this, rather than the postsynaptic loss of γ-2, caused the observed increase in stellate cell EPSC rectification. We therefore asked whether the increased EPSC rectification remained when stg/stg stellate cells were innervated by normal γ-2-containing granule cells. We examined dissociated cultures of cerebellar neurons prepared from stg/stg Gad2-egfp mice or their control littersmates. In these cultures, stellate cells express eGFP and can therefore be identified among granule cells. Neurons were isolated from postnatal day 7 (P7) mice, a developmental stage at which parallel fiber–stellate cell synapses contain mainly CP-AMPARs. To provide stg/stg stellate cells with normal excitatory input, we seeded both control and stg/stg cultures with a large excess of wild-type granule cells. After 7–9 d in vitro, under conditions that promote synaptic maturation (see Online Methods), mEPSCs from control cells displayed linear I-V relationships, suggesting a developmental shift to CI-AMPARs. In contrast, mEPSCs recorded from stg/stg stellate cells displayed marked inward rectification (Fig. 1c–f). This result strongly suggests that the altered rectification observed in stg/stg stellate cells in slices is indeed cell-autonomous. Moreover, these results argue strongly against the simple replacement of γ-2-containing CP-AMPARs by γ-7-containing CP-AMPARs. mEPSCs from control cells in culture displayed I-V relationships that were linear. As TARPs confer only a partial relief of polyamine block, such linear responses can arise only from CI-AMPARs. Thus, the difference between control and stg/stg stellate cells in culture is caused mainly by an altered CP-AMPAR/CI-AMPAR ratio. These results suggest that γ-2 is important for the expression of CI-AMPARs at these synapses.

Reduced qEPSC amplitude in stg/stg cells

We next recorded synaptic currents from stellate cells in slices in the presence of strontium (see Online Methods). In these conditions, parallel fiber stimulation evoked asynchronous transmitter...
Figure 2 Amplitude and kinetic properties of qEPSCs in control and stg/stg stellate cells. (a) Representative qEPSCs evoked in a control stellate cell by parallel fiber stimulation (arrow) in the presence of 5 mM SrCl2. The period immediately following stimulation is enlarged in the lower panel. Synaptic events occurring >10 ms after stimulation and exceeding the detection threshold (red dashed line) are indicated by arrowheads. (b) Amplitude distribution (open bars) of all selected events from the cell shown in a. Background variance is indicated by the baseline all-point amplitude distribution (gray bars) fitted with a Gaussian. The inset shows the average qEPSC. (c,d) Data are presented as in a for qEPSCs evoked in a stg/stg stellate cell. (e) Cumulative probability distributions for qEPSC amplitudes in control (blue) and stg/stg stellate cells (red). The averaged distributions are shown in bold with filled areas representing s.e.m. (f) Pooled data for qEPSC amplitudes and their coefficients of variation (n = 17 and 15 cells, from 14 and 13 animals, both ***P < 0.001). Box-and-whisker plots indicate the median value (red line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); open circles represent individual values. (g) Superimposed global mean control and stg/stg qEPSCs. Shaded areas denote s.e.m. (h) Pooled data for rise-time and decay measures (n = 18 and 8, *P < 0.05).

release, resulting in isolated quantal EPSCs (qEPSCs) that were readily identified, both in control and stg/stg cells (Fig. 2a–d). This allowed us to examine postsynaptic changes in isolation from possible presynaptic effects of the stargazer mutation37 and to measure synaptic currents without contamination by glutamate spillover onto surrounding extrasynaptic AMPARs38. To analyze the distribution of qEPSC amplitudes, we excluded any overlapping events that appeared to arise from the release of more than one quantum.

In control cells, individual qEPSCs displayed a wide range of amplitudes (typically up to 150 pA at −80 mV; Fig. 2a,b). In contrast, in stg/stg stellate cells, qEPSCs showed much less variation, with events having amplitudes that were typically less than 50 pA (Fig. 2c,d). Notably, the amplitude distributions were clearly distinct from the background noise (Fig. 2b,d), indicating that small events were adequately resolved. Across cells, the amplitude distributions from stg/stg cells were consistently narrower than those from control cells (Fig. 2e), with a reduced coefficient of variation (Fig. 2f). Although the average amplitude of qEPSCs was markedly reduced in stg/stg stellate cells (Fig. 2f,g), the kinetic parameters were altered only slightly, with a small, but significant, increase in rise time (P = 0.026) and no change in decay time (P = 0.72) (Fig. 2g,h and Supplementary Table 1).

Increased prevalence of CP-AMPARs at stg/stg synapses

Consistent with the inward rectification of control eEPSCs, the peak conductance of qEPSCs recorded in control cells at +60 mV was significantly less than that at −80 mV (−35%, P = 0.036; Fig. 3a–c). In addition, there was a visible reduction in the frequency of qEPSCs at positive potentials (Fig. 3d). In stg/stg cells, the peak conductance of qEPSCs was not significantly different from positive and negative potentials (P = 0.059; Fig. 3b,c), but the decrease of qEPSC frequency at positive potentials was much greater than that in control (Fig. 3d and Supplementary Table 1).

The reduced frequency of qEPSCs at +60 mV could arise if some events were suppressed by intracellular polyamine blocker. Alternatively, events might be less readily detected as a result of the lower driving force. We therefore made additional recordings with spermine-free intracellular solution. In these conditions, qEPSC frequency was similar at positive and negative potentials (the relative frequencies at +60 versus −80 mV were 1.13 ± 0.07 in control and 1.12 in stg/stg, n = 4 and 2, respectively). Notably, the background noise was similar at the two voltages: the s.d. of the baseline current in control cells (see Online Methods) was 2.8 ± 0.3 pA at −80 mV versus 3.2 ± 0.4 pA at +60 mV (n = 6). The corresponding values for stg/stg stellate cells were 2.4 ± 0.1 and 2.7 ± 0.21 pA (n = 5). Thus, the reduction in qEPSC frequency at +60 mV appears to be dependent on the presence of intracellular spermine, suggesting that some qEPSCs are mediated mainly (or solely) by CP-AMPARs.

If CP- and CI-AMPARs occur in similar relative proportions at all synapses in a cell, the qEPSCs detected at +60 mV would arise from those synapses that generate the largest events at −80 mV. Thus, the rectification measured from such events would match the average rectification measured from qEPSCs. However, our results suggest that this is not the case, as the rectification calculated using all of the events detected at +60 mV and a matching number of the largest events at −80 mV (see Online Methods) was considerably less than the rectification of eEPSCs and was not different between stg/stg and control synapses (Supplementary Table 1). This suggests that there are populations of synapses in both control and stg/stg stellate cells with different sensitivity to polyamines. However, synapses containing strongly rectifying CP-AMPARs are prevalent in stg/stg stellate cells, with a large proportion of synapses failing to generate any detectable current at +60 mV.

To estimate the relative proportion of CP-AMPARs across synapses, we calculated the rectification index as the ratio of summed qEPSC peak conductances from equal numbers of sweeps at +60 mV and −80 mV. This method takes into account the full range of possible rectification at individual synapses, including those at which no
currents could be detected at +60 mV as a result of a complete block by spermine (rectification index of 0). We found that the rectification index in stg/stg stellate cells was reduced to one quarter of that seen in control cells (Fig. 3e and Supplementary Table 1). These rectification values were comparable to the rectification measurements from eEPSCs, and confirmed an increased expression of CP-AMPARs at parallel fiber synapses in stg/stg stellate cells.

We reasoned that if the proportion of qEPSCs mediated by CP-AMPARs is greater in stg/stg stellate cells, QEPSCs should display a higher single-channel conductance 14,40,41. Notably, the decrease in channel conductance almost exactly matched the reduction in synaptic AMPARs in stg/stg synapses (Fig. 4e). Such a reduction in AMPAR-mediated currents could arise from a decrease in AMPAR surface expression, a change in the single-channel conductance or a modification of channel gating properties. To determine the conductance properties of synaptic AMPAR channels, we performed peak-scaled nonstationary fluctuation analysis on qEPSCs (ps-NSFA; Online Methods and Fig. 4a–d).

Synaptic AMPARs in stg/stg stellate cells displayed weighted mean conductance values ~50% lower than those in control cells (Fig. 4e). This was unexpected; our results indicate that these synapses contain mainly CP-AMPARs, and we would therefore predict that the receptors would exhibit a high single-channel conductance14,40,41. Notably, the decrease in channel conductance almost exactly matched the reduction that we observed in the peak amplitude of qEPSCs (Fig. 2f and Supplementary Table 1). As γ-2 does not affect the peak open probability of AMPARs14, our results suggest that the number of receptors activated by a quantum of transmitter is similar at stg/stg and control stellate cell synapses, despite the marked differences in mean qEPSC amplitudes. The low single-channel conductance and high sensitivity to block by internal spermine (at +60 mV) of synaptic AMPARs in

**Figure 3** Enhanced block by intracellular spermine and extracellular PhTx-433 of parallel fiber-evoked qEPSCs in stg/stg stellate cells. (a) qEPSCs recorded at −80 mV (left) and +60 mV (right) from a control stellate cell. Arrowheads indicate events occurring >10 ms after stimulation and exceeding the detection threshold. Asterisks in the upper sweep denote events arising from apparent superimposition of two qEPSCs. (b) qEPSCs recorded at −80 mV (left) and +60 mV (right) from a control stellate cell. Arrowheads indicate events occurring >10 ms after stimulation and exceeding the detection threshold. Asterisks in the upper sweep denote events arising from apparent superimposition of two qEPSCs. (c) Equivalent traces recorded from a representative stg/stg stellate cell. (c) Pooled data showing voltage-dependent changes in qEPSC peak conductance in control and stg/stg stellate cells. Note the much smaller conductance at −80 mV of stg/stg compared with control qEPSCs (n = 6 and 5 cells, from 5 animals each, **P < 0.01) and the significantly reduced conductance at +60 mV in control cells (n = 5, *P < 0.05). Box-and-whisker plots indicate the median value (red line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); open circles represent individual values. (d) Pooled data showing the greater depolarization-induced reduction in qEPSC frequency in stg/stg stellate cells (**P < 0.01). (e) Pooled data showing rectification index (see Online Methods). Note the significantly increased rectification in stg/stg stellate cells (**P < 0.01). (f) Time course of the effect of bath-applied PhTx-433 (10 μM) on qEPSC charge per parallel fiber stimulus in a representative control stellate cell. Right, box-and-whisker plots of the charge transfer measured over time periods 1 and 2 (left, gray lines) before and after PhTx-433 application (**P < 0.0001). (g) Time course of PhTx-433 action in a representative stg/stg stellate cell. Note the large fraction of failures in time period 2 and its effect on the qEPSC charge transfer per stimulus (right, ***P < 0.0001). (h–j) Pooled data showing the effect on control and stg/stg stellate cells of PhTx-433 on qEPSC charge per stimulus (n = 5 cells from 4 animals and 6 cells from 5 animals, respectively, *P < 0.05), qEPSC peak amplitude and qEPSC frequency (**P < 0.01).
Extrasynaptic γ-7–associated CP-AMPARs in stg/stg cells

Given that AMPARs diffuse in the neuronal membrane and are continuously exchanged between synaptic and extrasynaptic sites, one might expect that extrasynaptic CP-AMPARs in stg/stg stellate cells would also lack an associated TARPs. To investigate this and to determine the properties of extrasynaptic AMPARs, we used ultrafast application of glutamate onto somatic outside-out patches (10 mM, 100 ms; see Online Methods). Glutamate-evoked macroscopic currents were kinetically similar in stg/stg and control cells (Fig. 5a–c), with peak currents that displayed marked inward rectification (+60/−60 mV; Fig. 5d). As at synaptic sites, rectification was greatest in patches from stg/stg stellate cells. Notably, the rectification in somatic patches from control mice was indicative of the presence of a substantial population of extrasynaptic CP-AMPARs. This finding differs from earlier studies, which have suggested that extrasynaptic AMPARs in stellate cells are calcium impermeable. This is probably because these studies did not use the NMDAR antagonist d-2-amino-5-phosphonopentanoic acid (d-AP5) to limit elevation of the intracellular calcium concentration during slicing. NMDAR activation is known to increase GluA2 expression in stellate cells and is therefore likely to increase basal expression of extrasynaptic CI-AMPARs.

Having established that CP-AMPARs are prevalent in extrasynaptic patches of both control and stg/stg stellate cells, we next applied NSF to somatic currents to determine whether these displayed properties of TARPed or TARPlless receptors. The estimated weighted mean single-channel conductance of the extrasynaptic AMPARs was similarly high in stg/stg and control cells (stg/stg, 27.5 ± 1.2 pS; n = 9; control, 26.2 ± 3.2 pS; n = 8; Fig. 5e), consistent with the view that extrasynaptic CP-AMPARs are TARPed in stg/stg cells. However, these conductance values are lower than those that we obtained with γ-2- or γ-7–associated recombinant CP-AMPARs (Supplementary Table 2). This likely reflects the presence of multiple AMPAR subtypes (CP- and CI-AMPARs) in the extrasynaptic membrane of both stg/stg and control stellate cells. To address this issue and to provide direct evidence as to whether TARPed CP-AMPARs were indeed present extrasynaptically, we next turned to single-channel analysis.

In both control and stg/stg patches, we resolved single-channel events following the initial glutamate-evoked macroscopic current (Fig. 6a–g). We reasoned that, even if a variety of AMPARs were present, the most readily detectable events would be those mediated by CP-AMPARs. We therefore used single-channel recordings from the soma of control and stg/stg stellate cells to analyze the currents that displayed marked inward rectification (+60/−60 mV).

Figure 4 Single-channel conductance of synaptic AMPARs is reduced in stg/stg stellate cells. (a) Parallel fiber-evoked qEPSCs recorded in a representative control stellate cell at −80 mV. Individual events (thin gray traces) were aligned at their point of steepest rise and averaged (thick blue line). The lower panel is a color-coded image of all events. (b) Corresponding current-variance relationship. The dashed line indicates the background current variance. The weighted-mean unitary current (i) and the number of channels open at the peak (N) were estimated from the parabolic fit. (c,d) Data are presented as in a and b, but for a representative stg/stg stellate cell. Note the typically smaller qEPSC amplitude. (e) Pooled data showing the reduced single-channel conductance in stg/stg stellate cells (n = 18 cells from 16 animals and 8 cells from 7 animals, **P < 0.01). Box-and-whisker plot indicates the median value (red line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); open circles represent individual values.

Figure 5 Extrasynaptic AMPARs in stg/stg stellate cells exhibit increased rectification and large single-channel conductance. (a) Representative averaged current evoked by ultrafast application of 10 mM glutamate (100 ms) to an outside-out somatic patch (+60 mV) evoked from a stellate cell in a slice from a control mouse. Inset shows corresponding current-variance plot. Symbols denote mean variance in each of ten equally spaced amplitude bins. Vertical error bars denote s.e.m. The weighted-mean single-channel current (i) and the number of channels in the patch (N) are calculated from a weighted parabolic fit to the data. (b) Data are presented as in a, but for a stg/stg stellate cell. (c) Pooled data showing the similar desensitization time course (t_des) of the currents from control and stg/stg stellate cells (n = 11 cells from 9 animals and 8 cells from 6 animals). Box-and-whisker plot indicates the median value (red line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); open circles represent individual values. (d) Pooled data showing the greater rectification of extrasynaptic AMPARs in stg/stg stellate cells (n = 9 and 10 cells, from 7 animals each, **P < 0.05). Rectification index was calculated as the ratio of mean peak currents at +60 and −60 mV. (e) Pooled data showing the large single-channel conductance determined in both control and stg/stg stellate cells (n = 9 and 8 cells, from 7 and 5 animals).
by large-conductance CP-AMPARs, which should be unambiguously distinguished by their susceptibility to block by intracellular spermine. Indeed, although clear single-channel currents were observed at −60 mV, only brief, flickery openings were seen at +60 mV, consistent with a voltage-dependent channel block (Fig. 6h). In control cells, chord conductances of single-channel events at −60 mV ranged from 11.5–52.7 pS, with an average of 35.2 ± 1.8 pS (n = 8; Fig. 6a–c,g). In stg/stg cells, the corresponding values ranged from 9.1–56.7 pS, with an average of 31.6 ± 2.2 pS (n = 7; Fig. 6d–g and Supplementary Table 2).

The large openings recorded in stg/stg stellate cells almost certainly arise from γ-7–associated CP-AMPARs. Our experiments on recombinant receptors confirmed that γ-7 enhances the conductance of CP-AMPARs to the same extent as γ-2 (Supplementary Fig. 1f). Notably, only TARPed CP-AMPARs displayed openings larger than 40 pS (see Supplementary Fig. 1b). Such large channel events were...
recorded in six of seven stg/stg patches, suggesting that TARPed CP-AMPARs contribute substantially to extrasynaptic currents. Furthermore, most extrasynaptic CP-AMPARs in stg/stg cells would appear to be TARPed, as average single-channel conductances were similar in control and stg/stg patches (Supplementary Table 2).

The mean conductance of directly resolved single-channels in control stellate cells (27.6 ± 1.3 pS, n = 8) was ~30% higher than our estimate obtained from NSFA in the same patches (P = 0.008). Although not statistically significant, a similar trend was apparent in patches from stg/stg cells (25.9 ± 3.6 pS, n = 7, P = 0.078). Notably, when applied to homogenous populations of recombinant CP-AMPARs in heterologous cells (both TARPed and TARPPless), these two methods of analysis yielded very similar conductance estimates (Supplementary Table 3). It is likely that the lower estimate obtained with NSFA in stellate cell patches reflects a small proportion of low-conductance CI-AMPARs that contributed to the weighted-mean conductance, but were not resolved during selection of single-channel events (Fig. 6a,d). Our results suggest that, as in control stellate cells, stg/stg cells express mostly TARPed CP-AMPARs in their extrasynaptic membrane, together with some CI-AMPARs. The increased rectification that we observed in stg/stg patches is consistent with the view that CP-AMPARs are co-assembled with γ-7, which is less effective than γ-2 at relieving polyamine block (Supplementary Fig. 1a).

As 6-cyano-7-nitroquinazoline-2,3-dione (CNQX) has been shown to act as a partial agonist on TARPP-associated AMPARs, we sought to confirm the presence of such AMPARs in the plasma membrane of stg/stg stellate cells by recording CNQX-evoked responses. As expected, bath application of CNQX evoked clear inward currents in control cells (Fig. 7a). In contrast, CNQX failed to produce a current in stg/stg cells (Fig. 7b). This would seem to be inconsistent with the idea that extrasynaptic AMPARs are associated with a TARP. However, the action of CNQX on γ-7-associated AMPARs is not known. We therefore considered whether this TARP might differ from conventional ones (γ-2, γ-3, γ-4 and γ-8) in its ability to confer partial agonist activity on CNQX. We transfected tsA201 cells with AMPAR subunits (GluA1, GluA3 or GluA4) alone or with γ-2 or γ-7, and compared responses activated by fast application of glutamate or CNQX to outside-out membrane patches. Although glutamate produced large currents in each case, CNQX produced detectable currents only in those cells expressing γ-2 (Fig. 7c,d), providing a clear functional distinction between γ-7 and other TARPs. Together, the large single-channel conductance of CP-AMPARs in stg/stg somatic patches, their strong block by internal spermine and their failure to respond to CNQX support the view that extrasynaptic AMPARs in stg/stg stellate cells are predominantly calcium permeable and are associated specifically with γ-7.

TARPless AMPARs mediate mEPSCs in stg/stg granule cells

Our results suggest that, although γ-7-associated CP-AMPARs are expressed at the surface of stg/stg stellate cells, synaptic currents are mediated by channels displaying properties that are characteristic of TARPPless CP-AMPARs. However, this interpretation is based on correlative evidence. We therefore sought to test more directly the principle that TARP-association is not an absolute requirement for AMPAR localization at synapses by acutely disrupting γ-7 expression. Because stellate cells occur at relatively low density in culture, making it difficult to demonstrate TARP knockdown biochemically, we turned to cerebellar granule cells, the neurons in which the critical importance of TARPs in synaptic transmission was first revealed. Similar to stellate cells, granule cells express only γ-2 and γ-7 (refs. 2,5,29; see Fig. 3 of ref. 18) and lack synaptic currents in stg/stg mice.

We reasoned that if TARPPless AMPARs are capable of reaching synapses, the absence of mEPSCs in stg/stg granule cells must be a result of endogenous γ-7 interfering with this process. In which case, knockdown of γ-7 should make TARPPless AMPARs available for synaptic clustering. We transfected stg/stg granule cells with shRNA specific for γ-7 (see Online Methods) and compared them with untransfected cells. As expected, no mEPSCs were detected in the control untransfected stg/stg granule cells (Fig. 8a). In contrast, mEPSCs (arising at granule cell–granule cell synapses) were readily detected in >90% of treated cells (Fig. 8b), occurring at an average frequency of 2.0 ± 0.5 Hz (n = 25 cells). These results confirm the principle that TARPPless AMPARs can cluster at stg/stg synapses.

DISCUSSION

We found that TARPs differentially control the expression of CP- and CI-AMPARs in cerebellar stellate cells. Our results suggest that γ-2 is pivotal for the efficient expression of CI-AMPARs at parallel fiber synapses, but is not essential for accumulation of CP-AMPARs at these sites. We found that CP-AMPARs associated with the atypical TARP γ-7 at the surface of stg/stg stellate cells. Unexpectedly, these assemblies failed to accumulate at synapses, and synaptic transmission in stg/stg stellate cells instead appeared to be mediated by TARPPless CP-AMPARs. We confirmed the ability of AMPARs to accumulate at synapses in the absence of TARPs by rescuing synaptic transmission in stg/stg granule cells following knockdown of TARP γ-7. Overall, our results suggest that γ-2 shapes synaptic transmission in cerebellar stellate cells in several ways: it promotes the synaptic expression of CP-AMPARs, enhances AMPAR channel conductance and attenuates block of CP-AMPARs by internal polyamines. Furthermore, as γ-7 is normally enriched at stellate cell synapses, our results suggest that γ-2 promotes the synaptic clustering of γ-7-associated AMPARs.
Loss of γ-2 increases prevalence of synaptic CP-AMPARs

We found a marked increase in the rectification, and an enhanced block by PhTx-433, of EPSCs in stg/stg cells from stg/stg mice, indicating an increased prevalence of synaptic CP-AMPARs. Our data from cultured stellate cells suggest that this change in AMPAR subtype is cell-autonomous and results directly from the loss of postsynaptic γ-2. Observations in other cerebellar neurons are consistent with our interpretation of the role of γ-2 in stellate cells.

Similar to stellate cells, Golgi cells receive excitatory input from granule cells and express γ-2 and γ-7 (refs. 18, 29). In addition, they express γ-3 (refs. 2, 18), a TARPS closely related to γ-2. Although I-V relationships for eEPSCs are linear in control Golgi cells, and remain so in γ-3 knockout and stg/stg mice, they become inwardly rectifying in the absence of both TARPs45, supporting the view that γ-2 (or the closely related TARPS γ-3) is required for the efficient expression of CI-AMPARs, but not CP-AMPARs. In fact, it seems to be the inability of cerebellar granule cells to express CP-AMPARs that results in the loss of synaptic currents in stg/stg animals (D. Studniarczyk, M.F. and S.G.C.-C., unpublished observations).

Low-conductance CP-AMPARs underlie EPSCs in stg/stg cells

As well as being implicated in virtually every stage of AMPAR trafficking, including biosynthesis, surface delivery and synaptic clustering, TARPs remain co-assembled with surface AMPARs and determine many of their key functional properties. It is therefore unexpected that AMPARs appear to be TARPPless at stg/stg parallel fiber–stellate cell synapses. Our evidence in support of this view comes from both rectification index and single-channel conductance measurements. Although the desensitization and deactivation rates of recombinant AMPARs are typically decreased by the presence of a TARPS10,15, we found no difference in qEPSCs kinetics between stg/stg and control synapses. This is perhaps not surprising, as the kinetics of synaptic currents depend not only on intrinsic AMPAR properties, but also on the waveform of glutamate in the synaptic cleft46. As synaptic maturation is likely affected by the absence of γ-2, and the subsequent deficit in brain-derived neurotrophic factor in stg/stg cerebellum47,48, an altered glutamate concentration waveform may obscure any change in qEPSC decay associated with a change or loss of TARp. Indeed, we found that the rise time of qEPSCs was slightly, but significantly, slowed in stg/stg stellate cells. However, both rectification index and single-channel conductance measurements are independent of the glutamate waveform, and therefore reliably characterize functional changes in synaptic AMPARs. Regarding AMPAR channel conductance, our experiments in heterologous cells indicate that γ-2- and γ-7-associated CP-AMPARs display similarly high single-channel conductances. Our recordings of qEPSCs at stg/stg synapses suggest that they are mediated by channels that have a low conductance and are strongly rectifying, a combination of properties that is unique to TARPPless CP-AMPARs.

The reduction in glutamate-evoked whole-cell current in stg/stg stellate cells was greater than would be expected from the observed decrease in single-channel conductance of synaptic receptors; it likely reflects a reduced density of extrasynaptic AMPARs, as well as a decrease in the number of functional synapses. The latter would be consistent with our need to employ higher intensity parallel fiber stimulation to evoke EPSCs in stg/stg stellate cells. Despite this overall reduction in surface expression, the number of synaptic AMPARs activated during a qEPSC was similar at stg/stg and control synapses. Thus, our results suggest that AMPARs will accumulate at normal density at some synapses even in the absence of an associated TARp. The retention of TARPPless CP-AMPARs at synapses in stg/stg stellate cells suggests that the receptors are binding directly to postsynaptic PDZ-containing proteins. Indeed, there is good evidence that the C-tail PDZ-binding motif of GluA3 interacts directly with the anchoring protein GRIP, and the clustering of a fraction of CP-AMPARs at parallel fiber–stellate cell synapses is thought to depend on this specific interaction24,35.

Exclusion of γ-7-associated AMPARs from stg/stg synapses

In stg/stg stellate cells, a large proportion of extrasynaptic AMPARs exhibit a high single-channel conductance, similar to that seen with recombinant TARPPed CP-AMPARs. As no TARp other than γ-2 or γ-7 is found in wild-type stellate cells, and as the other main AMPAR accessory proteins, the cornichons, appear to not associate with surface AMPARs in cerebellar neurons49, it is highly likely that extrasynaptic CP-AMPARs are co-assembled with γ-7 in stg/stg stellate cells. This view is supported by our finding of a strong block of stg/stg cells by intracellular spermine and the absence of CNQX partial agonism. Nevertheless, our data also suggest that TARPPless AMPARs are expressed at synapses. The presence of both γ-7-associated and TARPPless AMPARs at the surface of stg/stg stellate cells must reflect some selectivity in TARp association. Indeed, such regulated assembly is indicated by the minimal co-immunoprecipitation of γ-7 and γ-2 in cerebellar extracts from wild-type mice5.

TARP γ-7 has been shown to cluster at parallel fiber–stellate cell synapses, where it interacts directly with PSD-95 (refs. 3, 29). However, our results suggest that this is unlikely to be the case in the absence of γ-2. Consistent with this interpretation, western blot analysis has revealed a pronounced reduction of γ-7 in the postsynaptic density fraction from the cerebellum of γ-2 knockout mice59. Furthermore, quantitative analysis of post-embedding immunogold labeling from these mice shows a ~70% reduction in γ-7 across parallel fiber synapses on stellate, basket and Golgi cells (M. Yamasaki and M. Watanabe, personal communication). The loss at parallel fiber–stellate cell synapses is potentially even greater, as the remaining γ-7 immunoreactivity likely reflects its presence at synapses onto γ-3-containing Golgi cells. Additional findings also support our proposal that γ-7 does not efficiently accumulate at synapses in the absence of γ-2. Cerebellar granule cells are severely affected by the lack of γ-2, displaying no clear synaptic currents and only residual whole-cell currents5. Overexpression of γ-7, despite promoting surface expression of AMPARs, does not rescue synaptic currents31. In fact, our finding that synaptic currents in stg/stg cerebellar granule cells were rescued by γ-7 knockdown suggests that γ-7-associated AMPARs may be excluded from the postsynaptic membrane in these cells.

While our manuscript was in revision, a study describing AMPAR-mediated currents in stg/stg stellate cells was published60. Although this study focused on synaptic plasticity, it also described increased AMPAR rectification at both synaptic and extrasynaptic sites. However, as the authors did not observe any increase in PhTx-433 sensitivity, they concluded that the increase in rectification was unlikely to be a result of a change in GluA2 content, and that stg/stg mice do not display a specific defect in GluA2 trafficking. These conclusions are in marked contrast with our finding of a clear increase in the prevalence of CP-AMPARs at stg/stg synapses. Although the reason for such a difference is unclear, our results indicate that a change in CP-AMPAR properties contributes to the observed increase in rectification (at both synaptic and extrasynaptic sites). Notably, the identification of differentially distributed low- and high-conductance CP-AMPARs in stg/stg stellate cells led us to suggest the presence of TARPPless and γ-7-associated CP-AMPARs at synaptic and extrasynaptic sites, respectively, both of which are more effectively blocked by spermine than are γ-2-associated CP-AMPARs.
In conclusion, our data indicate that the loss of γ-2 in stg/stg stellate cells induces quantitative and qualitative changes in synaptic transmission that cannot be compensated for by the remaining TARP γ-7. Our results extend the idea that TARPs interact with specific AMPAR subtypes, display distinct patterns of expression in the neuronal membrane and differentially modify AMPAR functional properties. However, they challenge the view that TARPs are essential for the expression of functional AMPARs at synapses.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Experiments were performed by C.B. [stellate cell recordings], D. Studniarczyk [granule cell recordings] and D. Soto [tsA201 cell and stellate cell recordings]. M.F.E., C.B., D. Soto and D. Studniarczyk analyzed the data. S.G.C.-C. and M.F.E. supervised the project. All of the authors contributed to the design and interpretation of experiments. C.B., M.F. and S.G.C.-C. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Stargazer mice were bred from +/stg (with a C57BL/6 background) and categorized according to phenotype: stg/stg (smaller size, head tossing, unsteady gait) and control. To enable identification of GABAergic stellate cells in dissociated cerebellar cultures, we used Gad2-eGFP (also known as GAD65-eGFP) mice. Cultures were prepared from stg/stg Gad2-eGFP or control littermate mice. Cultures were from individual P7 mice, and tail samples were used for stg/stg genotyping to enable culture identification. All procedures for the care and treatment of mice were in accordance with the Animals (Scientific Procedures) Act 1986.

Cerebellar slices. Recordings were made in coronal slices (200 μm) cut from the cerebellar vermis of P18–31 mice. The slicing solution contained 85 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 4 mM MgCl₂, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 64 mM sucrose and 25 mM glucose (pH 7.3 when bubbled with 95% O₂ and 5% CO₂). To prevent NMDAR-mediated cell damage, we included 20 μM D-AP5 (Tocris Bioscience). Slices were viewed using a fixed stage upright microscope (Zeiss Axioskop FS or Olympus BX51 WI with differential interference contrast—infared or oblique illumination) and recordings (23–26 °C) were made from visually identified interneurons in the outer third of the membrane layer, presumptive stellate cells.

The extracellular solution contained 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, and 25 mM glucose (pH 7.3 when bubbled with 95% O₂ and 5% CO₂). To block NMDA and GABA receptors, we added 20 μM D-AP5 and 20 μM SR-95531 (Ascent Scientific). Pipettes for whole-cell and outside-out patch recording were pulled from thick-walled borosilicate glass (1.5 mm outer diameter, 0.86 mm inner diameter, Harvard Apparatus), coated with Silgard resin (Dow Corning 184) and fire-polished to a final resistance of ~5 or ~7 MΩ, respectively. The internal solution contained 128 mM CsCl, 10 mM HEPES, 10 mM TEACl, 2 mM MgATP, 1 mM CaCl₂, 2 mM NaCl, 1 mM N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide (QX314) and 0.1 mM mepivaine tetramethochloride (both Tocris Bioscience) (pH 7.4 with NaOH). Currents were recorded at 22–24 °C using an Axopatch 200B amplifier, filtered at 5 kHz and digitized at 20 or 50 kHz (pClamp10 and Digidata 1440A, Molecular Devices). Series resistance (12–20 MΩ) and input capacitance (2.5–6.5 pF) were read directly from the amplifier settings used to minimize the current responses to 5-mV hyperpolarizing voltage steps. Series resistance was typically compensated by 60–85%.

Dissociated cultures. Dissociated cultures containing cerebellar stellate cells were prepared from P7 stg/stg Gad2-eGFP mice or their control littermates. Cells were plated on poly-l-lysine–coated glass coverslips together with a tenfold excess of cerebellar neurons dissociated from wild-type C57BL/6 mice. Cells were maintained in a humidified atmosphere at 37 °C (5% CO₂) in Basal Medium Eagle (Gibco) supplemented with 10% fetal bovine serum (vol/vol, Gibco), 100 mg ml⁻¹ gentamicin and 2 mM l-glutamine. Cells were maintained in high glucose conditions when bubbled with 95% O₂ and 5% CO₂. To block NMDA and GABAA receptors, we added 20 μM D-AP5 and 20 μM SR-95531 (Ascent Scientific). Pipettes for whole-cell and outside-out patch recording were pulled from thick-walled borosilicate glass (1.5 mm outer diameter, 0.86 mm inner diameter, Harvard Apparatus), coated with Silgard resin (Dow Corning 184) and fire-polished to a final resistance of ~5 or ~7 MΩ, respectively. The internal solution contained 128 mM CsCl, 10 mM HEPES, 10 mM TEACl, 2 mM MgATP, 1 mM CaCl₂, 2 mM NaCl, 1 mM N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide (QX314) and 0.1 mM mepivaine tetramethochloride (both Tocris Bioscience) (pH 7.4 with NaOH). Currents were recorded at 22–24 °C using an Axopatch 200B amplifier, filtered at 5 kHz and digitized at 20 or 50 kHz (pClamp10 and Digidata 1440A, Molecular Devices). Series resistance (12–20 MΩ) and input capacitance (2.5–6.5 pF) were read directly from the amplifier settings used to minimize the current responses to 5-mV hyperpolarizing voltage steps. Series resistance was typically compensated by 60–85%.

Excised somatic patches. Outside-out patches were excised from the soma of identified stellate cells. Currents were filtered at 10 kHz and digitized at 50 kHz. Ultra-fast application of glutamate was achieved using an application tool made from theta glass (2 mm outer diameter, Hilgenberg GmbH) pulled to a tip opening of ~200 μm, and moved by a piezoelectric translator (Physik Instrumente) mounted on a PatchStar micromanipulator (Scientifica). To determine channel properties from macroscopic responses, we applied glutamate (10 mM, 100-ms duration, 1 Hz) and calculated the ensemble variance of all successive pairs of current responses. The single-channel current (I) and total number of channels (N) were determined by plotting this ensemble variance (σ²) against mean current (Ī) and fitting with the equation

\[ \sigma^2 = I - \frac{I^2}{N} + \sigma^2_b \]

The weighted mean single-channel conductance was calculated from the single-channel current and the holding potential. \( P_{o,max} \) was estimated by dividing the average peak current by \( I_N \).

Recombinant receptors. tsA201 cells were grown according to standard protocols and transfected with DNA encoding AMPAR subunits, TARP γ-2 and γ-7, and eGFP using Lipofectamine 2000 (Invitrogen) as previously described. Cells were perfused with external recording solution containing 145 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES and 11 mM glucose (pH adjusted to 7.4 with NaOH). Glutamate application and NSFA was carried out as described above.

Single-channel analysis. Channel openings in the tail of macroscopic patch currents (both stellate cells and tsA201 cells) were analyzed using QuB (ver. 2.0.0.7, http://www.qub.buffalo.edu/). Records were filtered at 4 kHz and pre-processed with piecewise linear baseline correction. Individual channel events were selected by eye and, for each selected record, an all-point amplitude histogram was generated and fitted with two Gaussians to determine the amplitude of the single-channel current. On average, 50 channel events were measured from each patch.
Kinetic analysis. The decay of averaged EPSCs was described by one or, more often, two exponential functions. When fitted with two exponentials, the weighted time constant of decay (τ_{w, decay}) was calculated as the sum of the fast and slow time constants weighted by their fractional amplitudes.

Data presentation and statistical analysis. Data are presented in the text as mean ± s.e.m. The n number indicates the number of cells. Differences between control and stg/stg data were examined using non-parametric Wilcoxon rank sum tests (unpaired) or Wilcoxon signed rank tests (paired), as appropriate. Group comparisons of data from recombinant receptors were performed using a non-parametric Kruskal-Wallis rank-sum test followed by pair-wise Wilcoxon rank-sum tests with Holm’s sequential Bonferroni correction or one-way ANOVA and Tukey’s HSD test (as indicated). Results were considered significant with P < 0.05. Statistical tests were performed using R 2.13.1 (the R Foundation for Statistical Computing; http://www.r-project.org/).

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