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Cell class-specific regulation of neocortical dendrite and spine growth by AMPA receptor splice and editing variants

Mohammad I. K. Hamad[1,*], Zhan-Lu Ma-Högemeier[2], Christian Riedel[1], Claudius Conrads[1], Thomas Veitinger[1], Tim Habijan[1], Jan-Niklas Schulz[1], Martin Krause[3], Marcus J. Wirth[1], Michael Hollmann[2] and Petra Wahle[1]

SUMMARY

Glutamatergic transmission converging on calcium signaling plays a key role in dendritic differentiation. In early development, AMPA receptor (AMPAR) transcripts are extensively spliced and edited to generate subunits that differ in their biophysical properties. Whether these subunits have specific roles in the context of structural differentiation is unclear. We have investigated the role of nine GluA variants and revealed a correlation between the expression of flip variants and the period of major dendritic growth. In interneurons, only GluA1(Q)-flip increased dendritic length and branching. In pyramidal cells, GluA2(Q)-flop, GluA2(Q)-flip, GluA3(Q)-flip and calcium-impermeable GluA2(R)-flip promoted dendritic growth, suggesting that flip variants with slower desensitization kinetics are more important than receptors with elevated calcium permeability. Imaging revealed significantly higher calcium signals in pyramidal cells transfected with GluA2(R)-flip as compared with GluA2(R)-flop, suggesting a contribution of voltage-activated calcium channels. Indeed, dendritic growth induced by GluA2(R)-flip in pyramidal cells was prevented by blocking NMDA receptors (NMDARs) or voltage-gated calcium channels (VGCCs), suggesting that they act downstream of AMPARs. Intriguingly, the action of GluA1(Q)-flip in interneurons was also dependent on NMDARs and VGCCs. Cell class-specific effects were not observed for spine formation, as GluA2(Q)-flip and GluA2(Q)-flop increased spine density in pyramidal cells as well as in interneurons. The results suggest that AMPAR variants expressed early in development are important determinants for activity-dependent dendritic growth in a cell type-specific and cell compartment-specific manner.

KEY WORDS: Dendritogenesis, Spinogenesis, AMPA receptors, Neocortex, RNA splicing and editing, Two-photon calcium imaging, Rat

INTRODUCTION

Dendrites receive and integrate synaptic inputs, and during development dendritic trees grow under the control of neuronal activity. Studies have provided compelling support for the concept that dendritic arbor maturation is mediated by neuronal activity. AMPA-type and NMDA-type glutamate receptors (AMPARs and NMDARs) play a central role. In frog optic tectum, AMPARs regulate experience-dependent dendritic growth, and a neutralizing peptide that reduces GluA2 expression decreases dendritic complexity (Haas et al., 2006). A number of studies have emphasized the importance of NMDARs. Blockade with the NMDAR antagonist APV reduces the dendritic growth rate normally triggered by visually evoked synaptic activity (Rajan and Cline, 1998; Sin et al., 2002). NMDAR activation accelerates dendritic growth or stabilizes existing arbors (Wu and Cline, 1998). Although the NMDAR subunits GluN2B and GluN2A are both important, neurons overexpressing GluN2B display higher branch dynamics than neurons overexpressing GluN2A (Ewald et al., 2008). Furthermore, a single-cell knockout study has shown that hippocampal pyramidal cells lacking the GluN2B subunit fail to prune extra dendrites, that hippocampal granule and pyramidal cells display a reduced spine density, and that cortical spiny stellates fail to orient dendrites towards the barrels (Espinosa et al., 2009).

In rodents, the contribution of AMPARs has been studied in spinal cord motoneurons. Rat motoneurons strongly express the AMPAR subunit GluA1(Q)-flip, which controls dendritic complexity largely by altering the branching pattern rather than the dendritic length, and the calcium permeability of the subunit is important (Inglis et al., 2002; Jeong et al., 2006; Prithviraj et al., 2008; Zhang et al., 2008). For example, motoneurons in mice lacking GluA1 or cultured rat motoneurons with a knockdown of GluA1 display a reduced dendritic complexity (Zhang et al., 2008). The action of GluA1 depends on normal network connectivity and seems non-cell-autonomous (Zhang et al., 2008). NMDARs contribute to motoneuron dendritic plasticity (Inglis et al., 1998) and overexpression of GluN3B promotes elongation, branching and the growth of filopodia (Prithviraj and Inglis, 2008). In cortex, NMDARs play an important role in dendritic reorganization (Iwasato et al., 2000; Lee et al., 2005).

Cortical pyramidal neurons and the morphofunctionally diverse GABAergic interneurons differentially express a mixture of AMPARs encoded by *GluA1-4* (also known as *Gria1-4*) (Hollmann and Heinemann, 1994). Each GluA gene is subject to alternative splicing into the flip and flop isoforms (Sommer et al., 1990). Flip-containing receptors are more efficiently activated and desensitize with slower kinetics (Mosbacher et al., 1994; Sommer et al., 1990). During early development, flip variants are prominently expressed, but towards adulthood they become replaced by flop-containing subunits (Monyer et al., 1991). The GluA2 receptor is subject to RNA editing, which changes a single amino acid from glutamine (Q) (calcium-permeable) to arginine (R) (calcium-impermeable) (Burnashev et al., 1992; Seeburg et al., 1998). The presence of GluA2(R) in heteromeric AMPARs renders the channel virtually impermeable to calcium (Sommer et al., 1991). For example, fetal

[1]Department of Developmental Neurobiology, Ruhr University Bochum, 44780 Bochum, Germany. [2]Department of Biochemistry I – Receptor Biochemistry, Ruhr University Bochum, 44780 Bochum, Germany. [3]Department of Zoology and Neurobiology, Ruhr University Bochum, 44780 Bochum, Germany.

*Author for correspondence (mohammad.hamad@rub.de)

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neurons often coexpress calcium-permeable unedited GluA2(Q) and calcium-impermeable edited GluA2(R) and therefore display AMPARs with considerable calcium permeability. Perinatally, *GluA2* transcripts are edited, such that GluA2-containing channels are calcium-impermeable (Burnashev et al., 1992; Sommer et al., 1991). GluA receptors are differentially expressed in neocortical neuron classes and increase after birth (Monyer et al., 1991; Pellegrini-Giampietro et al., 1991). Pyramidal cells mainly express GluA2 and GluA3 and little GluA1, whereas inhibitory interneurons express mainly GluA1 and little GluA2 and GluA3 (Geiger et al., 1995; Jonas et al., 1994).

For cortical pyramidal and GABAergic neurons, it is not known whether the AMPAR subunits are equipotent for the induction of dendritic growth or whether their action depends on the context: cell class, laminar compartment or dendritic compartment. From a biophysical point of view, we initially hypothesized that the calcium-permeable subunits would be the most potent in both cell classes. In addition, we hypothesized that subunits with longer channel open times (flip) might play a more important role than hitherto believed. The present study shows that selected GluA splice and editing variants contribute in a cell class-specific manner to dendritogenesis and spine growth. Moreover, evidence is presented that NMDARs contribute to dendritogenesis in both cell classes.

MATERIALS AND METHODS

Organotypic cultures and pharmacological treatment

Organotypic slice cultures (OTCs) of rat visual cortex were prepared as described (Wirth and Wahle, 2003) from pigmented Long Evans rats at the day of birth (six to eight animals for every culture batch, two to four batches per experiment). Slices from every individual animal were allocated to all experimental conditions tested with the batch. For pharmacological treatment, one daily pulse of the AMPAR antagonist CNQX (10 μM; Sigma, Steinheim, Germany), the VGCC blocker nifedipine (10 μM; Sigma, Deisenhofen, Germany), or the NMDAR antagonist APV (DL-2-amino-5-phosphonovaleric acid, 50 μM; Sigma, Deisenhofen, Germany) was added to the medium from 5-10 days in vitro (DIV) concurrent with the overexpression.

Expression plasmids and biolistic transfection

At 5 DIV, cultures were transfected using a Helios Gene Gun (BioRad, München, Germany) as described (Wirth et al., 2003). Cartridges were prepared by coating 10 mg gold particles (1 μm; BioRad) with 10 μg endotoxin-free plasmid encoding enhanced green fluorescent protein (pEGFP-N1; Clontech, Heidelberg, Germany) as reporter alone, or in combination with 10 μg pcDNA3.0 plasmid encoding one of the following AMPAR variants: GluA1(Q)-flip, GluA1(Q)-flop, the dominant-negative mutant GluA1(R)-flip (Pei et al., 2009), GluA2(Q)-flip, GluA2(Q)-flop, GluA2(R)-flip, GluA2(R)-flop, GluA3(Q)-flip or GluA3(Q)-flop. The constructs lacked a fluorescent tag to avoid potential problems with trafficking and functional membrane insertion, and cells transfected with these constructs were used for morphometric analysis, immunohistochemical detection of overexpressed receptor protein, dendritic injury assay and calcium imaging. To assess the degree of coexpression, selected GluA variants fused with EYFP (Ma et al., 2007) were coexpressed together with DsRed2 (Clontech) for 5 DIV followed by confocal microscopy analysis. DsRed2 also served as morphological marker during calcium imaging.

Detection of edited versus unedited GluA2(Q) subunits during development

The extent of editing of the *GluA2* transcript was detected as previously described with some modification (Paschen and Djuricic, 1995). Samples were taken from the E15.5 rat occipital cortex separated into ventricular plus intermediate zone and cortical plate plus marginal zone, from visual cortex at P1, P2, P5 and P10, and from visual cortex OTC at 1, 2, 5, 7, 8 and 10 DIV. The mRNA was prepared using a Dynabead mRNA Direct Kit (Dynal, Hamburg, Germany). cDNA libraries were synthesized with Sensiscript reverse transcriptase (20 U/μl; Qiagen, Hilden, Germany) at 37°C for 60 minutes. PCR was performed to amplify a product across the edited region of *GluA2* using the first-strand cDNA as template. PCR products were precipitated overnight at –80°C with 0.3 M sodium acetate and one volume of isopropanol, collected by centrifugation, and subjected to *Bbv*I restriction (NEB, Schwalbach, Germany) overnight in NEB1 buffer. *Bbv*I recognizes the non-edited GCAGC sequence but not the edited GCGGC sequence of *GluA2*. The amplification product originating from non-edited mRNA is cut into 190 and 81 bp fragments, whereas the fraction originating from edited mRNA remains uncut at 271 bp.

Immunohistochemistry and confocal microscopy

At 10 DIV, OTCs were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 and warmed to 36°C for 2 hours. For morphometric reconstructions, immunostaining for EGFP was performed as described (Wirth and Wahle, 2003) using a mouse antibody against GFP (1:1000; Sigma-Aldrich, Schnelldorf, Germany). The DAB reaction product was intensified with osmium tetroxide followed by dehydration and coverslipping. Protein overexpression was demonstrated with rabbit

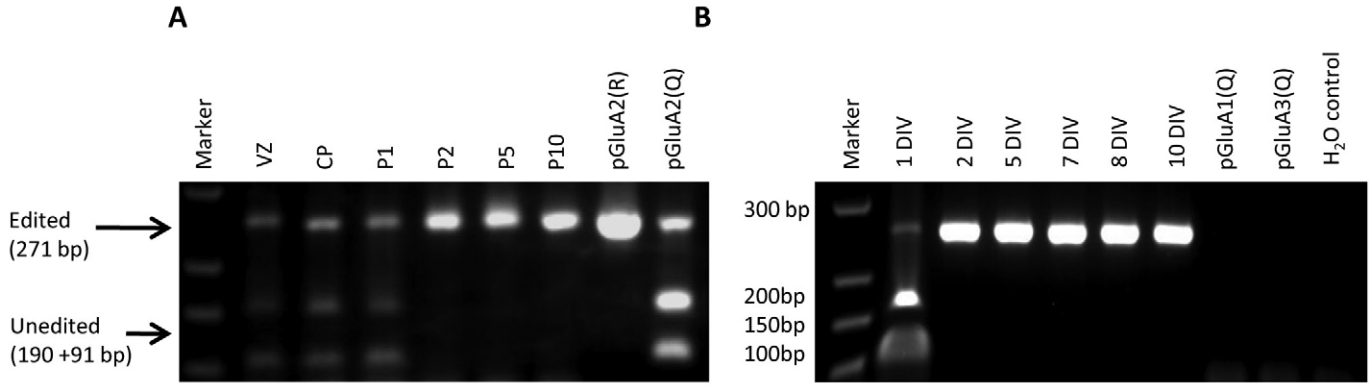

Fig. 1. Evaluation of RNA editing of the *GluA2* subunit in the rat cortex. *GluA2*-specific reverse transcriptase PCR was performed with cDNA synthesized from mRNA isolated from ~300 μm cortex slices followed by digestion with *Bbv*I. (**A**) Edited *GluA2(R)* is represented by the band at 271 bp, which is resistant to *Bbv*I. Unedited *GluA2(Q)*, as represented by digest products of 190 bp and 91 bp, was present in moderate amounts at E15.5 in ventricular/intermediate zone (VZ) and cortical plate/marginal zone (CP) and was detected until the first postnatal day (P1). *GluA2(R)* plasmid DNA served as a negative control template, and *GluA2(Q)* served as positive control template. (**B**) Analyses of organotypic slice culture (OTC) prepared at P0 show that unedited *GluA2(Q)* is present at 1 DIV but thereafter became undetectable. As further controls, no products were detected when plasmid DNA encoding GluA1(Q) or GluA3(Q) was amplified with *GluA2*-specific primers. In the H_2O control lane, template was omitted.

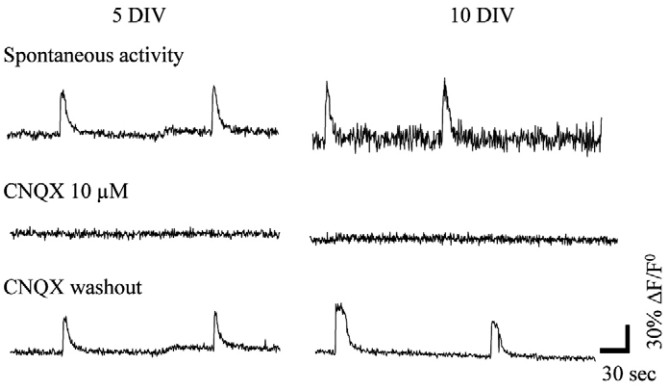

Fig. 2. Glutamatergic signaling in OTCs. Calcium signals, expressed as $\Delta F/F^0$, in spontaneously active OTC at 5 DIV and 10 DIV as revealed by two-photon imaging. Sensitivity to CNQX indicated that GluA receptors contribute to the calcium signals (12 OTCs, six randomly selected areas of interest per OTC), suggesting the occurrence of glutamatergic transmission during the 5-10 DIV time window permitted for dendritic growth.

antibodies against GluA1, GluA2 and GluA3 (1:300; Alomone Labs, Jerusalem, Israel) and Alexa Fluor 594 secondary antibody (Molecular Probes, Karlsruhe, Germany) and mounted with Vectashield (Vector Labs, Burlingame, CA, USA). Fluorescence was analyzed with a Leica TCS SP2 AOBS confocal microscope and 63× objective (Leica, Mannheim, Germany).

AMPA-induced dendritic injury assay

In heterologous expression systems, overexpressed GluA subunits are faithfully transported to the plasma membrane. To confirm this for cortical transfectants, we coexpressed GluA subunits together with EGFP for 5 days. At 10 DIV, cultures were challenged for 15 minutes with 100 μM AMPA to induce receptor-mediated dendritic injury. The 15-minute time point had been determined empirically by imaging transfectants for more than 60 minutes after AMPA stimulation. Cultures were fixed, immunostained for EGFP, and the percentage of transfectants displaying dendritic beading was determined by two observers blinded to the conditions (10-14 OTCs per condition from two preparations). We also performed these experiments without AMPA stimulation to rule out the possibility that the overexpression of GluA subunits evokes cell death via endogenous glutamate signaling present in the OTCs.

Neuron reconstruction and statistical analysis

EGFP-immunostained cells were reconstructed with the Neurolucida system (MicroBrightField, Williston, VT, USA) at 1000× magnification using established criteria that easily distinguish between pyramidal cells and interneurons based on their dendritic and axonal pattern (Karube et al., 2004; Wirth et al., 2003). In brief, layer II/III pyramidal cells reside in the outer third of the OTCs with apical dendrites branching into layer I. Pyramidal cells of layers V/VI reside in the deeper two-thirds of the OTC and were scored when apical dendrites terminated in middle layers; large layer V pyramidal neurons were omitted. All pyramidal cells had axons descending to the white matter. Interneurons were multipolar with sparsely spineous or aspiny dendrites and axons branching within the dendritic tree into horizontal or columnar arbors. For pyramidal cells, apical dendritic length and segment number, the mean length, mean segment number of all basal dendrites (≥20 μm) and the number of primary dendrites were assessed. For interneuronal dendrites (≥20 μm), we calculated mean dendritic length, mean number of dendritic segments and number of primary dendrites. With camera lucida drawings at 1000× magnification and a trained observer blinded to the conditions, we determined the density of short stubby, thin and mushroom spines; filopodia were omitted. For pyramidal cells, spines were assessed along basal dendrites starting from the cell body (on average 250 μm, two to three dendrites per cell) and along several oblique secondary branches arising from the proximal part of the apical dendrite; the tuft was avoided.

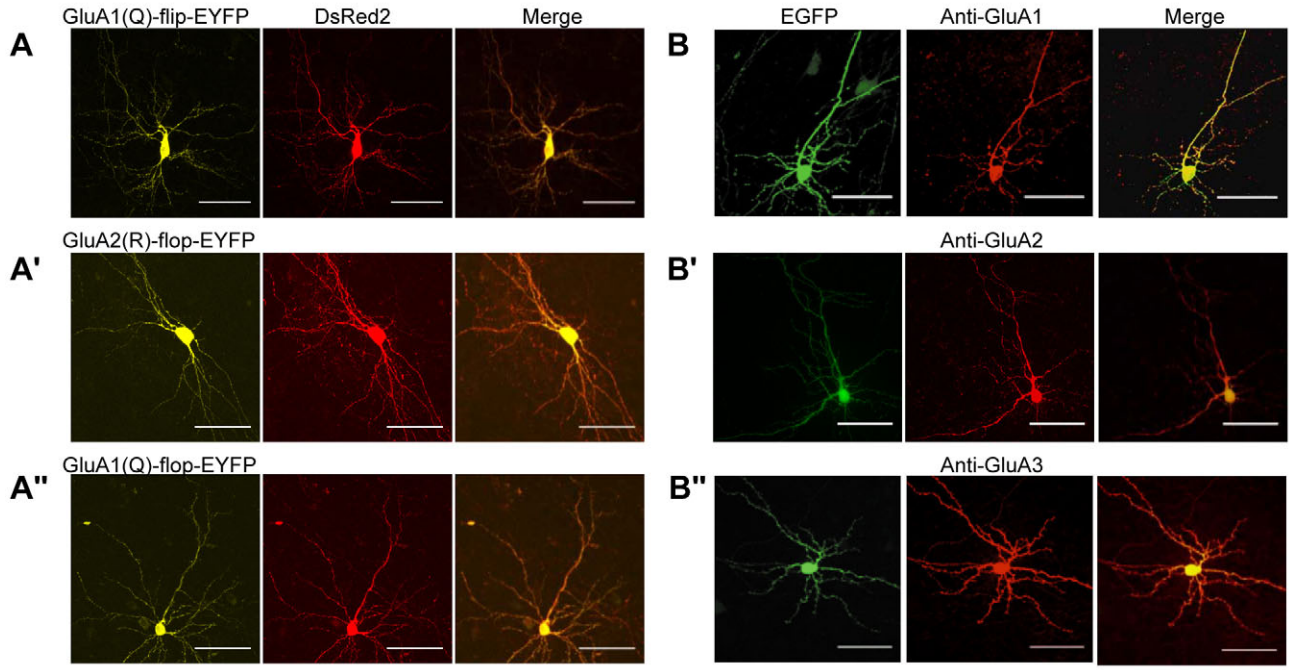

Fig. 3. GluA subunit expression in OTCs. (**A-A″**) Confocal images (taken at 63× magnification) show representative interneurons transfected with EYFP-tagged GluA1(Q)-flip and DsRed (A) or GluA2(R)-flop and DsRed2 (A′) and a pyramidal cell transfected with EYFP-tagged GluA1(Q)-flop and DsRed2 (A″). On average, 90% of the transfectants simultaneously displayed EYFP-tagged GluA and DsRed2. (**B-B″**) Immunostaining with specific antibodies against GluA1, GluA2 and GluA3. Confocal images (taken at 63× magnification) show pyramidal cells transfected with GluA1(Q)-flop (B) or GluA2(R)-flop (B′) and an interneuron transfected with GluA3(Q)-flip (B″) in combination with DsRed2. A high level of GluA protein was present in somata and apical and basal dendrites of transfected pyramidal cells and interneurons. Scale bars: 50 μm.

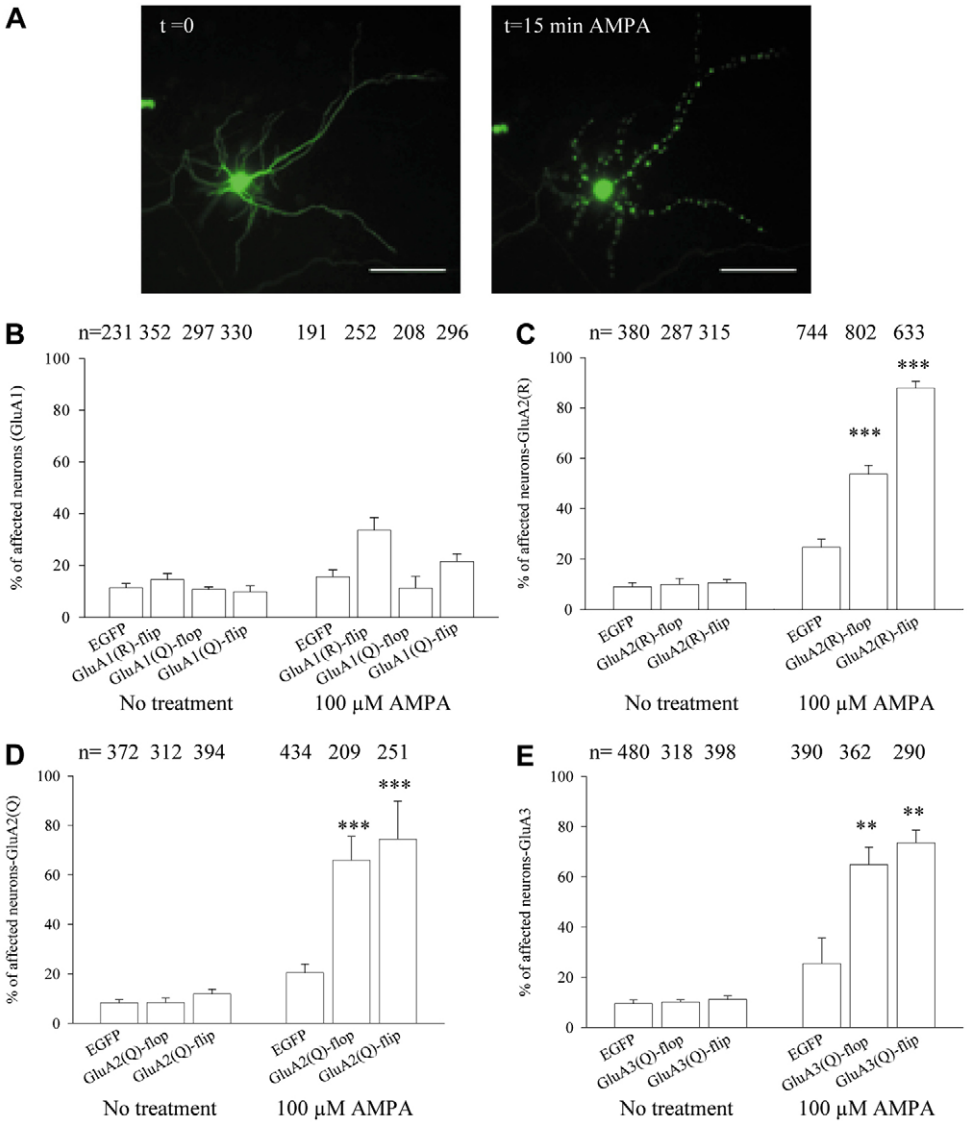

Fig. 4. AMPA-induced excitotoxicity in GluA-overexpressing neurons. (**A**) Pyramidal cell cotransfected with EGFP and GluA2(R)-flip imaged before and 15 minutes after 100 µM AMPA application. Beading of dendrites indicates functional GluA2 expression. (**B-E**) Percentages of cells (mean ± s.e.m.) displaying dendritic beading in untreated and 100 µM AMPA-treated OTCs transfected with EGFP (control) or GluA1 (B), GluA2(R) (C), GluA2(Q) (D) or GluA3(Q) (E). In total, 9528 neurons were assessed. Numbers of analyzed cells per group are given above the bars. AMPA provoked massive dendritic injury in neurons overexpressing flip or flop variants of GluA2(R), GluA2(Q) or GluA3 (C-E). ***, $P<0.001$; **, $P<0.01$; for treatment versus EGFP control group. Scale bars: 30 µm.

Statistical analysis was performed with Sigma Stat version 2.03 for Windows (SPSS, Chicago, IL, USA). One-way ANOVA followed by Bonferroni correction for multiple comparisons was employed when normality and equal variance passed. When normality failed, Kruskal-Wallis ANOVA on ranks followed by Dunn's correction for multiple comparisons was used. Non-parametric Mann-Whitney U-tests were employed to assess the statistical significance of differences between the treatment group and the control.

Two-photon calcium imaging

OTCs were loaded with 10 µM Oregon Green 488 BAPTA-1 AM (OGB-1 AM; dissolved in 20% pluronic acid/DMSO; Molecular Probes, Eugene, OR, USA) diluted in 200 µl self-conditioned culture medium for 1 hour at 36°C. Excess dye was washed out with oxygenated ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, pH 7.4). OTCs were incubated for another hour for recovery, then transferred to the recording chamber mounted on a fixed stage of an upright microscope, and continuously perfused with oxygenated ACSF (3-5 ml/minute at 32±2°C). Fluorometric recordings were made using a custom-built two-photon laser-scanning microscope equipped with a Ti:sapphire laser system (Spectra-Physics, Mountain View, CA, USA) for mode-locked laser light (pulse width less than 100 femtoseconds; repetition rate, 80 MHz), a laser-scanning system attached to a movable objective system on an upright microscope (Sutter Instruments, Lambrecht/Pfalz, Germany), and a water-immersion objective (20×, 0.8 NA; Zeiss, Oberkochen, Germany).

The DsRed2-transfected neurons were identified by two-photon excitation at 940 nm and emission collected through a 610/675 nm bandpass filter. Scattered infrared light was blocked by means of a 680 nm barrier filter (Chroma Technologies, Rockingham, VT, USA) mounted in front of the detector. To monitor calcium changes, the excitation wavelength was then changed to 820 nm and the laser power was reduced (<80 mW) and emission was collected through a 535/550 nm bandpass filter. Images were acquired at a rate of four frames/second and analyzed using MacBiophotonics ImageJ software and ScanImage 3.0 (Pologruto et al., 2003). In the OGB-1 AM recording channel, individual cell somata were selected as the area of interest (AOI) and the mean intensities in the AOIs in each frame were determined. Raw data delivered in the form of a linear 16-bit intensity scale were first plotted as fluorescence intensity versus time. The background fluorescence measured near an AOI was then subtracted from these raw data. The F^0 (resting fluorescence) image was generated by averaging the fluorescence intensities of 10-50 frames from the image stack in a time window when there was no activity (by visual inspection). Subsequently, data were normalized to the mean fluorescence intensities [$\Delta F/F^0=(F-F^0)/F^0$], allowing the comparison of data across experiments.

RESULTS

The unedited GluA2(Q) isoform is present early in developing cortex

The developmental profiles of AMPAR subunit expression have been described (Monyer et al., 1991); however, it has remained unclear for the neocortex when the unedited variant of GluA2 becomes downregulated. Therefore, a PCR approach was used to determine the presence of unedited *GluA2(Q)* mRNA in early postnatal rat visual cortex. The expression of GluA2(Q) has been determined in whole rat brain lysates followed by sequencing (Burnashev et al., 1992; Sommer et al., 1990). The authors estimate that from E14-P0, ~1% of GluA2 exists in the unedited form, whereas at P8 this has dropped to below 0.01%. We used the PCR approach followed by *Bbv*I restriction (Paschen and Djuricic, 1995). Using cDNA from neocortex in vivo and in vitro, our data showed that the unedited *GluA2(Q)* mRNA is detectable at E15.5, with stronger expression in the cortical plate sample, and that it remains detectable until P1 (Fig. 1A). Similarly, in OTCs the unedited *GluA2(Q)* mRNA was present at 1 DIV but undetectable thereafter (Fig. 1B). Using *GluA1* and *GluA3* plasmid DNA in addition to *GluA2* as template, we controlled for potential cross-amplification of *GluA1* or *GluA3* sequences with *GluA2* primers, which might occur owing to the considerable sequence homology around the edited region (Bettler et al., 1990). Specific PCR products were produced only when *GluA2* plasmid DNA was used as template (Fig. 1B), indicating that *GluA2* is specifically amplified. These results suggested that a certain, albeit declining, level of unedited GluA2(Q) protein is present during the early postnatal period.

Glutamatergic signaling is present in early postnatal OTCs

The overexpressed GluA receptor subunits can only promote dendritic growth when activated by endogenous glutamate signaling. However, the excitatory drive in young OTCs is rather low before 10 DIV (Klostermann and Wahle, 1999). To test whether OTCs exhibit spontaneous glutamatergic activity between days 5 and 10, we employed two-photon calcium imaging in the absence of exogenous stimulation. We observed that synchronized calcium waves reminiscent of cortical early network oscillations appear at ~5 DIV and recruit the entire network (Fig. 2). The events were highly correlated across the culture and required AMPARs, as application of the AMPAR antagonist CNQX abolished the activity (Fig. 2). This suggested the existence of functional glutamatergic signaling in OTCs during the time period allowed for dendritic growth.

Coexpression efficiency and immunohistochemical detection of GluA subunits

We employed the gene gun-mediated overexpression approach to test the role of the single amino acid change at the Q/R editing site and the role of the alternatively spliced flip and flop sequences in dendritic growth. All of the subunits are normally expressed and most are developmentally upregulated (Traynelis et al., 2010) during the time of analysis, between 5 and 10 DIV, which covers the major dendritic growth period. The degree of subunit coexpression was determined using two plasmids encoding DsRed2 and various EYFP-tagged GluA subunits (Fig. 3A; n=287 neurons). We found a ~90% coexpression efficiency for two independent plasmids, confirming our previous findings (Wirth and Wahle, 2003). Next, we tested for the presence of overexpressed receptor proteins using specific antibodies against GluA1, GluA2 and GluA3. After 5 days of expression, the proteins were highly expressed in somata, dendrites and spines of pyramidal cells and interneurons (Fig. 3B).

Agonist-induced excitotoxicity reveals a higher cell surface density of AMPARs

An agonist-induced dendritic injury assay was used to determine whether GluA subunit transfectants indeed display a higher cell surface density of AMPARs (Fig. 4A). Dendritic injury is a hallmark of excitotoxicity. Activation of glutamate receptors causes an influx of sodium and calcium, which results in an increase in intracellular NaCl concentration and causes the osmotic movement of both extracellular and intracellular water, resulting in focal dendritic swelling accompanied by disruption of the cytoskeleton. This swelling occurs along the dendrites in a distal-to-proximal fashion (Greenwood and Connolly, 2007). GluA-transfected neurons and control EGFP-transfected neurons in untreated OTCs rarely displayed dendritic beading in the absence of the exogenous agonist (~10%, n=4466 neurons; Fig. 4B-E), suggesting that endogenous glutamate does not cause dendritic injury in neurons overexpressing GluA subunits. The 10% affected neurons were mostly layer II/III pyramidal cells with weak beading in distal apical dendritic branches. Distal apical dendritic branches are rich in GluA (Pettit et al., 1997), have AMPAR-containing functional synapses early in development (Rumpel et al., 2004), and are equipped with signal-amplifying mechanisms (Waters et al., 2003) and therefore seem particularly sensitive. This sensitivity was not elicited by GluA overexpression because it was also observed in EGFP control transfectants. By contrast, within 15 minutes, the application of 100 μM AMPA provoked dendritic beading (Fig. 4B-E; n=5062 total neurons). The number of affected EGFP-only control transfectants increased by only a small extent. Of the neurons overexpressing flip or flop variants of GluA2(R), GluA2(Q) or GluA3(Q), however, 50-90% were affected (Fig. 4C-E).

AMPA failed to affect GluA1(Q)-flop transfectants, but a certain increase in the proportion of affected Glu1(Q)-flip transfectants was observed (Fig. 4B). This suggested that the affected subset of GluA1(Q)flip neurons might be non-pyramidal neurons. Indeed,

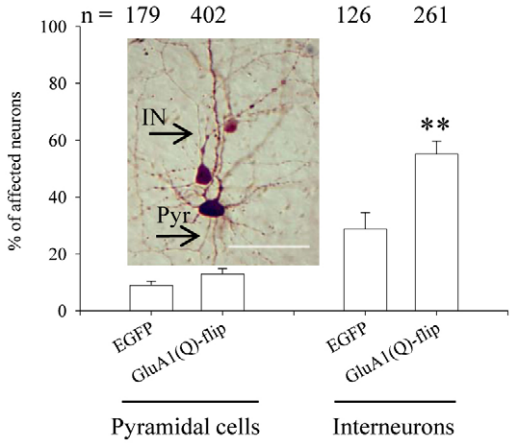

Fig. 5. The AMPA sensitivity of GluA1(Q)-flip transfectants is limited to interneurons. A pyramidal cell (Pyr) next to an interneuron (IN). Arrows indicate axons. Note that the interneuron exhibited dendritic beading after stimulation with 100 μM AMPA, whereas the pyramidal cell remained unaffected. Shown are the percentages of pyramidal cells and interneurons with dendritic beading after stimulation with AMPA (mean ± s.e.m.; the number of cells analyzed is given above the bars). OTCs were transfected with EGFP (control) or GluA1(Q)-flip. **, P<0.01; for treatment versus EGFP control group. Scale bar: 30 μm.

GluA1(Q)-flip-transfected pyramidal cells were unaffected, whereas 60% of GluA1(Q)-flip-transfected interneurons displayed dendritic injury (Fig. 5). Cells overexpressing the mutant GluA1(R)-flip variant were moderately affected, suggesting that expression of this mutant subunit might become lethal to neurons (Fig. 4B). Taken together, the results suggest that, compared with EGFP-transfected control neurons, GluA-transfected neurons have higher levels of receptor protein on the cell surface, where it can serve to induce dendritic growth.

Selected GluA2 and GluA3 variants regulate pyramidal cell dendritic growth

To test the role of GluA splice and editing variants in pyramidal cell growth, we overexpressed individual GluA variants at 5 DIV and allowed 5 days for expression. Our quantitative morphometric analysis (Fig. 6A and Table 1; n=945 total reconstructed neurons) revealed that, in pyramidal cells, GluA3(Q)-flip, GluA2(Q)-flip and GluA2(Q)-flop were able to promote apical dendritic elongation and branching. This was according to the prevailing hypothesis because the subunits can assemble into calcium-permeable receptors. GluA3(Q)-flip enhanced apical dendritic length and branching only in pyramidal cells of layers II/III, but GluA3(Q)-flop failed to do so (Fig. 6A and Table 1). Intriguingly, the calcium-impermeable variant GluA2(R)-flip strongly promoted apical dendritic growth, whereas GluA2(R)-flop did not (Fig. 6A and Table 1). None of the GluA variants promoted basal dendritic growth (Fig. 6A and Table 1). Overexpression of GluA1(Q)-flop, GluA1Q)-flip and the mutant GluA1(R)-flip failed to increase dendritic complexity (Fig. 6A and Table 1). In line with this, AMPA failed to induce dendritic injury in pyramidal cells overexpressing GluA1 subunits.

Together, these observations suggest that pyramidal cells induce dendritic growth via the receptor variants that they naturally express at high levels (GluA2 and GluA3, but not GluA1) and that flip variants with a longer channel open time are very efficient regulators.

GluA1(Q)-flip exclusively regulates interneuronal dendritic growth

Similar to the analyses of pyramidal cells above, we investigated the role of GluA variants in interneuron dendritic growth. Interneurons highly express GluA1, which forms homomeric channels (Isaac et al., 2007; Jonas et al., 1994). Only GluA1(Q)-flip, but not GluA1(Q)-flop and none of the GluA2 and GluA3 variants, induced dendritic growth (Fig. 6B and Table 2; n=654 total reconstructed interneurons). Similarly, in spinal cord motoneurons it has been shown that GluA1(Q)-flip, but not a dominant-negative GluA1, promotes dendritic growth and reorganization (Prithviraj et al., 2008). To test whether the glutamine in the editing site of GluA1 plays a role we transfected the mutant GluA1(R)-flip. The point mutation (Q to R), which

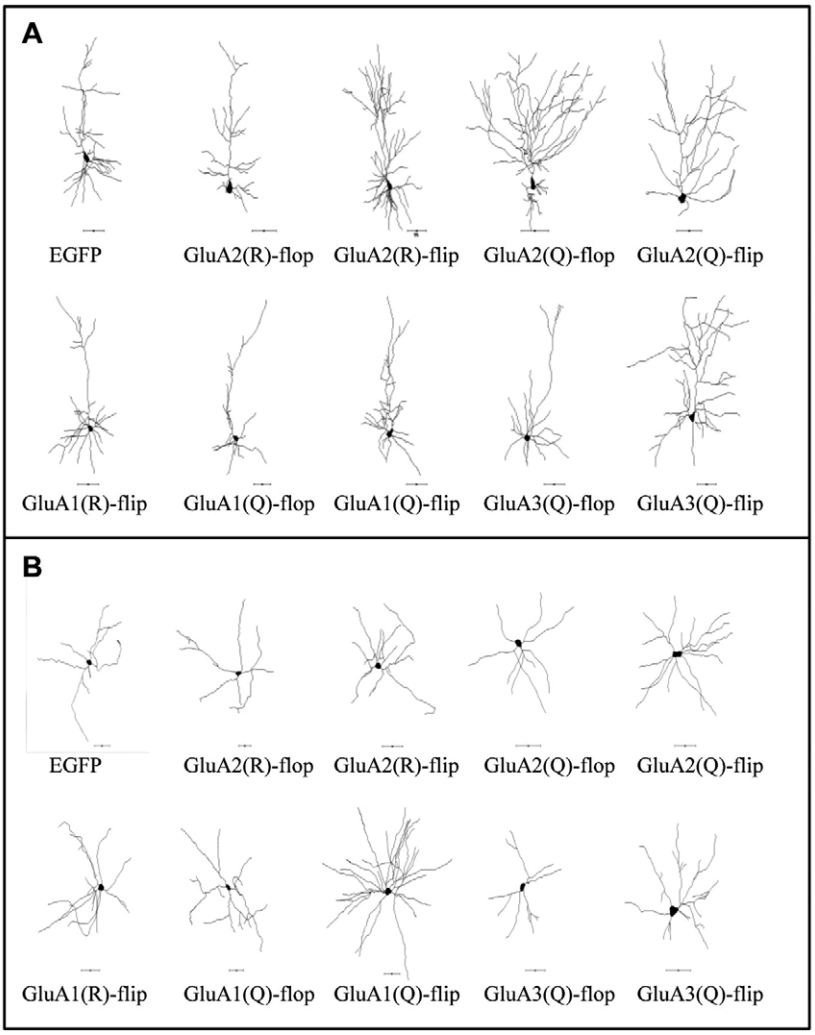

Fig. 6. Reconstruction of pyramidal cells and interneurons overexpressing GluA variants. Representative Neurolucida reconstructions of (**A**) layer II/III pyramidal cells and (**B**) interneurons transfected with plasmids encoding the indicated GluA subunit together with an EGFP plasmid, or EGFP alone as control. The dendritic trees closely match in length and branch number the mean values reported in Table 1. Scale bars: 50 μm.

Table 1. Quantitative morphometric analysis of pyramidal neurons

	Pyramidal cells in layers II/III		Pyramidal cells in layers V/VI	
Subunit	ADL (*n*) Segments	BDL Segments	ADL (*n*) Segments	BDL Segments
Control for GluA1	1174±58 (51) 38.9±2	212±13 7±0.4	1042±47 (51) 24.8±1.5	264±15 7.1±0.4
GluA1(Q)-flop	1286±69 (28) 41.8±4.4	235±22 7.8±0.8	1036±52 (46) 24.3±1.8	281±19 6.7±0.4
GluA1(Q)-flip	1252±84 (44) 33.8±2.8	212±15 7.7±0.6	1054±59 (37) 27±2.1	251±20 6.6±0.4
GluA1(R)-flip	1097±67 (39) 34.2±2.1	249±17 7.8±0.6	1053±58 (40) 24.4±1.8	222±14 6.1±0.4
Control for GluA2(R)	1056±65 (32) 33.8±2.2	235±40 5.7±0.7	996±47 (46) 28.3±1.8	226±21 7.3±0.6
GluA2(R)-flop	1084±81 (28) 33.6±2.3	180±21 5.8±0.6	963±118 (40) 27.1±1.7	191±15 6.5±0.6
GluA2(R)-flip	1464±87*** (36) 48.8±3.3***	211±21 7.2±0.6	1308±58*** (33) 36.8±2.3**	226±23 8.6±0.7
Control for GluA2(Q)	1115±66 (29) 35.3±2.7	203±22 6.8±0.8	1007±74 (37) 22.8±1.7	234±14 6.4±0.4
GluA2(Q)-flop	1605±111*** (29) 53.2±4.1***	233±25 8.2±0.9	1235±43* (25) 29.8±2.2**	258±31 6.9±0.8
GluA2(Q)-flip	1370±67** (28) 46±3.3**	245±30 7.8±0.7	1222±88** (32) 31.1±2.4**	282±25 7.6±0.7
Control for GluA3	1201±76 (44) 36.5±2.4	211±18 7.2±0.5	1076±43 (51) 24.5±1.8	274±21 7.3±0.5
GluA3(Q)-flop	1219±77 (28) 34.7±2.8	205±15 6.5±0.6	1071±64 (37) 26.5±2.4	227±15 6.3±0.5
GluA3(Q)-flip	1780±105*** (47) 47.5±2.8**	240±14 8.1±0.5	1239±69 (36) 28.7±2.5	317±27 8.1±0.8

Mean (± s.e.m.) of apical and basal dendritic length and segment number of layer II/III and layer V/VI pyramidal cells overexpressing the GluA subunits indicated. ADL, apical dendritic length (μm); BDL, mean basal dendritic length (μm). The number of cells reconstructed per group (*n*) is given in parentheses. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; for treatment versus the EGFP control group.

eliminates the calcium permeability, eliminated the growth-promoting action (Fig. 6B and Table 2). These observations support the hypothesis that the two neocortical cell classes, i.e. pyramidal cells and interneurons, promote dendritic growth via the GluA receptors that they naturally express, and further suggest that, for interneurons, prolonged channel open times and elevated calcium permeability are required for AMPAR-mediated dendritic growth.

NMDARs and voltage-gated calcium channels (VGCCs) mediate AMPAR-induced dendritic growth

The profound effects on pyramidal cell dendritic growth of the calcium-permeable GluA2(Q) subunits had been expected, but the effect of the calcium-impermeable GluA2(R)-flip subunit was unanticipated. The prolonged cationic influx through flip-containing AMPARs can result in the activation of NMDARs and/or VGCCs, which further prolong the excitation (Traynelis et al., 2010). The possibility existed that the transfection of particular GluA subunits could have led to an overall increase in network activity. Activity induces the somatic growth of neurons; however, the soma sizes of the GluA transfectants (data not shown) and the level of dendritic beading in the untreated condition (Fig. 4) were not different from those of EGFP control transfectants, which argued against an overall enhanced level of electrical activity. To test for a higher level of activity in individual transfectants we recorded the intensity of calcium transients using two-photon calcium imaging in pyramidal cells overexpressing GluA2(R)-flip or GluA2(R)-flop. Indeed, GluA2(R)-flip-overexpressing pyramidal neurons exhibited significantly higher calcium transients than GluA2(R)-flop-transfected and EGFP-transfected control pyramidal cells (Fig. 7). This implicated NMDARs and/or VGCCs as mediators downstream of AMPARs.

To examine the involvement of NMDARs and VGCCs, we treated GluA2(R)-flip-overexpressing neurons with the NMDAR antagonist APV and the VGCC blocker nifedipine. Both

Table 2. Quantitative morphometric analysis of interneurons

Subunit	MDL (*n*)	MDS	PD
Control for GluA1	345±16.3 (62)	7.1±0.4	4.9±0.2
GluA1(Q)-flop	357±24 (39)	6.3±0.4	4.8±0.2
GluA1(Q)-flip	459±26.5*** (61)	8.6±0.7**	4.5±0.3
GluA1(R)-flip	367±27.5 (25)	7.6±0.7	4.4±0.2
Control for GluA2(R)	352±20 (56)	7.5±0.4	4.5±0.2
GluA2(R)-flop	315±21 (42)	6.3±0.4	5.1±0.2
GluA2(R)-flip	321±19 (46)	6.6±0.7	4.7±0.2
Control for GluA2(Q)	368±17 (66)	7.7±0.7	4.9±0.2
GluA2(Q)-flop	380±18 (65)	8.0±0.7	4.6±0.2
GluA2(Q)-flip	405±19 (57)	7.6±0.7	4.6±0.2
Control for GluA3	359±31 (34)	7.5±0.7	5.1±0.3
GluA3(Q)-flop	408±42.5 (30)	7.8±0.7	4.3±0.2
GluA3(Q)-flip	356±23.7 (42)	7.8±0.7	4.7±0.2

Mean (± s.e.m.) of dendritic length and segment number of interneurons overexpressing the GluA subunits indicated. MDL, mean dendritic length (μm); MDS, mean dendritic segments; PD, number of primary dendrites. The number of cells reconstructed per group (*n*) is given in parentheses. ***, $P<0.001$; **, $P<0.01$; for treatment versus the EGFP control group.

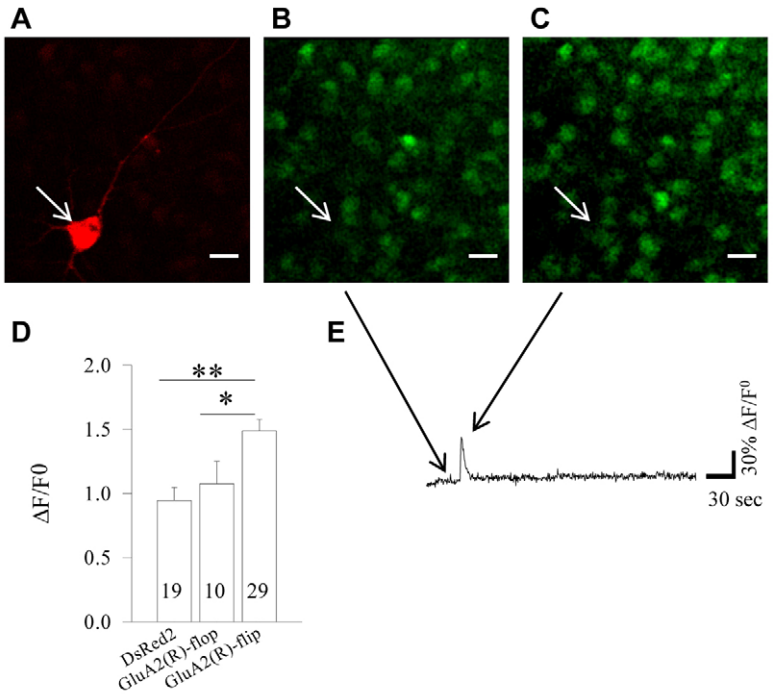

Fig. 7. Enhanced calcium signaling in GluA2(R)-flip transfectants. (**A-C**) Two-photon fluorometric calcium signals were recorded in GluA2(R)-flop-transfected and GluA2(R)-flip-transfected pyramidal neurons coexpressing DsRed2 (A). A pyramidal cell is shown displaying baseline (B) and maximal peak (C) intracellular calcium concentration. (**E**) A calcium imaging trace, expressed as $\Delta F/F^0$, records the calcium signals seen in the arrowed pyramidal cell in B and C. (**D**) In spontaneously active OTCs, higher calcium peak signals (~40%; mean ± s.e.m.) are recorded in GluA2(R)-flip-transfected pyramidal cells than with control DsRed2 alone. **, $P<0.01$; *, $P<0.05$; for treatment versus EGFP control group. The number of cells analyzed is given within the bars. Scale bars: 10 μm.

inhibited the growth-promoting action of GluA2(R)-flip in pyramidal cells (Fig. 8A and Table 3A; *n*=183 total reconstructed neurons). In interneurons, growth-promoting action was exclusive to GluA1(Q)-flip. Surprisingly, interneurons also required NMDARs and VGCCs for dendritic growth, as GluA1(Q)-flip overexpression was no longer effective in the presence of APV and nifedipine (Fig. 8B and Table 3B; *n*=184 total reconstructed neurons).

Finally, to show that the AMPARs are important, we treated GluA2(R)-flip-transfected and GluA1(Q)-flip-transfected cells with 10 μM CNQX. The competitive antagonist CNQX binds AMPARs with high affinity at the glutamate-binding site, thereby precluding their activation by glutamate. CNQX prevented the growth induced by the overexpression of GluA2(R)-flip in pyramidal cells (Fig. 8A and Table 3A) and the growth induced by GluA1(Q)-flip in interneurons (Fig. 8B and Table 3B). This emphasizes that glutamate signaling and activation of AMPARs is required as a first step, followed by the activation of NMDARs and VGCCs, to promote dendritic growth.

GluA2(Q)-flip and GluA2(Q)-flop increase spine density

GluA2(Q)-containing AMPARs have been reported to increase the spine density of hippocampal neurons (Passafaro et al., 2003). We tested whether the GluA variants alter the spine density in cortical neurons (Fig. 9; *n*=2414 neurons). Spine density on oblique secondary apical branches of pyramidal cells decreased in GluA2(R)-flip and GluA3(Q)-flip transfectants (Fig. 9A), suggesting that these dendritic growth-promoting subunits (see Fig. 6A and Table 1) do not support the concurrent production of new spines. By contrast, pyramidal cell transfectants expressing the growth-promoting subunits GluA2(Q)-flip and GluA2(Q)-flop displayed apical dendritic spine densities at the level of EGFP control neurons (Fig. 9A), suggesting that spines were added with ongoing dendritic growth (see Fig. 6A and Table 1). The basal dendritic spine density was similar to the EGFP control level for all GluA subunits (Fig. 9B). This was in accordance with the failure of all subunits to affect basal dendritic growth (see Fig. 6A and Table 1).

Intriguingly, the overexpression of both GluA2(Q) splice variants dramatically increased spine density in interneurons (Fig. 9C), and, in contrast to pyramidal cells, this occurred in the absence of dendritic growth (compare Fig. 6B and Table 2 with Fig. 6A and Table 1).

DISCUSSION

AMPAR subunits act in a compartment-specific manner

In 10 DIV pyramidal cells, the growth-promoting action of GluA2(R)-flip, GluA2(Q)-flip, GluA2(Q)-flop and GluA3(Q)-flip was restricted to apical dendrites. Several reasons could explain this cell compartment-specific action. Compared with basal dendrites, apical dendrites employ different molecular mechanisms during growth (Chow et al., 2009), they are more sensitive to glutamate and express more AMPARs, which increase in number with distance from the soma (Andrasfalvy and Magee, 2001; Pettit et al., 1997), they are preferentially equipped with calcium-permeable AMPARs (Kumar et al., 2002) and exhibit larger calcium transients (Svoboda et al., 1999; Waters et al., 2003). Pyramidal cells at ~10 DIV are electrotonically compact, and excitatory postsynaptic currents in the apical dendrite robustly evoke somatic depolarization (Atkinson and Williams, 2009), which is possibly required to activate transcriptional programs for differentiation (Konur and Ghosh, 2005; Lohmann and Wong, 2005). Apical dendritic branches were able to grow or stabilize spines in neurons overexpressing GluA2(Q)-flip and GluA2(Q)-flop, suggesting that the calcium permeability is important.

The AMPAR subunits induced neither growth nor a change in spine density in basal dendrites of pyramidal cells. Basal dendrites are reported to display a more protracted development, and between 5 and 10 DIV they could still be in a rather activity-

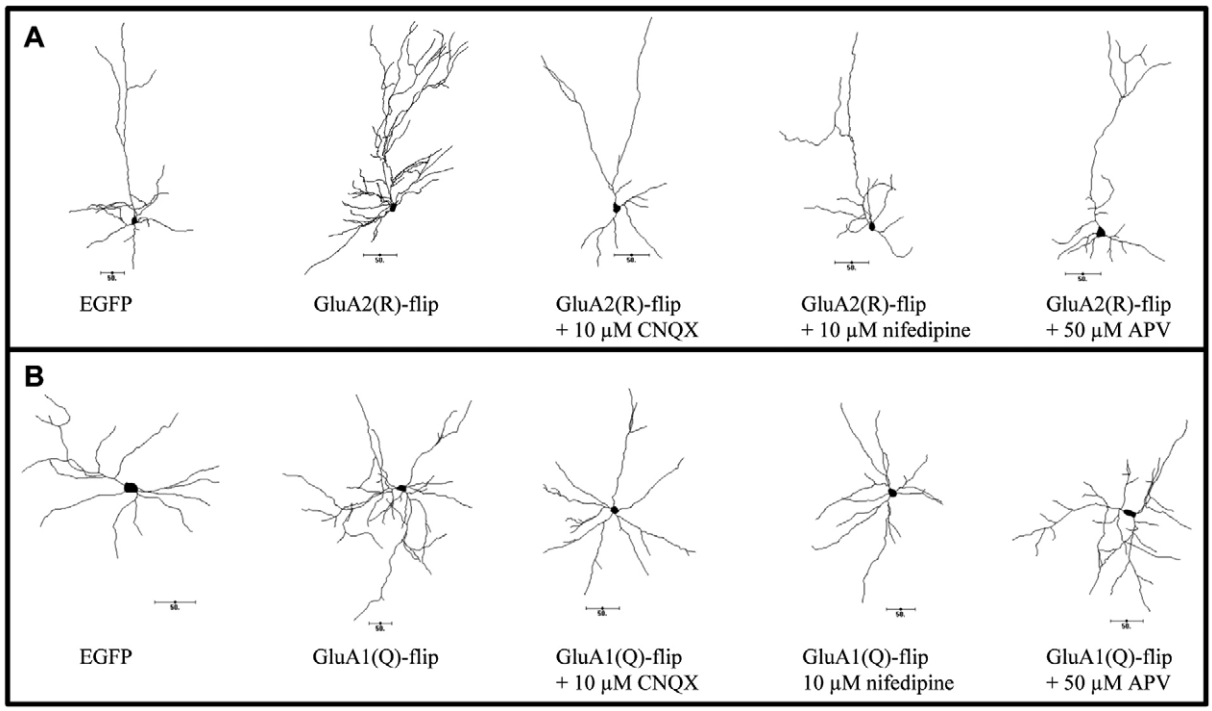

Fig. 8. Reconstruction of pharmacologically treated pyramidal cells overexpressing GluA2(R)-flip and interneurons overexpressing GluA1(Q)-flip. Representative Neurolucida reconstructions of (**A**) layer II/III pyramidal cells overexpressing GluA2(R)-flip and (**B**) interneurons overexpressing GluA1(Q)-flip in the absence or presence of CNQX, nifedipine or APV. The dendritic trees closely match in length and branch number the mean values reported in Table 3A (pyramidal cells) and Table 3B (interneurons). Scale bars: 50 μm.

independent phase of growth (Romand et al., 2011). However, basal dendrites at this age are growth competent and respond, for instance, to TrkB ligands (Wirth et al., 2003).

Another compartment-specific action was found for the GluA3(Q)-flip subunit, which effectively induced the apical dendritic growth of pyramidal cells in layers II/III, but not in layers V/VI. One reason for this distinction could be that the immature neurons of layers II/III exhibit a higher fraction of AMPAR-containing functional synapses, rather than silent synapses (Rumpel et al., 2004). Also, endogenous GluA3(Q)-flip is highly expressed in layers II/III (Sommer et al., 1990). This subunit is able to generate AMPARs that lack GluA2, which are of particular importance for neurons during synaptic potentiation.

The importance of flip and (Q) variants

We observed unedited *GluA2(Q)* transcripts in vivo and in OTC up until the first postnatal day, suggesting that some calcium-permeable GluA2 protein might well be present during the time when dendritic trees enlarge and synapses form by mechanisms not yet driven by sensory input (Romand et al., 2011). The immature brain employs unedited GluA2(Q) in addition to GluA2(R) to mediate the trophic actions of glutamate (Burnashev et al., 1992). The (Q) subunits exit the endoplasmic reticulum much faster, homotetramerize efficiently and traffic to the cell surface (Greger et al., 2007), where they generate large, inwardly rectifying currents that are presumably required for differentiation. In adult pyramidal cells, GluA2(R), by mass action at the level of the endoplasmic reticulum, restricts the assembly of calcium-permeable GluA1(Q)/GluA3(Q) homo- or heterotetramers (Brorson et al., 2004; Sans et al., 2003). Yet, pyramidal cells have significant numbers of calcium-permeable AMPARs that lack GluA2(R), in particular in apical dendrites (Kumar et al., 2002), which become incorporated into the cell membrane in an activity-dependent fashion (Cull-Candy et al., 2006). This could explain the high proportion of layer II/III pyramidal cells with mildly beaded apical dendrites observed in the dendritic injury experiments in the absence of AMPA treatment.

Contrary to our expectation, the overexpression of two unedited (Q) variants [GluA3(Q)-flop in pyramidal cells and GluA1(Q)-flop in interneurons] failed to induce dendritic growth, suggesting that calcium permeability alone is not sufficient. By contrast, all natural flip isoforms were highly effective in pyramidal cells and interneurons. This is likely to be due to the biophysical properties of the flip variants. AMPARs in adult neurons usually exhibit a rapid and virtually complete desensitization, which determines the decay times of the postsynaptic potentials. However, flip subunits are expressed at higher levels during development (Monyer et al., 1991). They are preferentially assembled and move more quickly to the cell surface than flop subunits. AMPAR flip subunit-containing receptors are more efficiently activated by glutamate and they desensitize with slower kinetics (Mosbacher et al., 1994; Sommer et al., 1990). GluA3(Q)-flip receptors, for example, recover more quickly from desensitization and display 4-fold longer channel open times than GluA3(Q)-flop (Pei et al., 2007; Pei et al., 2009).

It became clear, especially for interneurons, that GluA1(Q)-flip is a unique regulator of dendritic architecture. This result is unexpected because GluA1(Q)-flip and GluA1(Q)-flop receptor channels exhibit nearly the same kinetic parameters and both generate large, inwardly rectifying currents (Mosbacher et al., 1994). The dendritogenesis effect of GluA1(Q)-flip can be explained by the observation of Coleman et al. (Coleman et al.,

Table 3. Quantitative morphometric analysis of pharmacologically treated GluA2(R)-flip and GluA1(Q)-flip neurons

A. GluA2(R)-flip overexpression in pyramidal cells

			Pyramidal cells in layers II/III	
Subunit	Treatment	*n*	ADL Segments	BDL Segments
Control	None	40	1063±53	225±23
			27.8±2.5	6.4±0.6
GluA2(R)-flip	None	29	1369±77**	249±21
			38.8±3.9*	7.0±0.6
	10 μM CNQX	46	943±53	173±12
			28±2.1	5.1±0.3
	10 μM nifedipine	33	1002±53	229±24
			25.7±2.1	5.7±0.6
	50 μM APV	35	965±75	197±14
			24.4±2.6	5.5±0.3

B. GluA1(Q)-flip overexpression in interneurons

			Interneurons	
Subunit	Treatment	*n*	MDL	MDS
Control	None	41	340±17	7.8±0.5
GluA1(Q)-flip	None	39	487±33***	10.6±0.7**
	10 μM CNQX	30	349±30	9.7±0.9
	10 μM nifedipine	37	355±23	9.2±0.6
	50 μM APV	37	367±22	9.6±0.6

(**A**) Mean (± s.e.m.) of apical and basal dendritic length and segment number of layer II/III pyramidal cells overexpressing GluA2(R)-flip in the absence and presence of antagonists CNQX, nifedipine or APV. ADL, apical dendritic length (μm); BDL, mean basal dendritic length (μm). (**B**) Mean (± s.e.m.) of dendritic length and segment number of interneurons overexpressing GluA1(Q)-flip in the absence and presence of CNQX, nifedipine or APV. MDL, mean dendritic length (μm); MDS, mean dendritic segments. The number of cells reconstructed per group is given (*n*). ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; for treatment versus EGFP control group.

2006) that expression of GluA1(Q)-flip results in a 10-fold higher cell surface density than expression of GluA1(Q)-flop, indicating that flip subunits are trafficked more efficiently to the surface. After transfection of GluA1(Q)-flop we did not observe dendritic growth, possibly because GluA1(Q)-flop homotetramers are a disfavored configuration (Brorson et al., 2004). Calcium permeability also seems important for interneurons as the mutant GluA1(R)-flip failed to induce dendritic growth.

AMPAR subunits are not all equipotent and induce dendritic growth in a cell class-specific manner

Our results suggest that AMPAR subunits are not equipotent for the induction of dendritic growth. Similar to findings made in spinal motoneurons, the action depends on the context. We found that GluA1(Q)-flip is of unique importance for promoting the dendritic growth of interneurons, whereas GluA2(Q)-flip, GluA1(Q)-flop, GluA2(R)-flip and GluA3(Q)-flip promote the growth of apical dendrites of pyramidal cells. This suggested a cell class-specific and compartment-specific action of selected GluA subunits. A cell class-specific action of GluA subunits was not observed when spines were analyzed. Here, both GluA2(Q) variants were equipotent inducers of spine growth in both pyramidal cells and interneurons.

Our findings strongly oppose recently published findings arguing that the induction of dendritic growth of pyramidal cells does not depend on the AMPAR subunit composition. Chen et al. (Chen et al., 2009) show that overexpressed GluA1(Q)-flip and GluA2(Q)-flip are equipotent in putative cortical pyramidal cells, as both increase dendritic complexity. The discrepancy is likely to be due to differences in the approaches employed. Chen et al. used E17 dissociated neurons, transfection at 7 DIV and assessment by imaging at 8 DIV. This is ~5 days earlier than our time point of analysis. The classification of neurons is notoriously difficult in dissociated cultures because axonal patterns cannot be employed in the way that is possible in OTCs (see Materials and methods). Owing to a loss of polarity in dissociated neurons, apical and basal compartments were not assessed separately (Chen et al., 2009). Furthermore, the cellular responsiveness might differ in the absence of the normal 3D environment and it is unclear whether the overexpressed receptors are activated by endogenous glutamate as calcium signaling in the transfectants required stimulation with 100 μM exogenous glutamate (Chen et al., 2009). Moreover, Chen et al. report that both AMPAR subunits reduce filopodial motility and increase synapse formation. In our experiments, the GluA1(Q)-flip subunit failed to induce dendritic growth of pyramidal cells. With respect to the spine analysis, we and others (Passafaro et al., 2003) did not observe changes in spine density in GluA1(Q)-flip transfectants but, rather, that spine changes are mediated only by GluA2(Q) subunits.

NMDARs and VGCCs mediate dendritic growth triggered by selected AMPAR subunits

Our observation of the growth-promoting action of GluA2(R)-flip due to the enhancement of calcium transients suggested an involvement of VGCCs and/or NMDARs, which represent the two major pathways for calcium-mediated regulation of global dendritic growth (Lohmann and Wong, 2005). NMDARs are known to influence the differentiation of pyramidal cells. For example, blocking NMDARs until P21 in vivo results in prefrontal cortical pyramidal cells with smaller dendritic trees (Wedzony et al., 2005), blocking NMDARs in vitro abolishes the growth-promoting effect of BDNF (Finsterwald et al., 2010), and, in the absence of NMDARs, spiny stellate neurons fail to orient their dendrites towards the barrels (Espinosa et al., 2009; Iwasato et al., 2000). Moreover, hippocampal pyramidal neurons and granule cells lacking the NMDAR subunit GluNR2B have reduced spine density but display dendritic trees similar in length and branching to wild-type neurons, and the same has been observed for barrel cortex spiny stellate cells at P21 (Espinosa et al., 2009). Furthermore,

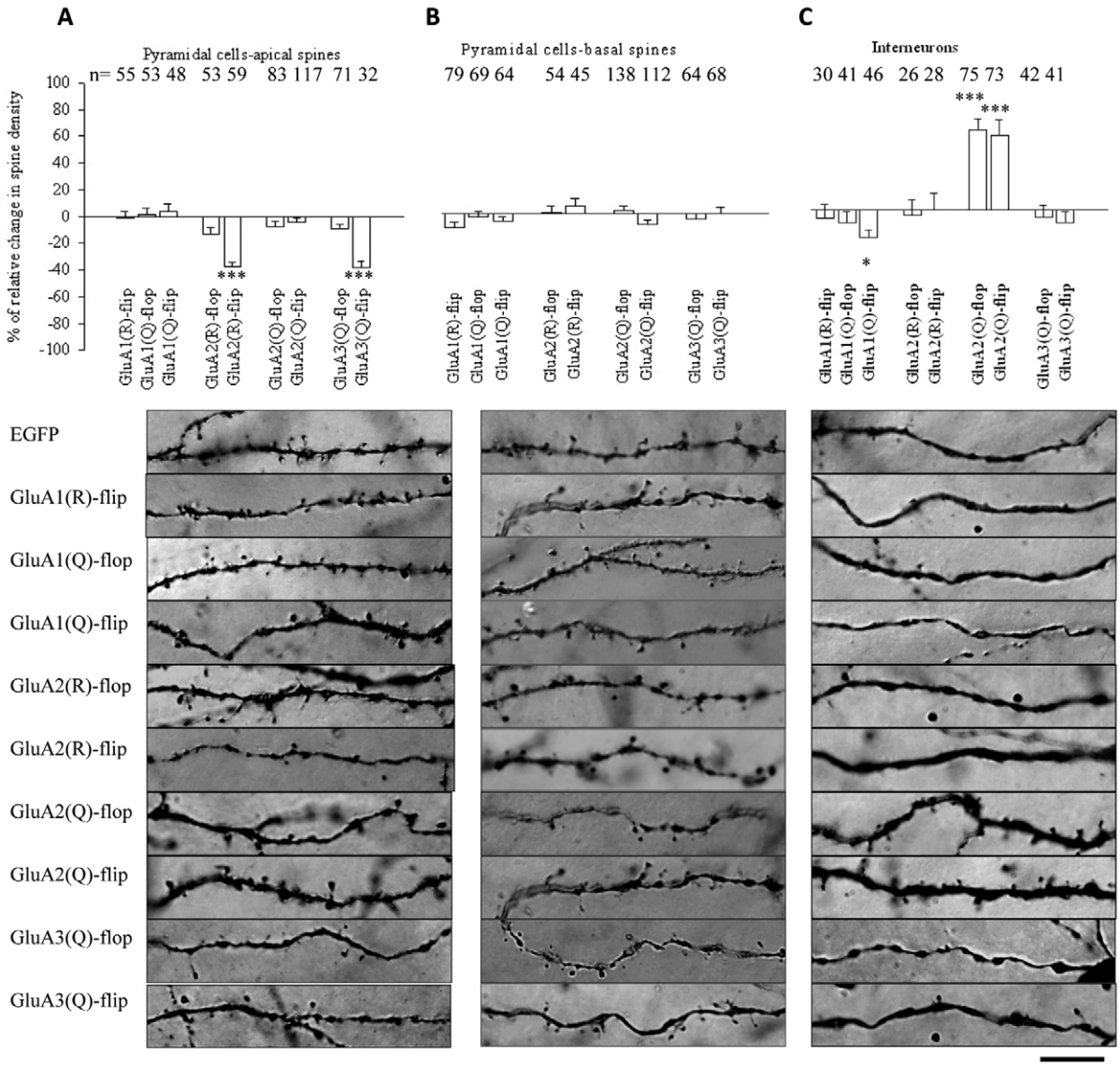

Fig. 9. Spine density of GluA-overexpressing neurons. (**A-C**) Relative change in spine density (number of spines per 100 µm dendrite) of GluA-transfected cells to EGFP-transfected control cells, expressed as mean percentage ±s.e.m. Beneath are shown photomicrographs (taken at 1000× magnification) for each experimental condition. The number of cells analyzed is given above the bars; in addition, 762 EGFP control cells were assessed but omitted from the normalized graphs. Layer II/III and layer V/VI pyramidal cells were assessed separately, but spine densities were not different and therefore data were pooled. For pyramidal cells, the basal dendritic spine density was not altered by GluA overexpression (B). For apical dendrites, the GluA2(Q)-flop and GluA2(Q)-flip transfectants had spine densities at control levels, suggesting that spines were added with ongoing dendritic growth (see Fig. 6A and Table 1) (A). By contrast, the GluA2(R)-flip and GluA3(Q)-flip transfectants displayed lower spine densities, indicating that these dendritic growth-promoting subunits (see Fig. 6A and Table 1) did not support the concurrent growth of new spines. For interneurons, GluA2(Q)-flip and GluA2(Q)-flop increased spine density (C). GluA1(Q)-flip elongated interneuronal dendritic length (see Fig. 6B and Table 2) and concurrently decreased spine density. ***, $P<0.001$; *, $P<0.05$; for treatment versus EGFP control group. Scale bar: 5 µm.

developmental analysis shows no differences in dendritic length and branching between wild-type and single-cell GluN2B knockout spiny stellate neurons (Espinosa et al., 2009). This indicates that GluN2B is unimportant specifically for spiny stellate neurons or that the dendritogenesis effect observed in the present study is mediated by other GluN2 subunits. Our data now reveal a role of NMDARs and VGCCs, as selective antagonists abolished the growth-promoting effect of GluA2(R)-flip in pyramidal cells.

The role of NMDARs in developing interneurons has remained elusive. Interneurons express NMDARs, and synaptic NMDAR-mediated excitatory postsynaptic currents increase robustly between P6 and P8-10 (Zhang and Sun, 2011). Later, interneurons downregulate NMDAR currents (Wang and Gao, 2010), which could explain why NMDARs play no role in the recruitment of feed-forward inhibition by adult thalamocortical inputs (Hull et al., 2009; Ling and Benardo, 1995). A recent study shows that depleting the GluN1 subunit from postnatal corticolimbic interneurons causes a schizophrenia-like phenotype, with a loss of Gad67 and parvalbumin expression and deficits in inhibition (Belforte et al., 2010). Our data now suggest that immature interneurons regulate dendritic growth via NMDARs and VGCCs downstream of GluA1(Q)-flip.

In summary, the results suggest that early postnatal pyramidal cells and interneurons employ AMPARs in a cell class-specific manner to induce dendritic growth. Second, neurons can potentially regulate their growth at the level of expression of flip-containing receptors. Third, both pyramidal cells and interneurons use NMDAR and VGCC signaling downstream of AMPARs to promote dendritic growth.

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Competing interests statement
The authors declare no competing financial interests.

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