RESEARCH ARTICLE

Type I TARPs promote dendritic growth of early postnatal neocortical pyramidal cells in organotypic cultures

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ABSTRACT

The ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionate glutamate receptors (AMPARs) have been implicated in the establishment of dendritic architecture. The transmembrane AMPA receptor regulatory proteins (TARPs) regulate AMPAR function and trafficking into synaptic membranes. In the current study, we employ type I and type II TARPs to modulate expression levels and function of endogenous AMPARs and investigate in organotypic cultures (OTCs) of rat occipital cortex whether this influences neuronal differentiation. Our results show that in early development [5-10 days in vitro (DIV)] only the type I TARP γ-8 promotes pyramidal cell dendritic growth by increasing spontaneous calcium amplitude and GluA2/3 expression in soma and dendrites. Later in development (10-15 DIV), the type I TARPs γ-2, γ-3 and γ-8 promote dendritic growth, whereas γ-4 reduced dendritic growth. The type II TARPs failed to alter dendritic morphology. The TARP-induced dendritic growth was restricted to the apical dendrites of pyramidal cells and it did not affect interneurons. Moreover, we studied the effects of short hairpin RNA-induced knockdown of endogenous γ-8 and showed a reduction of dendritic complexity and amplitudes of spontaneous calcium transients. In addition, the cytoplasmic tail (CT) of γ-8 was required for dendritic growth. Single-cell calcium imaging showed that the γ-8 CT domain increases amplitude but not frequency of calcium transients, suggesting a regulatory mechanism involving the γ-8 CT domain in the postsynaptic compartment. Indeed, the effect of γ-8 overexpression was reversed by APV, indicating a contribution of NMDA receptors. Our results suggest that selected type I TARPs influence activity-dependent dendritogenesis of immature pyramidal neurons.

KEY WORDS: Dendritogenesis, Glutamate receptors, Interneurons, Neocortex, Postnatal development, TARPs

INTRODUCTION

In the mammalian CNS, glutamate mediates most of the excitatory synaptic transmission through AMPARs (Hollmann and Heinemann, 1994). Glutamatergic signaling via selected AMPARs is also a major regulator of neuronal morphogenesis, in particular for modulating dendritic growth (Haas et al., 2006; Inglis et al., 2002; Hamad et al., 2011). Intriguingly, AMPA receptor flip splice variants with slower desensitization kinetics and prolonged channel open times are more efficient than flop variants and receptors with elevated calcium permeability (Hamad et al., 2011). In the present study, performed in organotypic cortex cultures, we employed TARPs as tools to address the role of endogenous AMPARs.

TARP proteins serve as auxiliary subunits for AMPARs. TARPs are widely expressed and developmentally regulated in the CNS. Type I TARPs γ-2 (stargazin), γ-3, γ-4 and γ-8 (also known as Cacng2, 3, 4 and 8, respectively) traffic AMPARs to the plasma membrane and modulate channel function. In heterologous cells, the TARPs γ-4 and γ-8 prolong the current rise time in response to brief applications of glutamate and slow GluA1 (also known as Gria1) receptor desensitization and deactivation to a greater extent than do the TARPs γ-2 and γ-3 (Cho et al., 2007; Milstein et al., 2007). The CTs of the TARPs are important for trafficking because they directly interact with the PDZ domains of PSD-95 (also known as Dlg4) to target the associated AMPARs specifically towards synaptic but not extrasynaptic sites (Chen et al., 2000). In hippocampal neurons, stargazin increases the number of AMPARs in the plasma membrane and dramatically increases the currents elicited by bath-applied AMPA (Schnell et al., 2002). The TARP subtype determines the kinetics of AMPARs when co-transfected in HEK293T cells (Milstein et al., 2007). For instance, type I TARPs increase AMPAR GluA1 maximum channel conductance (Shelley et al., 2012), and the desensitization properties of AMPA receptors are more strongly affected when co-expressed with γ-4 rather than γ-2 (Korber et al., 2007). In Xenopus oocytes, the co-expression of type I TARPs has been shown to potentiate AMPAR currents, and the extent of the TARP-mediated increase in agonist-induced responses is highly dependent on both the TARP and the AMPAR subunits (Kott et al., 2007). The type II TARPs γ-5 and γ-7 (also known as Cacng5 and Cacng7) do not traffic AMPAR, they only modulate AMPAR channel function (Kato et al., 2008). γ-5 is expressed in cerebellar neurons and has been shown to interact with GluA1, GluA4 (also known as Gria4) and edited GluA2 (also known as Gria2) (Soto et al., 2009). When expressed in neocortical neurons, γ-5 increases the rate of receptor deactivation and desensitization, and reduces the potency of glutamate and the steady-state currents of AMPARs (Kato et al., 2007). γ-7 in cerebellar neurons significantly enhances glutamate-evoked currents and modulates gating of AMPARs (Kato et al., 2007). The related 'non-TARP' isoforms γ-1 and γ-6 (also known as Cacng1 and Cacng6) have no auxiliary function for AMPARs (Tomita et al., 2003; Letts et al., 1998). Our present study demonstrates that only trafficking-competent type I TARPs promote dendritic growth. Specifically, for γ-8 we demonstrate the requirement of the CT domain and the importance of the endogenous γ-8.

RESULTS

Expression of TARPs in developing rat neocortex

Developmental TARP protein expression in rodent cortex (Tomita et al., 2003) involved a developmental increase of γ-2 and γ-8 from postnatal day (P) 4, an increase of γ-3 from P16, and a decrease of

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γ-4 from P16. Here, we analyzed the expression of TARP mRNAs in rat occipital cortex and in OTCs. Primer specificity was confirmed by PCR with all primer pairs on plasmid DNA encoding all eight γ subunit isoforms (Fig. 1A). The non-TARP γ-1 mRNA was absent at all stages (Fig. 1B,C). The non-TARP γ-6 mRNA was detected in cortex, but only the short splice isoform was expressed weakly from birth until P30 (Fig. 1B). The γ-5 mRNA was undetectable, whereas γ-7 mRNA was detected at moderate levels at all ages. The γ-2 mRNA was expressed from embryonic day (E) 15 until the adult stage (Fig. 1B). The γ-3 mRNA expression was low around birth, increased until P20, and declined afterwards. The γ-4 mRNA was abundant from E15 until P0, but was barely detectable after birth and was absent in the adult. The γ-8 mRNA was abundant at all developmental stages. The result showed that the *in vivo* mRNA profile from occipital cortex and protein profile from total cortex (Tomita et al., 2003) are not precisely matching. The TARP expression profile in OTCs (Fig. 1C) resembles the *in vivo* profile with two exceptions: γ-3 mRNA did not seem to be developmentally regulated and γ-4 mRNA remained at slightly higher levels after birth (Fig. 1B). Yet, the cultured cortex expressed the same set of TARP mRNAs as cortex *in vivo*.

γ-8 protein enhances somatic and dendritic expression of GluA2/3

Overexpression of γ-8 increases the number of AMPARs in the plasma membrane, whereas in γ-8$^{-/-}$ mice AMPAR-mediated synaptic transmission is severely impaired because of a loss of GluA2 and GluA3 (also known as Gria3) (Fukaya et al., 2006; Hashimoto et al., 1999; Menuz et al., 2009; Rouach et al., 2005). Previously, we have identified GluA2/3 subunits as the major regulators of pyramidal cell dendritic growth, whereas GluA1 regulates mainly dendritic growth of interneurons (Hamad et al., 2011). We now investigated the expression of GluA1 and GluA2/3 in γ-8-overexpressing pyramidal cells (Fig. 2). In comparison to control cells, γ-8-transfected pyramidal cells at 10 DIV showed higher expression of GluA2/3 protein in soma and dendrites (Fig. 2D), whereas the expression of the GluA1 subunit was not altered (Fig. 2E). Thus, the increase of GluA2/3 expression due to the overexpression of γ-8 confirms previous findings regarding the role of γ-8 in AMPARs trafficking and expression.

Agonist-induced excitotoxicity reveals high levels of functional AMPARs in pyramidal neurons overexpressing type I TARPs

AMPARs are abundantly expressed in rat cortex and developmentally upregulated (Traynelis et al., 2010) during the major dendritic growth period between 5 and 15 DIV. We hypothesized that overexpressed type I TARPs could shuttle more endogenous AMPARs to the plasma membrane, whereas overexpressed type II TARPs could only modulate the function of already existing AMPARs (Kato et al., 2008). These additional or more active receptors might cause a higher sensitivity to endogenous glutamate (ambient or released from developing synapses), which in turn will promote neuronal growth. We have previously shown (Hamad et al., 2011) that the higher the level of AMPARs is, the quicker a neuron responds to high concentrations of the agonist AMPA with focal dendritic swelling, a hallmark of dendritic injury (Fig. 3A,B). Neurons (pyramidal cells and interneurons) transfected with γ-5 and γ-7 did not have increased dendritic injury rates upon AMPA application at DIV 10 (Fig. 3C) and DIV 15 (Fig. 3D). With the assumption that transfected type II TARPs interact with already existing AMPARs, this observation implies that channel modulation did not cause an increased sensitivity to AMPA. The results for type I TARPs were different. At 5-10 DIV, the dendritic injury rate increased in pyramidal cells transfected with γ-4 and γ-8, but not γ-2 or γ-3 (Fig. 3E). At 10-15 DIV, a higher fraction of enhanced green fluorescent protein (EGFP)-only transfected pyramidal cells responded to the AMPA stimulation (Fig. 3G), indicating a developmental upregulation of endogenous AMPARs. At this time point, the dendritic injury rates in pyramidal cells transfected with γ-2, γ-3, γ-4 and γ-8 were dramatically increased (Fig. 3G). Such a high sensitivity to AMPA suggests that the transfected TARPs had increased the density of functional endogenous AMPARs. In interneurons, AMPA stimulation elicited dendritic injury in only a small fraction of neurons at DIV 10 (Fig. 3F) and in a larger fraction of neurons at 15 DIV (Fig. 3H), indicating a developmental upregulation of AMPARs also for this cell class. Interestingly, the rate of dendritic injury was only enhanced in γ-4-transfected interneurons at both developmental stages (Fig. 3F,H). TARPs do not interact with NMDA receptors (NMDARs) (Chen et al., 2000) or with kainate receptors (Chen et al., 2003). As expected, the application of 10 µM NMDA for 10 min to type I TARP transfectants did not elicit a significant change
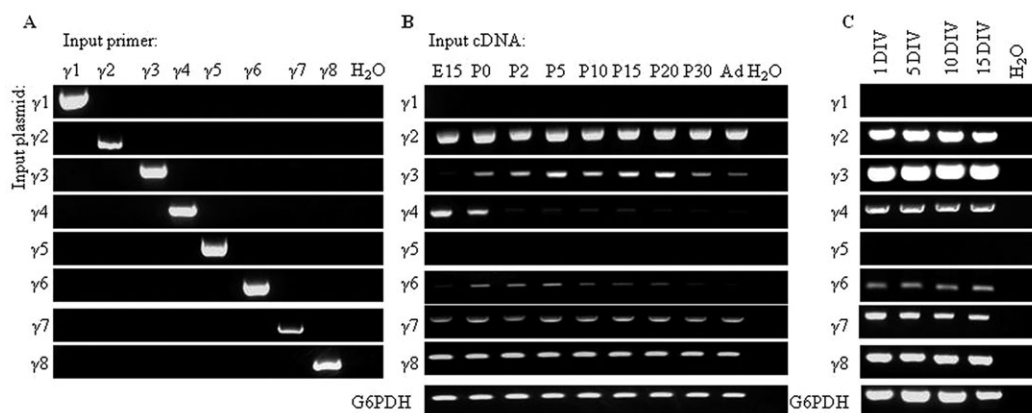

Fig. 1. Expression analysis of TARP mRNA in rat occipital cortex and OTCs. (A) The specificity of all primer pairs (input primer) was verified by cross-amplifying intended and unintended γ subunits using the plasmid cDNAs as template. Note that TARP primers amplify specifically the intended target plasmid. (B) RT-PCR analysis from *in vivo* tissue shows that all TARPs except γ-1 and γ-5 are expressed in occipital cortex. (C) RT-PCR analysis from OTCs shows that TARPs except γ-1 and γ-5 are expressed *in vitro*. The γ-6 primers recognize both the long and the short isoform, and were tested on a plasmid encoding the long isoform (442 bp amplicon); however, it detected only the short isoform (304 bp) *in vitro* and *in vivo*. Expression of the housekeeping gene G6PDH was used as an internal control. In the H$_2$O control lane, template had been omitted. Ad, adult.
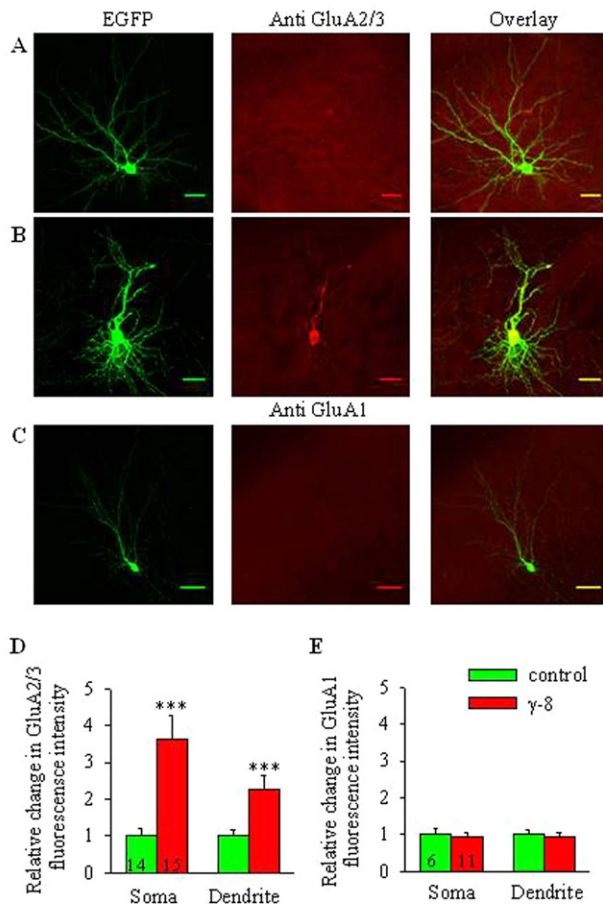
Fig. 2. Increased GluA2/3 immunoreactivity in γ-8-transfected pyramidal cells. (A-C) Confocal images (taken at 63× magnification) of representative pyramidal cells co-transfected with EGFP alone or EGFP and γ-8 and stained with antibodies against GluA2/3 or GluA1. (A) Pyramidal cell overexpressing EGFP alone (control) shows very low GluA2/3 expression. (B) Pyramidal cell overexpressing EGFP and γ-8 shows higher levels of GluA2/3 expression in soma and dendrites. (C) Pyramidal cell overexpressing EGFP and γ-8 shows low expression of GluA1. (D,E) Quantification of GluA2/3 immunofluorescence intensity (D) and GluA1 immunofluorescence intensity (E) in EGFP control and in γ-8-transfected cells; the average level of GluA expression in EGFP control cells was set to 1. Error bars represent s.e.m. Mann–Whitney U test; ***$P<0.001$. The numbers of cells analyzed is given within the bars. Scale bars: 30 μm.

in the dendritic injury rate compared with the control group (supplementary material Fig. S1). This confirms the specificity of TARPs towards AMPARs. Taken together, this experiment suggests that type I TARPs elicit the effects by enhancing AMPAR trafficking.

γ-8 regulates pyramidal cell dendritic growth in the early time window

To test the role of TARPs for dendritic growth, we overexpressed individual TARPs at 5 DIV and allowed 5 days for expression (Fig. 4). γ-8 was able to promote apical dendritic elongation and branching in pyramidal cells in layers II/III and layers V/VI (Fig. 4A). This confirms our previous finding that AMPARs regulate apical dendritic growth (Hamad et al., 2011) because they are preferentially distributed in the apical dendrites (Pettit et al., 1997). At 10 DIV, γ-2, γ-3 and γ-4 failed to increase apical length (Fig. 4A,B). Furthermore, type I TARPs had no effect on basal dendritic growth (Fig. 4A,B). The type II TARP γ-5 increases the rate of receptor deactivation, increases desensitization, and reduces

the potency of glutamate and the steady-state currents of AMPARs (Kato et al., 2007), and we expected that γ-5 would slow dendritic growth. By contrast, γ-7 enhances AMPAR currents (Kato et al., 2007) and we expected that γ-7 could phenocopy the effect of a transfection of AMPAR flip variants (Hamad et al., 2011). However, type II TARPs failed to induce dendritic growth (Fig. 4C).

γ-2 and γ-3 become efficient dendritic modulators at a later developmental stage

The expression of AMPARs in neocortical neurons reaches adult levels by the second to third postnatal week (Monyer et al., 1991). Indeed, AMPA-induced dendritic injury in EGFP-transfected control pyramidal cells increased significantly from 10 to 15 DIV, confirming that endogenous AMPARs become upregulated (Fig. 3). Considering the age-dependent effect of the TARPs in eliciting dendritic injury, a reason for the failure of γ-2 and γ-3 to modulate dendritic growth could be that the level of endogenous AMPAR proteins was too low at 5-10 DIV. Therefore, we tested the TARPs between 10 and 15 DIV. Like in the earlier time window, the overexpression of γ-8 promoted growth of apical dendrites of layer II/III pyramidal neurons, and the effect was stronger at 15 DIV than at 10 DIV (Fig. 4D). Moreover, at DIV 10, there was no effect on basal dendritic growth (Fig. 4D). Surprisingly, the overexpression of γ-2 and γ-3 now strongly increased apical dendritic growth of pyramidal cell in layers II/III, but not in layers V/VI (Fig. 4E). Basal dendrites tended to be longer and more branched, but the effect failed to reach statistical significance (Fig. 4E). This suggests that the effect of the TARPs depends on a certain amount or composition of AMPAR subunits, or requires a certain degree of neuronal maturation. Most intriguingly, the overexpression of γ-4 reduced apical dendritic elongation and branching in pyramidal cells in layers II/III and V/VI (Fig. 4D). This might be related to its subcellular localization, as γ-4 has been reported to localize mostly to extrasynaptic rather than synaptic membranes (Ferrario et al., 2011). To test this possibility, we characterized the subcellular localization of the overexpressed γ-2 versus γ-4 protein at 10-15 DIV (supplementary material Fig. S2). Indeed, γ-2 was intensely labeled in dendritic shafts and in spines, whereas γ-4 staining was intense in shafts and weak in spines. Together, these observations suggest that the localization of a TARP and the associated AMPAR in dendritic spines is a key factor for pyramidal cell dendritic growth. Type II TARPs failed to induce dendritic growth at 10-15 DIV (Fig. 4F). This suggested that the mechanisms by which type II TARPs modulate AMPARs are either too weak to become translated into a dendritic growth response, or that they modulate AMPARs that contain subunits not involved in dendritic growth. Taken together, pyramidal cell apical dendrite growth is stimulated only by TARPs that increase trafficking and expression of synaptic AMPARs.

The dendritic growth modulation of AMPARs has been shown to be mediated through NMDARs (Hamad et al., 2011). To examine whether the growth effect elicited by γ-8 requires NMDAR activation, we treated γ-8-transfected pyramidal cells with 50 μM of the competitive NMDAR antagonist (2R)-amino-5-phosphonovaleric acid (APV) at 5-10 DIV (supplementary material Fig. S3). APV inhibited the γ-8-induced growth effect, suggesting the involvement of NMDARs. Expectedly, the γ-8-induced growth effect requires the activation of AMPARs because apical dendritic length of γ-8 transfectants exposed to 10 μM of the competitive AMPAR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were not different from control. Together, the data show that γ-8 executes its growth-promoting effects directly through AMPARs with the contribution of NMDAR activation.
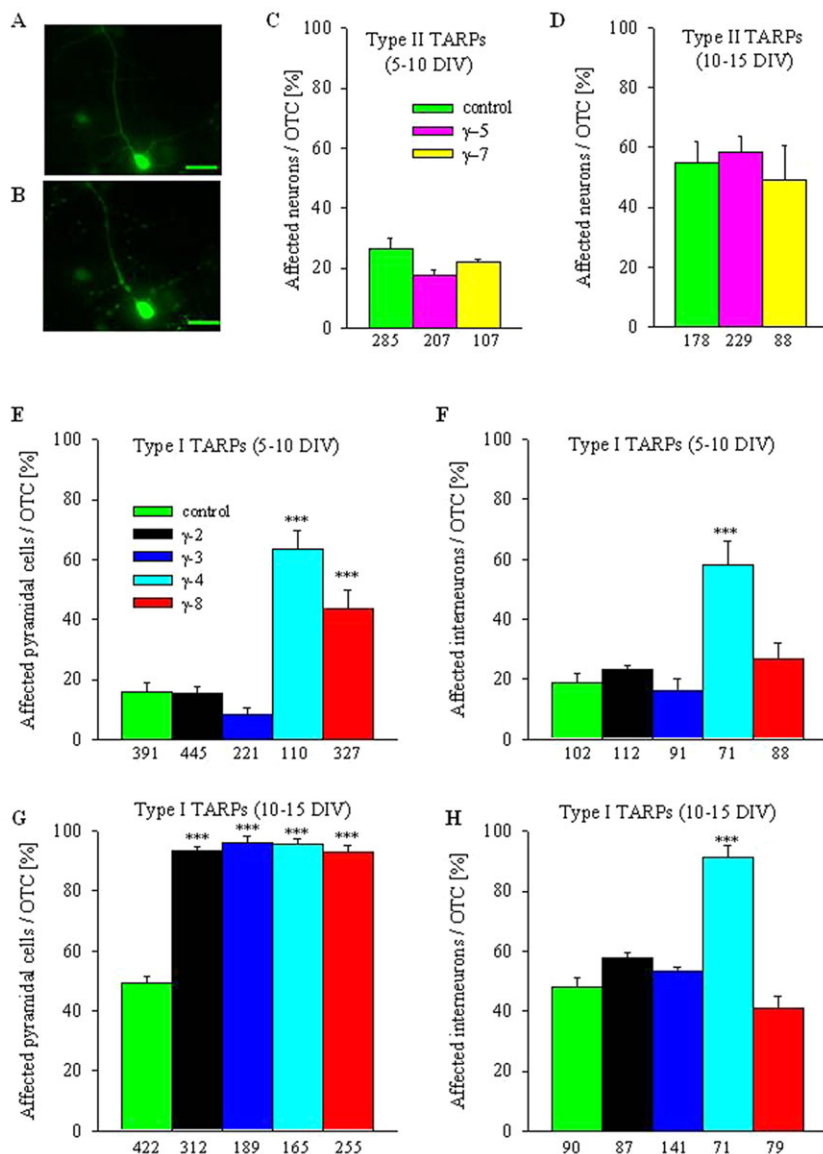
Fig. 3. Agonist-induced excitotoxicity in TARP-overexpressing neurons. (A,B) An example of dendritic injury in pyramidal cells overexpressing γ-8 before (A) and after (B) 15 min of AMPA stimulation. (C-H) Percentages of cells (mean±s.e.m.) displaying dendritic injury after 15 min stimulation with 100 µM AMPA. (C,D) Pyramidal cells and interneurons (pooled together) overexpressing EGFP alone (control) or with the indicated type II TARP at 5-10 DIV (C) and 10-15 DIV (D). (E,G) Pyramidal cells overexpressing EGFP alone (control) or with the indicated type I TARP at 5-10 DIV (E) and 10-15 DIV (G). (F,H) Interneurons overexpressing EGFP alone (control) or with the indicated type I TARP at 5-10 DIV (F) and 10-15 DIV (H). Mann–Whitney U test; ***$P<0.001$. The numbers of cells analyzed is given below the bars. Scale bars: 30 µm.

Type I TARPs γ-2, γ-3 and γ-8 increase dendritic complexity from proximal to distal

To confirm the effect of the type I TARPs on dendritic growth at 10 DIV and 15 DIV, dendritic complexity was assessed with Sholl analysis (Fig. 5). Representative examples of 10 DIV pyramidal cells from layer II/III are shown in Fig. 5A. We observed an increase in apical dendritic complexity proximal to the soma of γ-8-overexpressing pyramidal cells in both layers at 10 DIV (Fig. 5B,C). Furthermore, the total number of apical dendritic intersections in both layers was significantly higher in γ-8-transfected pyramidal cells. The Sholl analysis of basal dendrites confirmed the negative effect of γ-8 on basal dendrites at 10 DIV (Fig. 5D,E). Moreover, γ-2, γ-3 and γ-4 failed to modulate dendrites at 10 DIV. However, when overexpressed between 10 and 15 DIV, γ-2, γ-3 and γ-8 increased apical dendritic complexity between 100 and 200 µm from the soma (Fig. 5F). γ-4 reduced apical dendritic complexity (proximal and distal) in neurons of both layers (Fig. 5F,G). Basal dendritic complexity was not altered (Fig. 5H,I). These results suggest that the action of γ-2, γ-3 and γ-8 shift with age to more distal apical dendritic zones.

TARPs act in a cell class-specific manner

TARPs are enriched in excitatory neurons. For instance, stargazin has been reported to colocalize with GluA2 and PSD-95 at excitatory synapses, but not with glutamic acid decarboxylase GAD-65 (also known as Gad2), a presynaptic marker of inhibitory synapses (Tomita et al., 2003). However, stargazin is also expressed in hippocampal interneurons (Tomita et al., 2003) and specifically enriched in neocortical interneurons (Maheshwari et al., 2013; Tao et al., 2013). Co-expression studies have shown that stargazin enhances GluA1-mediated currents (Kott et al., 2007). Neocortical interneurons strongly express GluA1 (Geiger et al., 1995), and the GluA1(Q)-flip isoform, when overexpressed, promotes dendritic growth (Hamad et al., 2011). Unexpectedly, all TARPs failed to alter dendritic growth of interneurons at 5-10 and 10-15 DIV (supplementary material Fig. S4).

The CT domain of γ-8 is important for dendritic growth

For both AMPAR trafficking and channel modulation, the CT domain and the extracellular domain 1 (Ex1) domain of TARPs have been shown to be important (Sager et al., 2009; Tomita et al.,
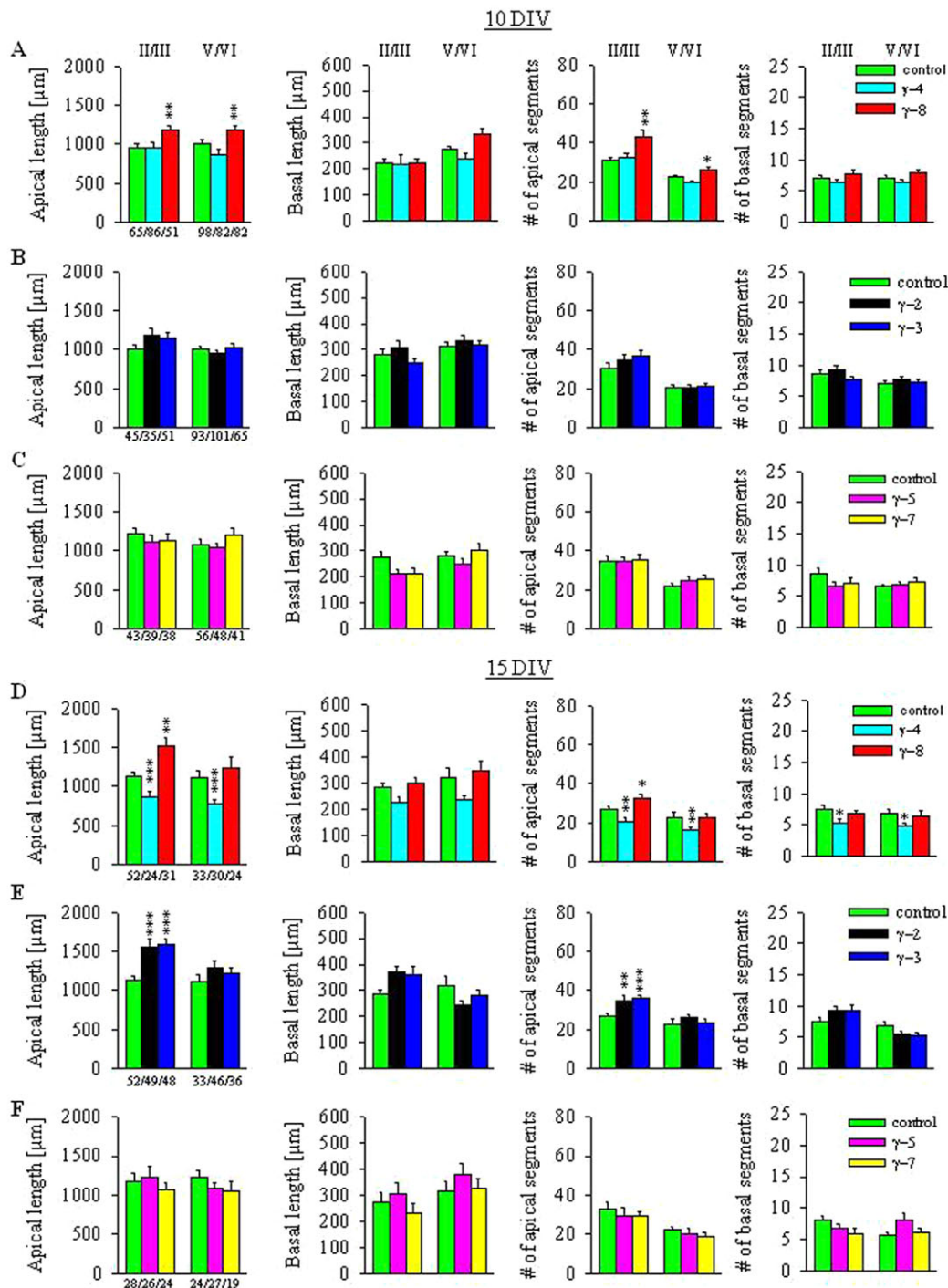
DEVELOPMENT

Fig. 4. Quantitative morphometric analysis of pyramidal neurons. Graphs showing the mean±s.e.m. of apical and basal dendritic length and segment number of layer II/III and V/VI cells overexpressing type I or type II TARPs in addition to EGFP as indicated. The analyses were carried out at two developmental time windows: 5-10 DIV (A-C) and 10-15 DIV (D-F). The number of cells reconstructed per group is given below the bars in the graphs for the apical length. (A,D) The type I TARP γ-8 increased dendritic length and branching in the early and the late time window. (B,E) The type I TARPs γ-2 and γ-3 failed to induce changes in the early time window (B), but increased dendritic length and branching at 10-15 DIV in layers II/III (E). (D) The type I TARP γ-4 decreased apical length and branching at 10-15 DIV. (C,F) The type II TARPs γ-5 and γ-7 had no effect on dendritic growth and branching at both developmental time windows. Mann–Whitney U test; ***$P<0.001$, **$P<0.01$, *$P<0.05$.

2005). γ-8 has a very long CT domain (Chu et al., 2001), and mice deficient for γ-8 showed a substantial loss and mislocalization of GluA1, GluA2 and GluA3 proteins (Rouach et al., 2005). Because γ-8 strongly promoted dendritic growth at both developmental stages, we studied the role of the CT and Ex1 domains by

replacing each independently with the homologous domain of γ-1 (Fig. 6A). For the agonist-induced excitotoxicity assay, γ-8 and its chimeras were overexpressed in OTCs between 5 and 10 DIV, followed by stimulation for 15 min with 100 µM AMPA. The overexpression of γ-1 did not result in an increase of the
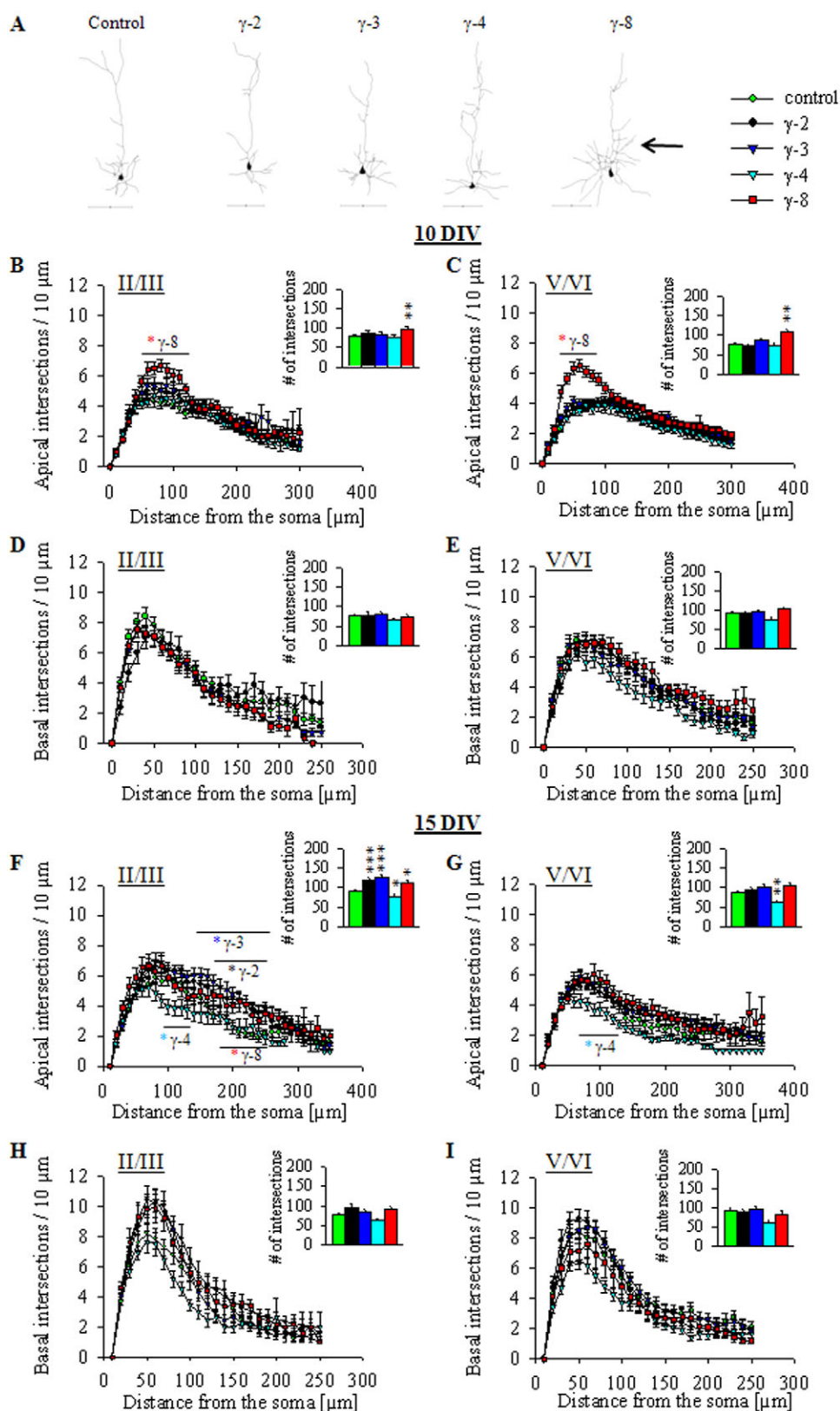
Fig. 5. Type I TARPs increase dendritic complexity. (A) Representative layer II/III pyramidal cells at 10 DIV overexpressing EGFP alone (control) or the indicated type I TARP. Only γ-8 increased proximal dendritic complexity (arrow). Scale bars: 100 μm. (B,C) Sholl analyses of apical dendritic intersections for layers II/III and layers V/VI at 5-10 DIV. (D,E) Sholl analyses show that basal dendrites of pyramidal cells in both layers were unaltered. (B-E, inserts) Total dendritic intersections were significantly increased in apical and not basal dendrites. (F) Sholl analyses of apical dendritic intersections for layers II/III at 10-15 DIV. (G) Sholl analyses of apical dendritic intersections for layers V/VI at 10-15 DIV show a decrease in distal dendritic complexity in γ-4 transfectants. (H,I) Sholl analyses for basal dendrites in both layers at 10-15 DIV were unaltered. (F-I, inserts) Total dendritic intersections were significantly increased in apical and not basal dendrites in γ-2, -3 and -8 transfectants and reduced in γ-4 in transfectants. The Sholl analyses were performed on the reconstructed cells from Fig. 4. Error bars represent s.e.m. Mann–Whitney U test; ***$P<0.001$, **$P<0.01$, *$P<0.05$.

dendritic injury rate compared with the EGFP control (Fig. 6B). Neurons overexpressing γ-8 again displayed dendritic injury after AMPA stimulation (Fig. 6B; confirming results shown in Fig. 3). Neurons overexpressing the γ-8-(CT)-γ-1 chimera displayed low rates of dendritic injury, similar to controls, whereas neurons overexpressing γ-8-(Ex1)-γ-1 displayed high dendritic injury rates. This suggests that transfectants show high sensitivity to AMPA only when expressing γ-8 with its natural CT domain because AMPA sensitivity remained at control levels in neurons expressing the γ-8-(CT)-γ-1 chimera.
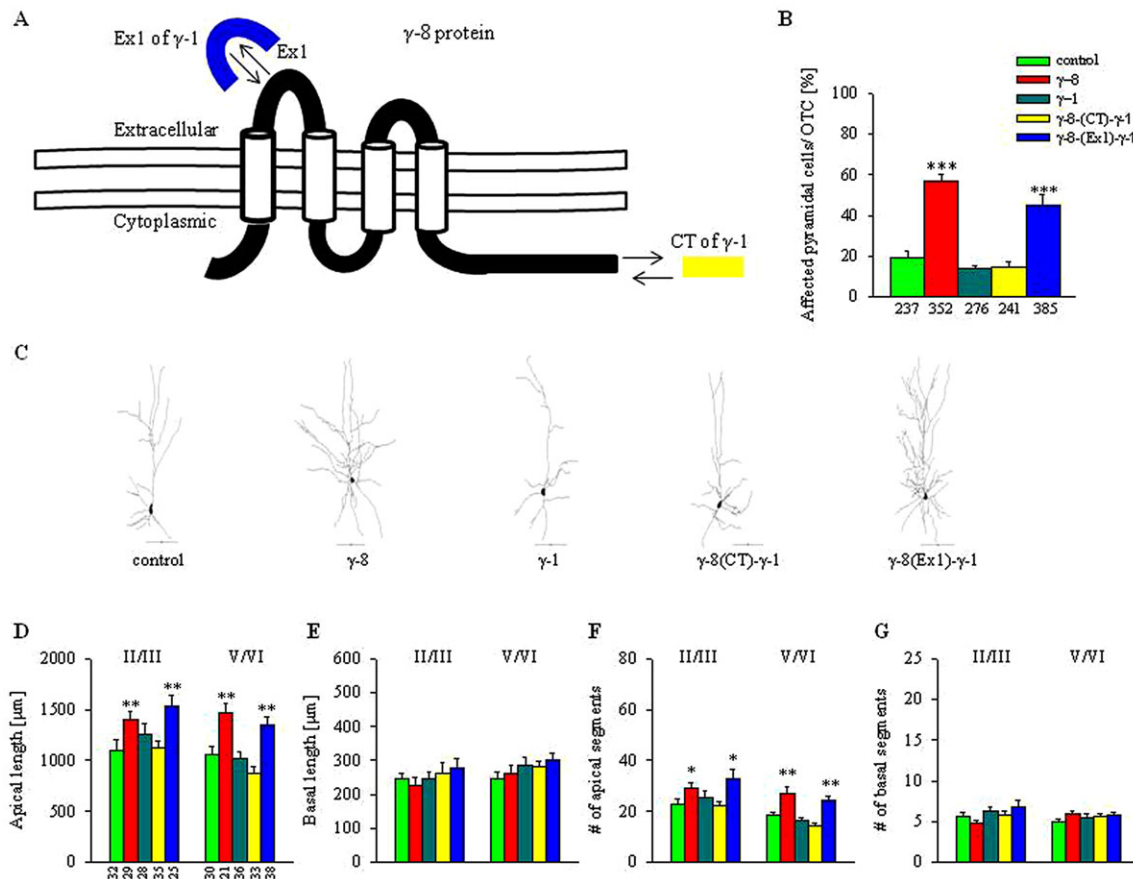
Fig. 6. The role of the CT domain of γ-8. (A) Schematic of γ-8 and the first extracellular domain (Ex1), and the intracellular cytoplasmic tail domain (CT). In the chimeras, the γ-8 CT and Ex1 domains have been replaced with the homologous domains of γ-1. (B) Percentage of cells (mean±s.e.m.) transfected at 5 DIV with EGFP alone (control) or EGFP plus γ-8 or γ-1, or the chimeras γ-8-(CT)-γ-1 or γ-8-(Ex1)-γ-1, which display dendritic injury after 15 min of 100 µM AMPA; cells were analyzed at 10 DIV. AMPA provoked massive dendritic injury in neurons transfected with γ-8 and the γ-8-(Ex1)-γ-1 but not γ-8-(CT)-γ-1 chimera. The number of cells assessed is given below each bar. (C) Representative Neurolucida reconstructions of layer II/III pyramidal cells overexpressing γ-8 or γ-1 or their chimeras at 5-10 DIV. Scale bars: 100 µm. (D-G) Mean±s.e.m. of apical and basal dendritic length and segment number of layers II/III and V/VI pyramidal cells overexpressing γ-8, γ-1 and the chimeras. The analyses were performed at 5-10 DIV. The number of cells reconstructed per group is indicated below the bars in D. Mann–Whitney U test; ***P<0.001, **P<0.01, *P<0.05.

Next, we overexpressed γ-1, γ-8 and its chimeras in OTCs at 5-10 DIV and analyzed dendritic morphology. γ-1 failed to alter dendritic growth in pyramidal cells (Fig. 6D,E). Overexpression of γ-8 again increased apical dendritic length and segment number. Overexpression of γ-8-(CT)-γ-1 abolished the growth-promoting effect. By contrast, γ-8-(Ex1)-γ-1 was able to promote apical dendritic growth (Fig. 6D,F). Basal dendritic length and branching was not affected (Fig. 6E,G). Taken together, these results suggest that γ-8-induced dendritic growth depends on its CT domain. This further supports the assumption that γ-8 influences dendritogenesis primarily by enhancing the trafficking of AMPARs. It is likely that this will result in increased excitability of the neurons.

γ-8 enhances calcium signaling

Next, we examined whether overexpression of γ-8 alters the excitability of the neurons by measuring calcium fluctuations in spontaneously active OTCs. We used two-photon imaging of calcium fluorescence signals between 8 and 10 DIV in pyramidal neurons co-transfected at 5 DIV with γ-8 or its chimeras and the TN-XXL construct, a troponin C-based calcium biosensor (Mank et al., 2008) (Fig. 7). This fluorescence resonance energy transfer (FRET) construct, with $F_{Citrine\,cp174}/F_{CFP}$ flanking the troponin-coding portion, signals upon

binding calcium in transfectants that were identified cyan fluorescent protein (CFP). Compared with control (Fig. 7A), pyramidal cells overexpressing γ-8 displayed significantly increased amplitudes of calcium fluorescence but the frequency was unaltered (Fig 7B,F,G). As a negative control, we showed that co-expressing γ-1 with TN-XXL did not alter amplitude or frequency of TN-XXL calcium fluorescence (Fig. 7C,F,G). Pyramidal cells expressing γ-8-(CT)-γ-1 displayed calcium fluorescence amplitudes similar to those of control transfectants (Fig. 7D,F,G). By contrast, neurons expressing γ-8-(Ex1)-γ-1 displayed calcium amplitudes similar to those seen in γ-8 transfectants (Fig. 7E-G). Our observations fit with previously published data showing that synaptic transmission and AMPARs are reduced in knock-in mice lacking the cytoplasmic tail domain of γ-8 (Sumioka et al., 2011). Our results indicate a change at the postsynaptic level and suggest that γ-8 requires its CT domain but not the Ex1 domain to enhance the excitability of pyramidal cells.

Knockdown of endogenous γ-8 affects dendritic complexity and calcium signaling in pyramidal cells

The current study demonstrated that overexpression of γ-8 causes an increase in dendritic complexity in pyramidal cells during early development. To examine the role of endogenous γ-8, we
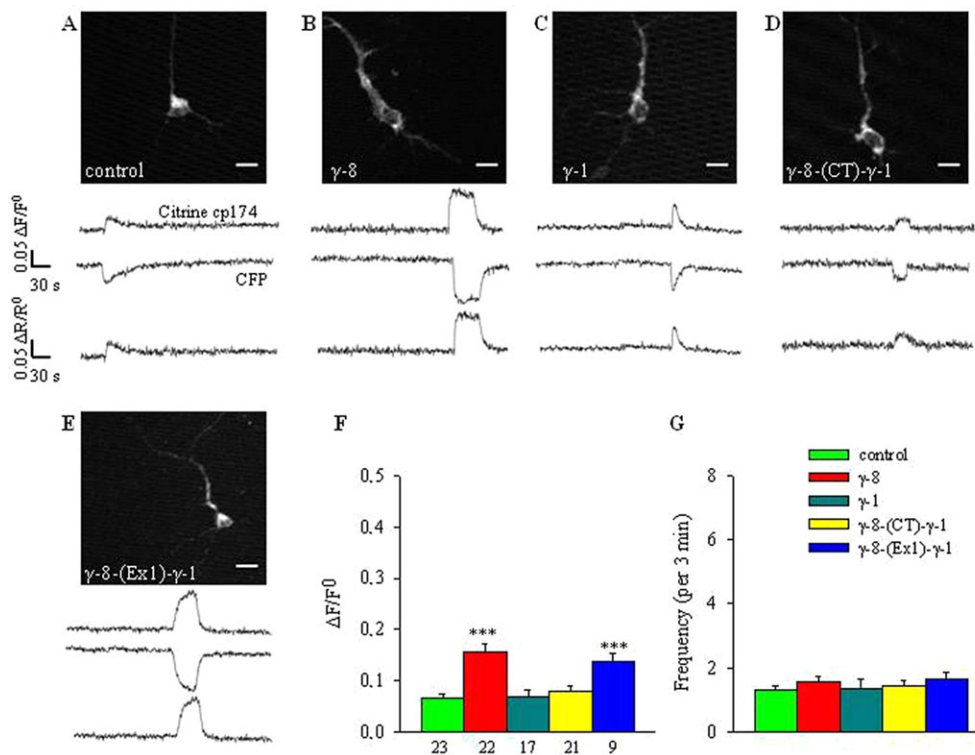
Fig. 7. The γ-8 CT domain is necessary for calcium signaling in pyramidal cells. (A-E) Single-cell two-photon fluorometric calcium signals were recorded in pyramidal cells overexpressing γ-8, γ-1 or γ-8 chimeras at 8-10 DIV using the TN-XXL construct as a FRET ratiometric indicator. Example images of recorded pyramidal cells at 20× magnification: control (A), γ-8 (B), γ-1 (C), γ-8-(CT)-γ-1 (D), γ-8-(Ex1)-γ-1 (E). Corresponding $\Delta F/F^0$ traces from Citrine cp174 and CFP channels and the $\Delta R/R^0$ traces are shown below each image. (F) Maximal $\Delta R/R^0$ recorded in spontaneously active pyramidal cells (fluorescence was converted into $\Delta[Ca^{2+}]i$). The γ-8 CT domain but not the Ex1 domain is required for increasing in amplitude of calcium transients. (G) The frequency (number of calcium events recorded in 3 min) was not different from control cells. The number of cells imaged for every condition is given below the bars in F. Error bars represent s.e.m. Mann–Whitney U test; ***$P<0.001$. Scale bars: 10 μm.

employed shRNAs to knock down the expression of γ-8. First, we characterized the most efficient shRNA construct for knocking down γ-8 directly at the protein expression level. For that, we co-expressed in HEK293 cells the γ-8-mCherry-N1 together with one of four shRNAs tagged with monster Green fluorescent protein (hMGFP) that target the γ-8 mRNA in HEK293 cells or a scrambled sequence as a negative control (supplementary material Fig. S5). The γ-8-mCherry-N1 fluorescence intensity was quantified to identify the most efficient shRNA construct. The most efficient construct was clone 3, which yielded ~70% knockdown efficiency (supplementary material Fig. S5). This shRNA and the scrambled shRNA were used for the experiments on calcium imaging and dendritic morphometry.

Next, we measured calcium fluctuations in pyramidal cells expressing the γ-8 shRNA or the scrambled shRNA (Fig. 8A,B). Pyramidal cells overexpressing γ-8 shRNA showed a significant reduction of the amplitude but not the frequency of calcium events (Fig. 8A,B). This suggests that endogenous γ-8 regulates calcium signaling in pyramidal cells.

To test the role of endogenous γ-8 in dendritic growth, we overexpressed the γ-8 shRNA or the scrambled shRNA in pyramidal cells at 5-10 DIV (Fig. 8C-G). The overexpression of γ-8 shRNA in pyramidal cells reduced apical dendritic length in both layers (Fig. 8D,E). Basal dendrites were not affected (Fig. 8F,G). Dendritic length and segments of interneurons were not affected by the expression of γ-8 shRNA (Fig. 8H), which shows that endogenous γ-8 is not involved in interneuronal maturation. Moreover, the Sholl analysis showed a significant decrease in dendritic complexity in the proximal zone of apical dendrites of γ-8 shRNA-expressing pyramidal cells in both layers in comparison with scrambled shRNA- and EGFP-only-transfected control cells (Fig. 8I,J). Basal dendritic complexity was not altered (Fig. 8K,L). This suggests that endogenous γ-8 is an important modulator of pyramidal cell apical dendritic growth during the early time window.

DISCUSSION

Glutamatergic signaling elicits membrane depolarization in neurons by activating AMPARs. Depending on size and kinetics, currents through AMPARs gate downstream processes that promote dendritic growth (Haas et al., 2006; Hamad et al., 2011; Inglis et al., 2002). We found that the dendritic growth effect elicited by the TARPs could have been further potentiated by the increased expression of the AMPAR subunits GluA2 and GluA3. For pyramidal cells, GluA2(R)-flip and GluA3(Q)-flip have previously been found to be the dendritogenically active AMPAR subunits (Hamad et al., 2011); they cause prolonged channel open times and/or additional calcium permeability. We suggest that γ-8 in young pyramidal cells mediates the trafficking of GluA2 and GluA3 subunits, including the flip variants, which are at high levels in immature neurons (Monyer et al., 1991).

The present study demonstrated a role of γ-8 for dendritic growth already between 5 and 15 DIV, which is in line with the developmentally early function reported for γ-8 (Menuz et al., 2009). The effects of γ-2, γ-3 and γ-8 were compartment-specific as apical but not basal dendritic growth was promoted. This matched our previous observations that overexpressed AMPAR subunits could regulate apical, but not basal, dendrites (Hamad et al., 2011). Indeed, the Sholl analyses revealed that γ-8 increases the complexity of proximal apical dendrites until 10 DIV, and that the action shifts to more distal zones until 15 DIV. In the later time window, γ-2 and γ-3 did the same. Thus, γ-2, γ-3 and γ-8 presumably traffic their cargo preferentially to apical dendrites and in particular to the distal zones. AMPARs are more enriched in apical dendrites and even increase in number with distance from the soma; thus, apical dendrites are more sensitive to glutamate than are basal dendrites (Pettit et al., 1997). Indeed, γ-8 overexpression rendered neurons highly sensitive to AMPA stimulation in both time windows whereas γ-2 and γ-3 did the same in the later time window. Moreover, γ-8 elicits its growth effects via AMPARs, which trigger the activation of NMDARs.
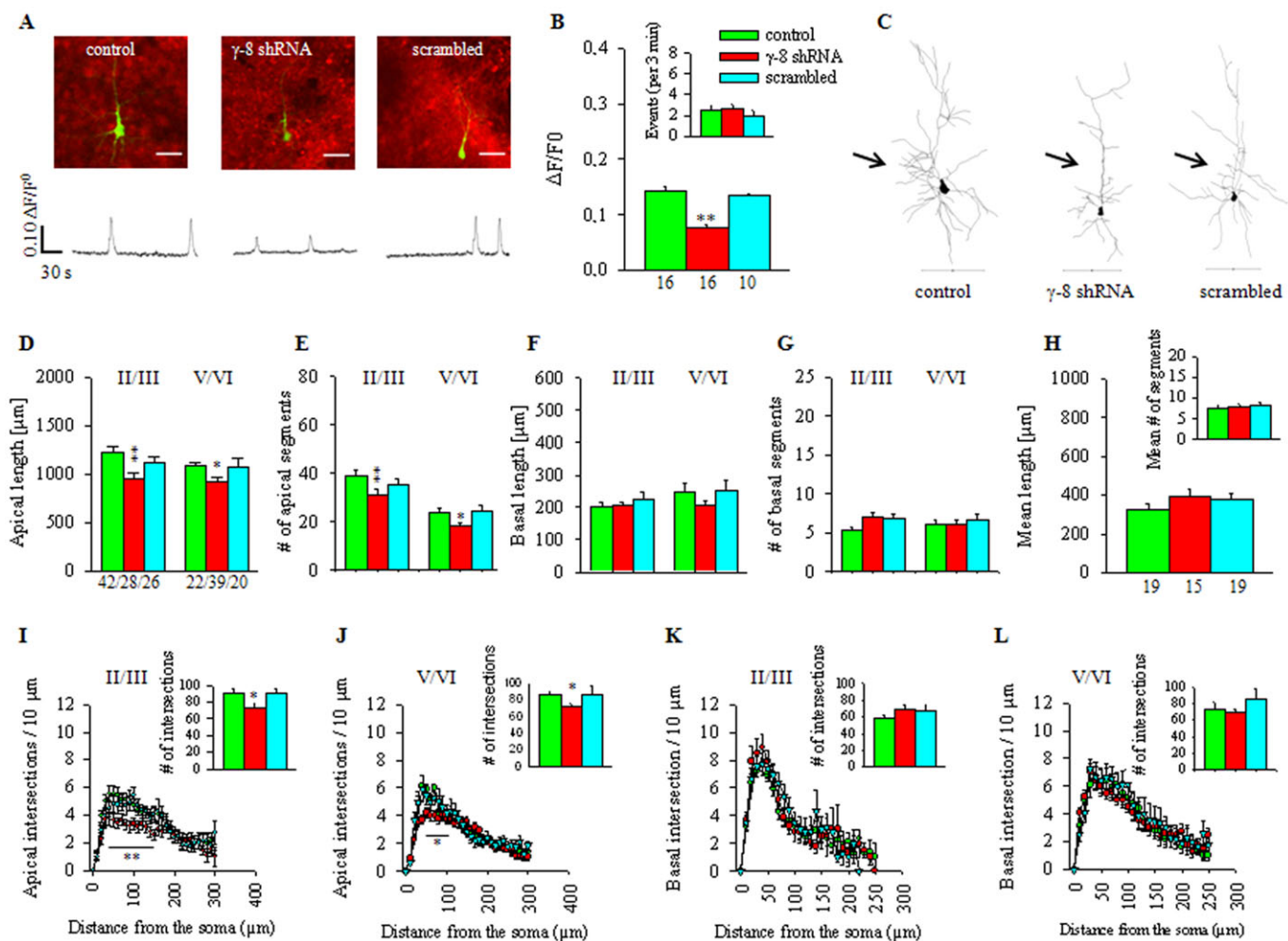
Fig. 8. Knocking down endogenous γ-8 reduces calcium signals, dendritic growth and dendritic complexity in pyramidal cells. (A) Overlay images at 10× magnification from control, γ-8-shRNA-transfected and scrambled shRNA-transfected pyramidal neurons loaded with Calcium Orange. Scale bars: 30 μm. Below the overlay images are traces displaying baseline and maximal peak of intracellular calcium events expressed as $\Delta F/F^0$. (B) The main graph shows the maximal increase in calcium signal expressed as $\Delta F/F^0$ and insert shows the frequency of calcium events per 3 min. (C) Examples of reconstructed pyramidal cells from the three conditions. Scale bars: 100 μm. Arrows indicate the proximal dendritic compartment. (D-G) The graphs indicate the mean±s.e.m. of apical length (D), apical segment number (E), basal dendritic length (F) and basal segment number (G) of layer II/III and V/VI pyramidal cells overexpressing γ-8, γ-8-shRNA or the scrambled sequence. The number of pyramidal cells analyzed is given below the bars in D; it is the same for E-G and also for the Sholl analysis (I-L). (H) As expected, in interneuronal dendritic length (H, main graph) and number of branches (H, insert) were not altered. The number interneurons analyzed is given below the bars. (I,J) Sholl analyses showed a reduction in dendritic complexity in proximal apical dendrites in layer II/III and V/VI pyramidal cells. (K,L) Basal dendrites in layer II/III and V/VI pyramidal cells were not altered. Indeed, the total dendritic intersections were significantly reduced in apical and not basal dendrites (I-L, inserts). Mann–Whitney U test; **$P<0.01$, *$P<0.05$.

Moreover, our results demonstrated for γ-8 that replacing its CT domain with that of a non-TARP eliminates its effects on agonist-induced excitotoxicity, dendritic growth, and potentiation of calcium transients in pyramidal cells, whereas replacing the Ex1 domain had no such effects. However, studies in heterologous expression systems have shown that the CT domain is additionally involved in AMPAR channel modulation (Turetsky et al., 2005). We attempted to address the role of AMPAR channel modulation by employing type II TARPs as they are known to modulate AMPARs without affecting trafficking. However, type II TARPs neither altered dendritic complexity nor evoked a higher sensitivity to AMPA. The failure of γ-5 might be due to the fact that it is not normally present in cortical neurons (Fukaya et al., 2005; Kato et al., 2007), although previous studies reported that overexpressed γ-5 alters AMPAR function in cortical neurons (Kato et al., 2007). γ-7 in cerebellar neurons localizes to the

postsynaptic density, significantly enhances glutamate-evoked currents, and modulates gating of AMPARs (Yamazaki et al., 2010). However, in neocortical neurons, γ-7 shows only weak interactions with AMPARs in immunoprecipitation experiments (Kato et al., 2007), which might be a reason why γ-7 failed to modulate dendritic growth in the present study.

A surprising finding was that the embryonic TARP γ-4, when overexpressed postnatally, can induce dendritic injury in both interneuronal and pyramidal cell transfectants. In addition, γ-4 reduced dendritic growth and proximal complexity at the later time window (10-15 DIV). We found γ-4 enriched in extrasynaptic rather than synaptic membranes, whereas γ-2 was also particularly enriched in spine heads. The mainly extrasynaptic localization of γ-4 and its associated AMPAR cargo presumably renders the cells more vulnerable to excitotoxicity. Furthermore, it is tempting to speculate that an excess of γ-4 in neurons aged 10-15 DIV not only

sequesters AMPARs in the extrasynaptic zones but impairs the proximo-distal diffusion and the redistribution of AMPARs to more distal synapses (Hoerndli et al., 2013).

With regard to interneurons, we expected strong modulatory effects. Interneurons express stargazin (Tomita et al., 2003) and neocortical interneurons are specifically enriched with γ-2 (Maheshwari et al., 2013; Tao et al., 2013). Moreover, all type I TARPs interact with GluA1 resulting in a slow-down of GluA1 receptor desensitization and deactivation (Cho et al., 2007; Korber et al., 2007; Milstein et al., 2007). GluA1 is highly expressed in neocortical interneurons compared with the pyramidal cells (Geiger et al., 1995), and GluA1(Q)-flip has been reported to be the only AMPAR subunit promoting dendritogenesis of interneurons (Hamad et al., 2011). Strikingly, however, γ-2 and the other TARPs failed to alter dendritic growth of interneurons. This might be due to an inefficient trafficking of GluA1(Q)flip compared with other AMPARs subunits. However, with the exception of γ-4, none of the TARPs rendered interneurons more sensitive to AMPA stimulation, and this suggested that in interneurons most TARPs failed to traffic AMPARs. The reasons remain to be clarified; possibly, transport molecules other than TARPs are more important in interneurons. Recently, Erbin (also known as Erbb2ip) has been identified as a co-factor that stabilizes the γ-2-AMPAR interaction specifically in interneurons. This suggests that in immature interneurons Erbin is a factor that limits the action of TARPs (Tao et al., 2013).

To this end, we can conclude that only the dual-function type I TARPs promote dendritic growth. It is likely that this requires an increase in the number of AMPARs for the following reasons: first, overexpressing selected AMPAR subunits increases dendritic complexity (Hamad et al., 2011; Inglis et al., 2002); second, knocking the number of AMPARs down drastically reduces dendritic complexity (Haas et al., 2006); third, overexpression of γ-8 increases the number of AMPARs in the plasma membrane whereas in γ-8$^{-/-}$ mice AMPAR-mediated synaptic transmission is severely impaired because of a loss of GluA2 and GluA3 (Fukaya et al., 2006; Hashimoto et al., 1999; Menuz et al., 2009; Rouach et al., 2005). The growth-promoting TARPs identified in the present study have one common feature: they enrich in the postsynaptic site or postsynaptic density fraction as revealed by electron microscopy and biochemistry (Ferrario et al., 2011; Fukaya et al., 2006; Kato et al., 2010). Morever, γ-8 tends to deliver AMPARs to synaptic sites (Sumioka et al., 2011). Together, these data suggest that only AMPARs that become localized to synaptic membranes are efficient modulators of dendritic growth. In summary, we propose that γ-2, γ-3 and γ-8 influence the dendritic architecture of developing neocortical pyramidal cells because they have a key role for trafficking and positioning of AMPARs. Thus, TARPs might be important for balancing two maturational processes: the increasing levels of AMPAR-mediated synaptic transmission and the activity-dependent shaping of dendritic trees.

MATERIALS AND METHODS

Organotypic cultures, expression plasmids and chimera generation

Roller-type OTCs were prepared from newborn Long Evans rat occipital cortex as described (Wirth and Wahle, 2003). Plasmids were prepared as endotoxin-free solutions (Qiagen). Enhanced green fluorescent protein (pEGFP-N1, Clontech) in pcDNA3.0 was used as reporter. The chimeras of TARP γ-8 with the cytoplasmic domain [CT; plasmid γ8-(CT)-γ1] and the extracellular domain 1 [Ex1; plasmid γ8-(Ex1)-γ1] of γ1 were generated via PCR-directed mutagenesis and tested with recordings in *Xenopus* oocytes as described (Sager et al., 2009). All TARP constructs were generated without a

fluorescent tag to avoid potential problems with trafficking and functional membrane insertion. They were cloned into pcDNA3.0 and employed for morphometry, dendritic injury assays, and calcium imaging. The degree of co-expression of two independent co-transfected plasmids was previously reported to be ~90% in OTCs (Hamad et al., 2011). For knockdown experiments, the γ-8 construct was subcloned into the pmCherry-N1 vector generating the γ-8-mCherry-N1 construct. The TN-XXL construct (Mank et al., 2008) was used for calcium imaging.

Biolistic gene gun transfection and pharmacological treatment
Cartridges were prepared as described (Wirth and Wahle, 2003). In brief, 10 μg endotoxin-free plasmid encoding EGFP as reporter alone, or in combination with 10 μg pcDNA3.0 plasmid encoding one of the eight γ proteins were used for gold particle coating. Transfection was performed using a Helios Gene Gun (Bio-Rad) as described (Wirth and Wahle, 2003) at 5 DIV or at 10 DIV. Five days were allowed for overexpression. For pharmacological treatment, one daily pulse of the AMPAR antagonist CNQX (10 μM) or the NMDAR antagonist APV (50 μM) (both from Sigma) was added to the medium from 5 to 10 DIV.

RT-PCR
Small tissue slabs of rat occipital cortex and of OTCs were harvested at matching developmental ages. The mRNA was extracted using a Dynabead mRNA Direct Kit (Dynal). cDNA was synthesized with Sensiscript reverse transcriptase (20 U/μl; Qiagen) at 37°C for 60 min. PCR primers were designed with the NCBI/Primer-BLAST program (supplementary material Table S1A). Glucose-6-phosphate dehydrogenase (G6PDH), a housekeeping gene, was used as input reference. Each primer pair was tested for crossreactivity with non-intended γ cDNAs.

Knockdown of γ-8 expression
The knockdown efficiency of γ-8 protein was quantified in HEK293 cells transfected with 2.5 μg plasmid DNA using Metafectene Pro (Biontex) as described (Ma et al., 2007). For selective knockdown of γ-8, shRNAs were custom-synthesized targeting rat γ-8 (supplementary material Table S1B) or as a scrambled shRNA, and inserted into the pGeneClip hMGFP vector (Qiagen). The efficiency and specificity of the shRNA constructs were determined by quantifying (using MacBiophotonics ImageJ) the fluorescence intensity of γ-8-mCherry-N1-expressing cells co-expressing one of four different γ-8-shRNA constructs or the scrambled shRNA.

Immunohistochemistry and laser confocal microscopy
EGFP immunohistochemistry was performed with mouse anti-GFP (1:1000; G6795, Sigma-Aldrich). The overexpressed TARPs γ-2 and γ-4 were detected with a primary antibody directed against the cytoplasmic domain of γ-2, which cross-reacts with γ-3 and γ-4 (1:300; 07-577, Millipore) and an Alexa Fluor 594 secondary antibody (1:500; A-10438, Molecular Probes). GluAs were detected with rabbit antibodies against GluA1, GluA2 and GluA3 (1:300; AGC-004, AGC-005 and AGC-010, respectively, Alomone Labs). Fluorescence was analyzed with a Leica TCS SP5 confocal microscope and a 63× objective (Leica) and fluorescence intensity was quantified using MacBiophotonics ImageJ software as follows: regions of interest (ROI) were placed in the soma and apical dendrite of the γ-8 transfected and the EGFP control neurons. Images were background-subtracted, and average pixel intensity in somata and dendrites were determined.

AMPA-induced dendritic injury assay
At 10 and 15 DIV, cultures overexpressing the TARPs were challenged for 15 min with 100 μM AMPA or for 10 min with 10 μM NMDA (Tocris) as described (Hamad et al., 2011). The percentage of transfectants displaying dendritic beading was determined by two observers blinded to conditions.

Neuron reconstruction and Sholl analysis
EGFP-immunostained cells were reconstructed (Neurolucida system, MicroBrightField) at 1000× magnification mostly by observers blinded to conditions. Criteria to distinguish pyramidal cells and interneurons were

based on dendritic and axonal patterns (Hamad et al., 2011; Karube et al., 2004). Sholl analysis of the number of dendrite intersections at 10 µm interval distance points starting from the soma was performed separately for apical and basal dendrites to identify the area where dendritic complexity changed (Sholl, 1953; Zagrebelsky et al., 2010).

Two-photon calcium imaging using the TN-XXL construct and confocal calcium imaging

OTCs were allowed to express for 3-5 days the calcium indicator plasmid TN-XXL, alone or together with γ-8 or γ-8 chimeras. Afterwards, the cultures were washed with oxygenated artificial cerebrospinal fluid (in mM: 125 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgSO$_4$, 25 NaHCO$_3$, 1.25 NaH$_2$PO4, 25 glucose, pH 7.4), and transferred to the recording chamber perfused with oxygenated artificial cerebrospinal fluid (3-5 ml/min at 32±2°C). Fluorometric calcium recordings were made using a custom-built two-photon laser-scanning microscope equipped with a Ti:Sapphire laser system as described previously (Hamad et al., 2011). TN-XXL was excited at 860 nm. For the separation of the emission spectra of CFP and Citrine cp174, a beam splitter (515 nm, Visitron Systems) and emission band pass filters for CFP (480/30 nm) and YFP (535/30) were used (AHF). For ratiometric analysis the ratio (R) of Citrine cp174 and CFP fluorescence intensities were calculated as follows: R=F$_{Citrine\ cp\ 174}$/F$_{CFP}$. Raw data delivered in the form of a linear 16 bit intensity scale were plotted as fluorescence intensity versus time. Pyramidal cell soma were chosen as the ROI. The background fluorescence measured near a ROI was then subtracted from these raw data. The baseline ratio (R$_0$) was calculated as an average of the first 20 frames in a time window in which there was no change in fluorescence signals (as judged by visual inspection). Subsequently, data were normalized to the mean ratio intensities using a custom-written MATLAB R2008a program [summary formula is $\Delta R/R = (R-R_0)/R_0$], allowing the comparison of data across experiments. Images were acquired at a rate of four frames per second and analyzed using MacBiophotonics ImageJ software and ScanImage 3.0 (Pologruto et al., 2003).

Confocal laser calcium imaging was carried out on cultures transfected with plasmids encoding the γ-8-shRNA-hMGFP or a scrambled sequence after loading with Calcium Orange (Molecular Probes) dissolved in 20% pluronic acid/DMSO and diluted in 200 µl of the culture's own medium. Fluorometric recordings were performed with a Leica TCS SP5 confocal microscope with a 10× objective. The (x, y, t) scanning mode was employed at 1400 Hz. The hMGFP construct was excited with the 488 nm laser line and the Calcium Orange dye was excited at 565 nm. Fluorometric data are expressed as $\Delta F/F^0$ (background-corrected increase in fluorescence divided by the resting fluorescence) as described previously (Hamad et al., 2011).

Statistical analysis

Statistical analysis was performed with SigmaStat2.03 (SPSS Incorporated). Non-parametric Mann–Whitney U tests were employed to assess statistically significant differences between the treatment group and the control.

Competing interests

The authors declare no competing financial interests.

Author contributions

M.I.K.H., M.H. and P.W. developed the concept and designed research; M.I.K.H., A.J., M.L., T.S. and P.W. performed experiments; C.S. and S.K. generated and functionally tested the TARP constructs in *Xenopus* oocytes; M.I.K.H., M.L., A.J., T.S., O.K. and P.W. analyzed the data. M.I.K.H., M.H. and P.W. wrote the paper.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099697/-/DC1

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DEVELOPMENT