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# AMPA GluR1 and GluR2 receptor subunits regulate dendrite complexity and spine motility in neurons of the developing neocortex

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## Abstract

Within neurons of several regions of the central nervous system, mature dendrite architecture is attained via extensive reorganization of arbor during the developmental period. Since dendrite morphology determines the firing patterns of the neuron, morphological refinement of dendritic arbor may have important implications for mature network activity. In the neocortex, a region of brain that is sensitive to activity-dependent structural rearrangement of dendritic arbor, the proportion of AMPA receptors increases over the developmental period. However, it is unclear whether changes in AMPA receptor expression contribute to maturation of dendritic architecture. To determine the effects of increasing AMPA receptor expression on dendrite morphology and connectivity within the neocortex, and to determine whether these effects are dependent on specific AMPA receptor subunits, we overexpressed the AMPA GluR1 and GluR2 subunits in cultured neocortical neurons at the time that AMPA receptors would normally be incorporated into synapses. Following expression of GluR1 or GluR2 we observed increases in the length and complexity of dendritic arbor of cortical neurons, and a concurrent reduction in motility of spines. In addition, expression of either subunit was associated with an increased density of excitatory postsynaptic puncta. These results suggest that AMPA receptor expression is an important determinant of dendrite morphology and connectivity in neocortical neurons, and further, that contrary to other regions of the central nervous system, the effects of AMPA receptors on dendrite morphology are not subunit-specific.

### Keywords

Dendrite branching; activity-dependent development; filopodia; glutamate receptor; PSD95; calcium

In many regions of the central nervous system, mature neuronal morphology is achieved through extensive reorganization of dendritic arbor in a manner that depends on the activity of

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glutamate receptors, although the cellular mechanisms underlying morphological plasticity in developing neurons remain obscure. Within several cortical regions of the developing brain, insertion of AMPA receptors into synapses is concurrent with a period of increasing network activity (Durand et al., 1996; Wu et al., 1996; Shi et al., 1999) and the initiation of activity-dependent developmental restructuring of dendrites (Rajan and Cline, 1998; Wong et al., 2000; Inglis et al., 2002; Haas and Cline, 2006). The presence of AMPA receptors in developing synapses may influence synaptic connectivity and neuronal network properties indirectly, via neuronal depolarization and activation of NMDA receptors and voltage-gated calcium channels in response to presynaptic glutamate release (Liu and Zukin, 2007); however, AMPA receptors may also mediate more direct effects on dendrite structure. During development, dendrites contain many transient protrusions such as filopodia, or thin spines (Harris, 1999; Dunaevsky et al., 1999). Filopodia have been proposed to be precursors to mature branch-points or spines (Vaughn et al., 1974; Niell et al., 2004; Yuste and Bonhoeffer, 2004; Zuo et al., 2005), and the motility of these elements can be altered by changing AMPA receptor activity (McKinney et al., 1999; Fischer et al., 2000; Richards et al., 2004). Loss of AMPA receptors from the postsynaptic cell has been shown to result in reduced dendritic branch stability (Haas and Cline, 2006), whereas overexpression of AMPA receptor subunits has been associated with increased numbers of branch segments (Inglis et al., 2002; Prithviraj et al., 2008). Since the morphology and complexity of neuronal dendrites influences the afferent innervation and firing patterns of a neuron (Purves and Hume, 1981; Schaefer et al., 2003; Vetter et al., 2001), the effect of AMPA receptors on dendrite structure during the developmental period may be an important determinant of mature network properties.

AMPA receptors are composed of homomeric or heteromeric combinations of four GluR1-GluR4 (or GluRA-D) receptor subunits, of which combinations lacking GluR2 are inwardly rectifying and calcium-permeable, whereas the presence of GluR2 confers linear rectification properties, calcium-impermeability, and lower conductance (Hume et al., 1991; Verdoorn et al., 1991). The proportion of GluR2-containing AMPA receptors is spatially and temporally diverse (Hack et al., 1995; Jakowec et al., 1995; Kumar et al., 2002; Lilliu et al., 2001; Parks, 2000; Pickard et al., 2000; Sugden et al., 2002), and the composition of AMPA receptors may regulate synaptic plasticity (Cull-Candy et al., 2006), although the role for AMPA receptors of different compositions in other developmental processes is unclear. Recent observations suggest that AMPA receptors have been shown to affect dendrite morphology in a subunit-specific manner: in motor neurons of the spinal cord, overexpression of GluR1 increased dendritic complexity via the addition of branch-points (Inglis et al., 2002) and increases the number of filopodia and excitatory synapses (Prithviraj et al., 2008), whereas GluR2 expression was associated with elongation of existing branch-points, and reductions in filopodia number. In contrast, GluR2 expression in the hippocampus increased spine density (Passafaro et al., 2003), whereas loss of GluR2 resulted in loss of dendritic branch segments. The role of AMPA receptor subunits in attaining mature dendritic architecture is therefore complex, and appears to be region-specific.

In this study, we overexpressed the AMPA subunits GluR1 or GluR2 in developing neurons cultured from rodent neocortex, to determine whether AMPA receptor expression is a determinant of neuronal morphology in this brain region. Within the cortex, expression of AMPA receptors is developmentally upregulated in such a manner that the proportion of GluR2 increases over the course of development (Kumar et al., 2002). We hypothesized that if AMPA receptor expression is a predictor of dendrite morphology, overexpression of GluR1 and GluR2 subunits would promote morphological refinement of neuronal dendrites of the neocortex in a subunit-specific manner.

# EXPERIMENTAL PROCEDURES

## Animals

All animal protocols used in this study were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee, and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For this study, timed-pregnant Sprague Dawley rats were obtained from Charles River, and housed and maintained on a 12/12 hour light/dark cycle, with unlimited access to food and water. Every effort was made to minimize the number of animals used and their suffering.

## Cortical Neuronal Cultures

Cortices were dissected from timed-pregnant embryonic day 17 (E17) Sprague Dawley rats, and dissociated with trypsin (0.25%) for 15 minutes, triturated via fire-polished Pasteur pipets, and resuspended in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin (10,000 U/ml) and 2mM glutamine. Cultures were plated ($10^5$ cells/coverslip) on glass-bottom culture dishes (12mm diameter glass bottom area; MatTek, Ashland, MA), pretreated with poly-D-lysine (100μg/ml) and laminin (10μg/ml). Twenty-four hours later, culture media was replaced with Neurobasal media supplemented with B27, 1% penicillin/streptomycin and 0.5mM glutamine. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) with the exception of poly-D-lysine (Sigma, St. Louis, MO).

## Transfection and Imaging of Cultured Neurons

To determine the effects of AMPA GluR1 and GluR2 subunits on parameters of cortical dendrite morphology, cells were transfected with pcDNA3 expressing $GluR1_{flip}$ or pRK5 expressing $GluR2_{flip}$ (100ng/coverslip) using Lipofectamine 2000 (Invitrogen), as previously described (Prithviraj et al., 2008). Neurons were visualized by the co-expression of green fluorescent protein (GFP) (50ng/coverslip); control neurons received 50ng of GFP alone. These concentrations were employed, because previous studies using these plasmids have demonstrated that transfected subunits are expressed at levels close to those of native subunits (Prithviraj et al., 2008; Robert et al., 2002), and confer predictable electrophysiological responses (Robert et al., 2002). Transfection of plasmids took place on the seventh day in vitro (DIV7) so that the effects of glutamate receptor expression could be measured before dendritic trees are fully established. This time-point also corresponds to the normal time period during which AMPA receptors are likely to be inserted into synapses (Lu et al 2006; Rumpel et al., 1998); we reasoned, therefore, that if AMPA receptor expression is an important determinant of dendrite morphology, we would be most likely to observe morphological effects of AMPA receptor expression at this time. Twenty-four hours after transfection, on DIV8, the media within the culture dish was replaced with a dye-free buffered external media (Robert et al., 2000), and the dish was placed on an inverted microscope (Zeiss Axiovert, 200M), and maintained at 37°C with plate and objective heaters. Time-lapse imaging of transfected cortical pyramidal neurons, identified by their characteristic shape and the presence of numerous spines and filopodia, was performed using Zeiss Axiovision image capture software. For each neuron, a series of 25 images was captured at 5 s intervals (total 2 minutes) using a CCD camera (Hamamatsu Orca; exposure ~10ms for each frame). These procedures were not observed to result in signs of injury such as swelling or blebbing, suggesting that cells remained viable throughout.

## Quantitative Analyses of Dendrite Morphology

Following image capture, indices of dendritic length and complexity were measured using Neurolucida (MBF Biosciences, Williston, VT), as described previously (Prithviraj et al., 2008). For each neuron, the following parameters were calculated: the number of primary

dendrites, branch-points and tips per neuron; number of filopodia and spines per neuron; total amount of dendritic arbor per neuron; and average length of branch segments, calculated by dividing the total amount of arbor by the number of branch segments (number of branch-points + number of tips). Statistical variance among treatment groups was estimated using ANOVA (Statview 5.0). Pairwise comparisons between treatment groups were performed post-hoc using Scheffé's F test.

To determine whether GluR1 or GluR2 expression resulted in intrinsic changes in geometry across the span of the dendritic tree, dendrites were also analyzed according to branch order (Inglis et al., 2002; Prithviraj et al., 2008); for these analyses, a dendritic process emerging from the cell body is considered a primary dendrite; once this bifurcates, two secondary dendrites are formed, and so on. Comparisons of the total amount of dendritic arbor per branch order, and the number of segments of each branch order, were made using Repeated Measures ANOVA. Pairwise comparisons of repeated measures were made between treatment groups using Scheffé's Post-hoc F test.

### Quantification of filopodium and spine motility

To determine whether the expression of AMPA receptor subunits promotes alterations in the stability of filopodia and spines, the motility of each process was analyzed from time-lapse images using Metamorph software. For these analyses, the position of the tip of each protrusion was recorded in the 25 successive frames captured during time-lapse imaging, and the deflection from the point of origin was calculated for the duration of imaging. The average distance and movement speed was calculated for each protrusion, and average motilities were calculated for each cell. Statistical analyses of the effects of treatment on the average filopodial motility per cell were made using ANOVA.

### Immunocytochemical detection of GluR1 and GluR2

Cultures transfected with GFP and GluR1 or GluR2 were processed immunochemically to confirm expression of AMPA receptor transgenes. Briefly, cultures were fixed by the addition of 4% paraformaldehyde in phosphate-buffered saline (PBS), for 20 minutes. Cells were rinsed in PBS for a further 30 minutes, and incubated overnight at 4°C with primary antibodies against GluR1 (rabbit polyclonal, Chemicon, Temecula, CA; 1:200 in 1% normal goat serum (NGS) with 0.1% TritonX-100) or GluR2 (rabbit polyclonal, Chemicon, Temecula, CA; 1:500 in 1% NGS with 0.1% TritonX-100). Cells were rinsed with PBS and incubated for 1 hour at room temperature in a rhodamine-TRITC-conjugated secondary antibody (goat anti-rabbit, 1:200; Chemicon, Temecula, CA) in PBS containing 1% NGS. Cells were rinsed with PBS and coverslipped, and immunoreactivity for GluR1 and GluR2 was detected by fluorescence microscopy and analyzed as described above.

To estimate the degree of transgene expression, mean fluorescence intensities were quantified for the cell body and dendrites of transfected cells, and compared with non-transfected cells within the same coverslip, as described previously (Robert et al., 2002). For each group, the fluorescence intensities of 5 cells were analyzed and compared statistically using Student's t-test (unpaired, two-tailed).

### Estimation of synaptogenesis

To estimate the degree of excitatory synaptogenesis following AMPA receptor expression, the density of puncta containing the excitatory postsynaptic density protein, PSD95 (El-Husseini et al., 2000; Marrs et al., 2001; Prange and Murphy, 2001; Niell et al., 2004) was determined using immunocytochemistry. To confirm the synaptic nature of PSD95, cells were co-labeled for the presynaptic marker, synaptophysin. For these experiments, neuronal cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) 24 hours after transfection with

GluR1 or GluR2, rinsed in buffer, and incubated overnight at 4°C in primary antibody against PSD95 (rabbit polyclonal, Invitrogen/Zymed, Carlsbad, CA; 1:500 in 1% NGS with 0.1% TritonX-100) and synaptophysin (mouse monoclonal, Chemicon, Temecula, CA; 1:5000 in 1% NGS with 0.1% TritonX-100). Cells were rinsed with PBS and incubated for 1 hour at room temperature in an Alexa Fluor 647 (goat anti-rabbit, 1:200; Molecular Probes, Carlsbad, CA) or an Alexa Fluor 594 (goat anti-mouse, 1:200; Molecular Probes, Carlsbad, CA) conjugated antibody, in PBS containing 1% NGS. Cells were rinsed with PBS and coverslipped, and immunoreactivity for PSD95 and synaptophysin was detected by confocal microscopy (Zeiss LSM 510) and imaged using lasers of 488nm, 543nm and 633nm wavelengths.

Due to culture densities required for maintaining healthy cells, it was often difficult to ascertain with complete certainty whether puncta were associated with transfected neurons or with dendrites of other neurons close by. Thus, a second series of experiments were performed using GFP-labeled PSD95 (GFP-PSD95; provided by Dr. Alaa El-Husseini, University of British Columbia, Vancouver). GFP-PSD95 was co-transfected with GluR1 or GluR2 (100ng of each plasmid) in DIV 7 neurons. Control neurons were transfected with 100ng of GFP-PSD95 alone. After 24 hours, neurons were fixed by the addition of 4% paraformaldehyde in PBS, and processed for synaptophysin immunocytochemistry as above. Neurons were subsequently placed on a fluorescent microscope, and Z-stacks of images were collected using Axiovision software.

From Z-stack tiff images, neuronal dendritic length was quantified using Neurolucida, and the position of each dendritic punctum recorded. A punctum was defined as a bright, clearly delineated area of fluorescence of at least twice the pixel intensity of the surrounding area. From these images, the total number of puncta per cell was recorded. Statistical comparisons of the number of puncta per unit length were made using ANOVA, with Scheffé post-hoc test for pairwise comparisons of treatment groups. To determine density of synaptic and extra-synaptic puncta, a representative 200μm segment of dendrite was selected from 5 cells from each treatment group and viewed at high magnification. From this segment, we recorded the total number of PSD95 puncta, total number of synaptophysin puncta, and the number of colocalized puncta. These numbers were used to calculate the percentage of total PSD95 puncta that were colocalized with synaptophysin.

## Measurement of Intracellular Calcium

Relative intracellular calcium concentrations were recorded in neurons transfected as described above, and imaged after 24 hours using fura-2. Prior to imaging, cultures were rinsed with the external media and then incubated for 35 minutes at 37°C with a 5μM solution of the acetoxymethyl ester form of fura-2 (Invitrogen, Carlsbad, CA) in external media. The fura-2 solution was then replaced with plain external media, held at 37°C and perfused with 100% $O_2$. Transfected neurons were identified by the presence of GFP, and the internal calcium concentration was determined by a ratio of the fluorescence intensity at a 340nm excitation (F340) to the fluorescence intensity at 380nm excitation (F380). Cellular calcium levels were measured in response to perfusing the cells with a solution of 100 μM glutamate and 10 μM MK-801 in external media. The cells were visualized on a fixed stage microscope (BX51WI; Olympus, Center Valley, PA). The light source was a xenon lamp controlled with high speed wavelength switchers (Lambda DG-4; Sutter Instruments, Novato, CA and Prior Scientific filter wheel system; Prior Scientific, Rockland, MA). Images were collected at 2s intervals (total 600 frames) using a CCD camera (QuantEM:512SC; Photometrics, Pleasanton, CA) controlled by IPLab software (BD Biosciences, Rockville, MD). Images were analyzed and the F340/F380 ratios calculated using ImageJ (NIH, Bethesda, MD). To correct for imaging

rig differences over time, the calcium spikes were normalized to a percentage of the maximum spike seen during a day of imaging.

### Statistical Analyses

Statistics for quantification of dendrite morphology and transgene expression were performed using ANOVA (Statview 5, SAS Inc). The exact F statistic and degrees of freedom were used to calculate probabilities; results were deemed significant if the p value was less than 0.05. For calcium imaging techniques, calcium concentrations were estimated ratiometrically, and the ratios compared using Kruskall-Wallis one way analysis of variance.

## RESULTS

### Detection of GluR1 and GluR2

Levels of GluR1 and GluR2 following transfection were estimated in 5 cells per group by measuring the mean fluorescence intensity, compared to surrounding non-transfected cells in the same coverslip (Supplementary Figure 1). Over-expression of GluR1 and GluR2 resulted in increased fluorescence intensity of 198% and 189% respectively, with reference to levels of immunofluorescence from surrounding, non-transfected cells. The increases in immunoreactivity were significant for both GluR1 ($t = 8.289$; $p < 0.0001$) and GluR2 ($t = 11.531$; $p < 0.0001$) in the cell bodies and dendrites of transfected neurons. These results are in line with similar levels of transgene expression reported previously using these expression plasmids (Prithviraj et al., 2008; Robert et al., 2002).

### Overexpression of GluR1 and GluR2 increases dendrite complexity

Twenty-four hours after transfection, at DIV8, overexpression of GluR1 or GluR2 in cortical neurons was associated with alterations in a number of indices of dendrite morphology (Table 1; Figure 1). Expression of either GluR1 or GluR2 increased the number of branch-points ($p<0.01$) and branch-tips ($p<0.01$; Scheffé post-hoc test) relative to control values, suggesting that expression of AMPA receptors affects dendrite complexity within cortical neurons. Overexpression of GluR1 and GluR2 was also associated with an increase in the total amount of arbor per cell ($p<0.02$), consistent with the increased number of branch segments. In contrast, there was no significant change in the number of primary processes extended from the cell body in response to GluR1 or GluR2 expression, suggesting that the effect of AMPA receptor subunits on branch segment numbers was limited to existing dendritic trees.

Analyses of dendrites according to their branch order revealed a homogeneous population of neurons (Figure 1), and demonstrated that the addition of segments in response to GluR1 and GluR2 expression occurred at third order and higher branch segments ($F_{2,78}=6.500$; $p=0.002$), a result consistent with addition of branch segments to newer regions of the dendritic tree. There was no Group X Order effect of treatment ($F_{12, 468}= 0.891$; $p=0.556$), suggesting that the patterns of changes in dendrite complexity in response to GluR1 and GluR2 were distributed similarly. Analyses of the total amount of arbor within each branch order confirmed that the increase in total arbor was distributed between second and higher orders of magnitude ($F_{2,78} = 3.926$; $p=0.024$), with no significant difference in distribution between treatment groups (Group X Order, $F_{12, 468}= 0.769$; $p=0.683$). These results imply that AMPA receptor expression leads to an increase in the formation and/or maintenance of new branch segments within existing dendritic trees in cortical neurons.

### Expression of GluR1 and GluR2 does not affect filopodia and spine numbers

In other regions of the central nervous system, the number of dendritic spines and filopodia are affected by the level of AMPA receptor activity (McKinney et al., 1999; Richards et al.,

2004). In hippocampal neurons, GluR2 expression has been associated with an increase in the number of spines (Passafaro et al., 2003), whereas in spinal motor neurons, GluR1 overexpression has been observed to increase the number of filopodia (Prithviraj et al., 2008). To determine whether GluR1 or GluR2 had significant effects on cortical filopodia and spines, these were identified from captured images and quantified according to their morphology, as previously defined for various populations of spines (Harris, 1999; Yuste and Bonhoeffer, 2004) (Table 2). At this developmental age, the majority of small dendritic protrusions were observed to be filopodia, although a small portion of processes identifiable as thin or stubby spines existed; the numbers of these are given in Table 2. Neither GluR1 nor GluR2 significantly altered the number of filopodia or spines, despite the apparent increase in the total number of filopodia following GluR2 (Table 2). Expression of GluR1 was associated with an overall reduction in the density of filopodia per cell (25%); however, most likely due to the inherent variability of these measures, this value did not reach significance. These results is in contrast to previous studies that show AMPA receptor expression may increase densities of spines and filopodia (Passafaro et al., 2003; Prithviraj et al., 2008), and suggest that the effects of AMPA receptors may be region-specific.

### GluR1 and GluR2 overexpression reduces filopodial motility

Increased spine and filopodial motility act to enlarge the volume of space occupied by a neuron over a period of time, and may be important for enhancing the chances of synaptic innervation by afferent axons (Ziv and Smith, 1996). Addition of AMPA agonists to spines reduces their motility (McKinney et al., 1999; Fischer et al., 2000; Richards et al., 2004), an observation that is consistent with the stabilizing effect of synaptogenesis within dendritic elements. To determine whether GluR1 or GluR2 overexpression altered motility of filopodia and spines within developing cortical neurons, motility of each dendritic protrusion was recorded from a series of time-lapse images (Figure 2), as described previously (Prithviraj et al., 2008). Compared to controls (average velocity = 0.138 ± 0.03 μM/s), expression of GluR1 and GluR2 each significantly reduced filopodial motility per cell (GluR1, average velocity = 0.067 ± 0.01 μM/s; GluR2, average velocity = 0.053 ± 0.01 μM/s; $F_{2,79}$=6.628; p=0.002) over the two minute imaging period employed; this reduction amounted to an approximately 54% reduction in response to GluR1 expression, and a 66% reduction in response to GluR2. These results are consistent with the hypothesis that enhanced AMPA receptor activity results in stabilization of cortical dendritic filopodia and spines.

### Expression of GluR1 and GluR2 increases the number of PSD95 puncta

Previously, using cultured spinal motor neurons, we have shown that expression of GluR1, but not GluR2, is associated with an increase in the density of puncta containing the postsynaptic density protein, PSD95 (Prithviraj et al., 2008), suggesting that GluR1 expression is coupled with an increase in excitatory synaptogenesis. To determine whether GluR1 or GluR2 expression affected excitatory synaptogenesis within cortical neurons, we examined densities of synapses labeled immunochemically with antibodies against PSD95 and the presynaptic marker, synaptophysin (Figure 3). In neurons transfected with GluR1 or GluR2, we observed an increase in puncta of PSD95 and synaptophysin, although we could not determine with certainty that these puncta were always associated with transfected neurons. Of interest, we observed that many PSD95 puncta were not apposed to synaptophysin, suggesting that these PSD95 puncta were not always innervated by a functional presynaptic terminal.

To better quantify whether numbers of PSD95 puncta were increased following GluR1 or GluR2 transfection, we employed a GFP-labeled PSD95 (GFP-PSD95), co-transfected with GluR1 or GluR2 (Figure 4), which allowed us to visualize PSD95 puncta located within individual transfected neurons. In these neurons, we observed a significant increase in the number of puncta ($F_{2,57}$=8.241; p<0.001), with significantly greater numbers of puncta in

response to both GluR1 (339 ± 31; $p<0.01$) and GluR2 (327 ± 20; $p<0.02$), compared to neurons transfected with GFP-PSD95 alone (219 ± 16). We also observed an increase in the number of PSD95 puncta per 100μm of dendrite length ($F_{2,57}=4.530$; $p<0.02$); GluR1 and GluR2 transfection were each associated with an increase in the density of GFP-PSD95 puncta, (22 ± 1.2, $p<0.01$ and 22 ± 1.4, $p<0.02$ respectively), compared to controls (17 ± 1.2).

To quantify the number of PSD95 puncta that were likely to be synaptic, we counted the number of synaptophysin puncta, and the number of these that were co-localized with PSD95 puncta from high magnification images of a region of dendrite (200μm) from 5 neurons per treatment group (Table 3). As with the endogenous label, PSD95 puncta were not always associated with a presynaptic terminal positively stained for synaptophysin, suggesting that many PSD95 puncta are asynaptic. However, we noticed that the number of puncta associated with a presynaptic terminal increased following expression of GluR1 (60 ± 9%) or GluR2 (80 ± 18%) compared with PSD95 alone (41 ± 8%), suggesting that overexpression of AMPA receptor subunits promotes the synaptic incorporation of PSD95 puncta.

### Expression of GluR1 or GluR2 increases intracellular calcium levels

AMPA receptors composed of GluR1 or GluR2 differ in their permeability to calcium and their ability to carry depolarizing current (Verdoorn et al., 1991; Hume et al., 1991). Levels of calcium have linked directly to neurite outgrowth and dendrite complexity (Spitzer, 2002; Redmond et al., 2005). We therefore examined levels of free cytosolic calcium to determine whether overexpression of GluR1 or GluR2 affected calcium levels within the cell, and whether these effects were subunit-dependent. Immediately following application of glutamate, we observed an increase in cell calcium levels in all neurons imaged, compared with baseline levels (Figure 5). Analysis of variance using the Kruskall-Wallis test indicated significantly different levels of calcium between groups ($p=0.0046$), with the greatest increase in calcium levels recorded in neurons transfected with GluR1 (64.4 ± 9.8% relative to baseline), compared with GluR2 (44.4 ± 9.9) and with GFP controls (17.2 ± 4.5). These results confirm that intracellular calcium increases to a greater extent in neurons transfected with GluR1 than with GluR2, and that transgene expression in these experiments is associated with a significant cellular response.

## DISCUSSION

In this study we investigated whether increasing AMPA receptor expression in developing neurons affects dendritic morphology and complexity of cortical neurons, and whether these effects were dependent on the presence of specific AMPA receptor subunits. Our main findings were that expression of either GluR1 or GluR2 increased dendritic complexity and length in developing cortical neurons; that neither GluR1 nor GluR2 significantly affected spine number within cortical neuronal dendrites, but expression of each subunit reduced motility of spines; that expression of GluR1 or GluR2 increased the number of PSD95 puncta; and that expression of GluR1 and GluR2 increased cytosolic calcium levels, but to different extents. These results suggest (1) that increasing the number of AMPA receptors within cortical neurons during development has significant implications for cellular activity, dendrite morphology, synaptic connectivity, and in turn, the network properties of cortical neuronal circuits; (2) in contrast to the observed effects elsewhere in the nervous system (Passafaro et al., 2003; Prithviraj et al., 2008), the subunit composition of AMPA receptors does not appear to be a critical determinant of dendrite morphology within the neocortex.

### AMPA receptors and dendrite complexity

While our results suggest that developmental regulation of AMPA receptor expression has important implications for dendrite morphology and connectivity within the developing

neocortex, the relationship between AMPA receptor activity and dendrite morphology appears to be complex: for example, reductions in AMPA receptor activity have been associated with diminished numbers of dendritic branch segments in developing visual neurons (Rajan and Cline, 1998; Wong et al., 2000; Haas et al, 2006). Conversely, increased activation of AMPA/kainate receptors has been shown to attenuate neurite outgrowth in retinal ganglion neurons (Bodnarenko et al., 1995), hippocampal neurons (Mattson et al., 1988) and spinal motor neurons (Metzger et al., 1998).

Some variance in the outcomes of these studies may be explained in part by differences in developmental stage of tissues and drug concentrations employed. Alternatively, linking AMPA receptors to region-specific intracellular effector systems would permit dendrite morphology to be controlled in a spatially-dependent manner. Indeed, the effects of GluR1 or GluR2 expression on dendrite morphology appear to be region-specific: in motor neurons, GluR1 expression enhances dendrite complexity (Inglis et al., 2002; Prithviraj et al., 2008), whereas GluR2 expression increases segment length (Prithviraj et al., 2008). In contrast, expression of GluR2, but not GluR1, increases spine density in hippocampal neurons (Passafaro et al., 2003). The effects of GluR2 have been attributed to a stabilizing mechanism between the extracellular portion of the receptor and the presynaptic terminal, and via intracellular interactions with ephrin receptor EphB, via the glutamate receptor interacting proteins GRIP1 and GRIP2 (Hoogenraad et al, 2005). GluR1, on the other hand, has been shown to interact specifically with the actin-binding proteins 4.1N (Shen et al., 2000) and SAP97 (Zhou et al., 2008), proteins which are also developmentally regulated (Douyard et al., 2007), and which may, therefore, underlie spatial differences in the effects of AMPA receptor subunits on dendrite morphology. Our finding that GluR1 and GluR2 produce similar effects on cortical dendrite morphology is therefore surprising. However, the composition of cortical AMPA receptors has been observed to change over the developmental period (Kumar et al., 2002), and developmental regulation of these signaling molecules may result in temporally-specific effects of AMPA receptor expression that we were not able to observe in the current study.

Neurite outgrowth is intrinsically linked to cellular calcium levels (Spitzer, 2002; Redmond et al., 2005), and it has been suggested previously that different levels of calcium entry via AMPA receptors may mediate outgrowth or retraction of dendrites via activation of specific downstream signaling molecules (Jeong et al., 2006). Our results confirm that overexpression of GluR1 and GluR2 has markedly different effects on intracellular levels; however in the neocortex at least, cellular calcium levels do not appear to predict dendrite morphology.

### AMPA receptors and filopodial dynamics

During early cortical development, the majority of dendritic spines are filopodia, which can extend or retract entirely in a matter of minutes (Harris, 1999; Jontes and Smith, 2000; Matus, 2000; Smart and Halpain, 2000). Filopodia have been suggested to be precursors to new branch segments (Vaughn et al., 1974; Niell et al., 2004) and may promote synaptogenesis (Ziv and Smith, 1996). However, we found that although the absolute number of filopodia increased in response to GluR2, this effect did not reach significance. Our data show that numbers of filopodia and spines per cell are inherently variable, thus hampering statistical analyses; however, in light of our previous results that GluR1 increases filopodia in developing motor neurons, this result was unexpected. Synaptic activity has been shown to promote an increase in the number of filopodia in hippocampal pyramidal neurons (Maletic-Savatic et al., 1999), although filopodia within different regions of neocortical dendrites have been shown to be differentially affected by glutamatergic activity (Portera-Cailliau et al., 2003), and glutamate may have bi-directional effects on spines (Korkotian and Segal, 1999). These observations attest to the complex relationship between neuronal activity and filopodial extension.

While the number of filopodia did not change significantly, we observed that expression of either GluR1 or GluR2 reduced spine motility over the imaging period we employed. In hippocampal neurons, activating AMPA receptors inhibits spine motility (Fischer et al., 2000) and prevents deafferentation-induced loss of spines (McKinney et al., 1999), suggesting that AMPA receptor activation is critical in the maintenance of dendritic elements that would otherwise be lost. Recent studies *in vivo* have confirmed that filopodial dynamics are related to the degree of afferent sensory information. For example, ocular deprivation increases the motility of dendritic spines within the visual cortex (Majewska and Sur, 2003; Oray et al., 2004), and whisker trimming has been associated with alterations in spine motility, such that previously stable spines were lost (Holtmaat et al., 2006). Thus, insertion of AMPA receptors into developing neurons may provide an important mechanism for stabilizing spine structure, leading to maintenance of dendritic elements in an activity-dependent manner.

### AMPA receptors and synaptogenesis

An important observation in our study is that expression of GluR1 or GluR2 enhanced the total number of PSD95 puncta, and increased the percentage of PSD95 puncta associated with a presynaptic terminal, suggesting that increasing the number of AMPA receptor subunits in neocortical dendrites may also facilitate synaptogenesis. In agreement with previous studies in neurons from the hippocampus (Charych et al., 2006; Gerrow et al., 2006), we observed a large proportion of PSD95 puncta that were not synaptic. Recently it has been suggested that preformed complexes of postsynaptic density proteins may be involved in the production of “hot spots” that facilitate synaptogenesis (Gerrow et al., 2006); thus it is possible that GluR1 and GluR2 expression in the current study lead to the formation of pre-assembled postsynaptic densities that may be later converted into synapses.

PSD95 may be involved directly in dendrite branch formation and maintenance: dendritic filopodia of non-spiny visual neurons are stabilized by the addition of PSD95 puncta (Niell et al., 2004), and dendrite complexity is regulated by proteins that modulate the synaptic localization of PSD95 (Charych et al., 2006; Akum et al., 2004). PSD95 interacts with proteins that bind microtubules (Passafaro et al., 1999), and is thus positioned to participate in AMPA receptor-mediated alterations in synaptic plasticity and dendrite morphology. It is possible, therefore, that the increase in GluR1 and GluR2 expression leads to recruitment of PSD95 to postsynaptic elements which can in turn facilitate synaptogenesis, and stabilization of filopodia and dendrites in an activity-dependent manner.

## CONCLUSION

In our study, we report that expression of AMPA receptor subunits GluR1 and GluR2 promote increased dendrite complexity, reduced motility of filopodia, and increased numbers of puncta containing the postsynaptic density protein PSD95. Unlike observations of hippocampal (Passafaro et al., 2003) and spinal motor neurons (Prithviraj et al., 2008), these results do not appear to be subunit-specific, suggesting that the effects of AMPA receptor expression on dendrite morphogenesis and spine motility are regionally discrete. Our results are important because they imply that increasing the proportion of AMPA receptors in dendrites of the developing neocortex permits regulation of dendrite morphology, a result that is likely to have important implications for network properties in the mature nervous system.

Altered dendrite morphology has been observed in several disease states, including schizophrenia (Black et al., 2004; Kolluri et al., 2005), Alzheimer’s disease (Schiebel, 1983) and Fragile X (Irwin et al., 2000), implying that changes in dendrite architecture contribute to the pathology of these diseases. Recovery of neuronal function after disease or injury may be limited by the ability to remodel dendritic arbor and to promote synaptogenesis. Our results provide greater insight into the molecular mechanisms underlying dendrite morphogenesis;

understanding these mechanisms and their molecular constituents may provide novel targets for new therapeutic strategies in the diseased brain.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations Used

**AMPA**
Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

**ANOVA**
Analysis of variance

**CCD**
Charge-coupled device

**DIV**
Days *in vitro*

**DMEM**
Dulbecco's Modified Eagle's Medium

**GFP**
Green fluorescent protein

**GluR1**
AMPA Glutamate receptor subunit 1

**GluR2**
AMPA Glutamate receptor subunit 2

**NGS**
Normal goat serum

**NMDA**
N-methyl-D-aspartate

**PBS**
Phosphate buffered saline

**TRITC**
Tetramethyl rhodamine iso-thiocyanate

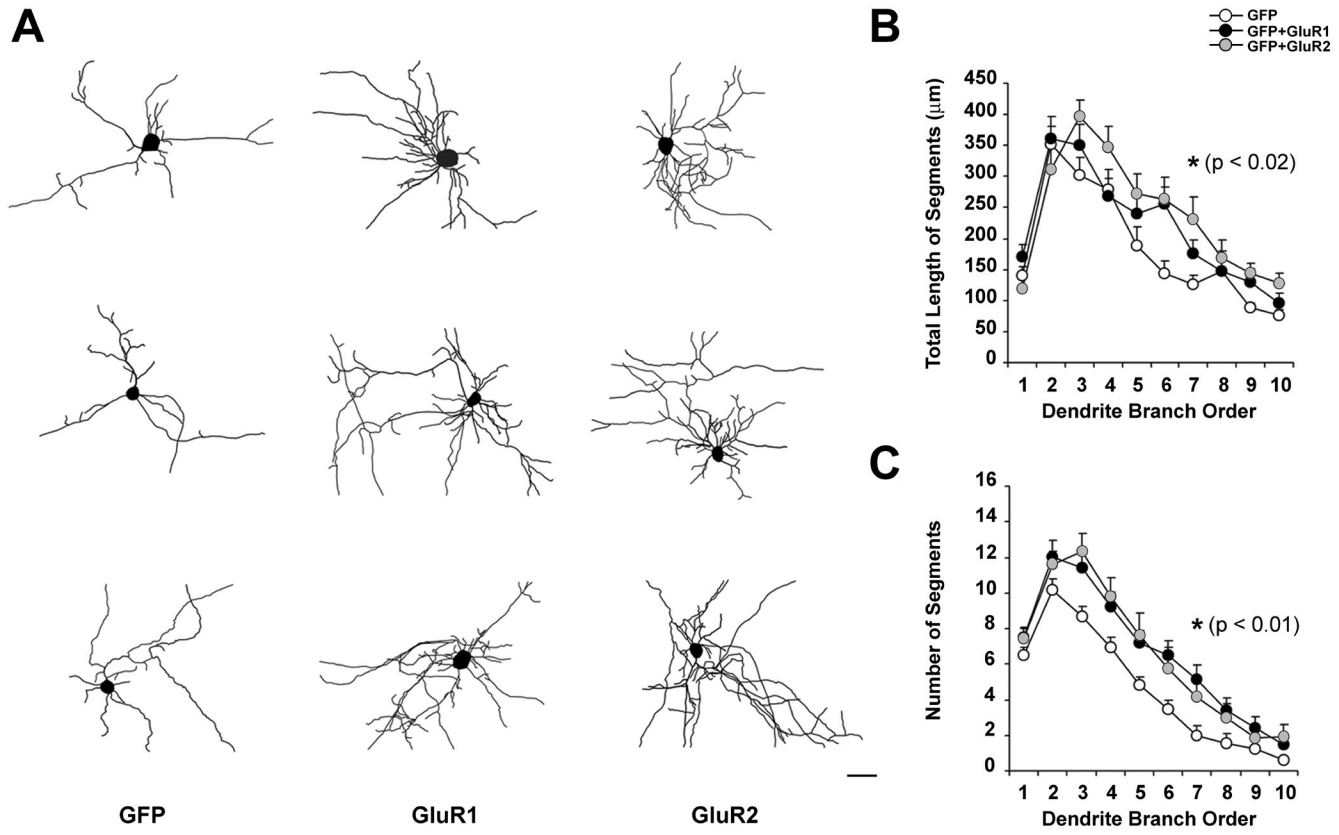


**Figure 1.**

Over-expression of both GluR1 and GluR2 causes an increase in the total amount and complexity of dendritic arbor of transfected cortical neurons. (A) Digitized camera lucida tracings of representative GFP-transfected control neurons (first panel), neurons co-transfected with GFP and GluR1 (second panel) or GluR2 (third panel). Scale bar represents 20μm. (B, C) Line graphs representing changes in the total dendritic arbor and number of dendritic segments, respectively, following over-expression of GluR1 or GluR2. Filled circles represent neurons co-transfected with GFP and GluR1 and shaded circles represent neurons co-transfected with GFP and GluR2. Unfilled circles represent GFP-expressing control neurons. Over-expression of GluR1 and GluR2 caused significant increases in total dendritic arbor ($F_{2,78} = 4.402$; $p = 0.0154$) and number of dendritic segments ($F_{2,78} = 6.286$; $p = 0.0029$) of transfected neurons, as compared to controls.

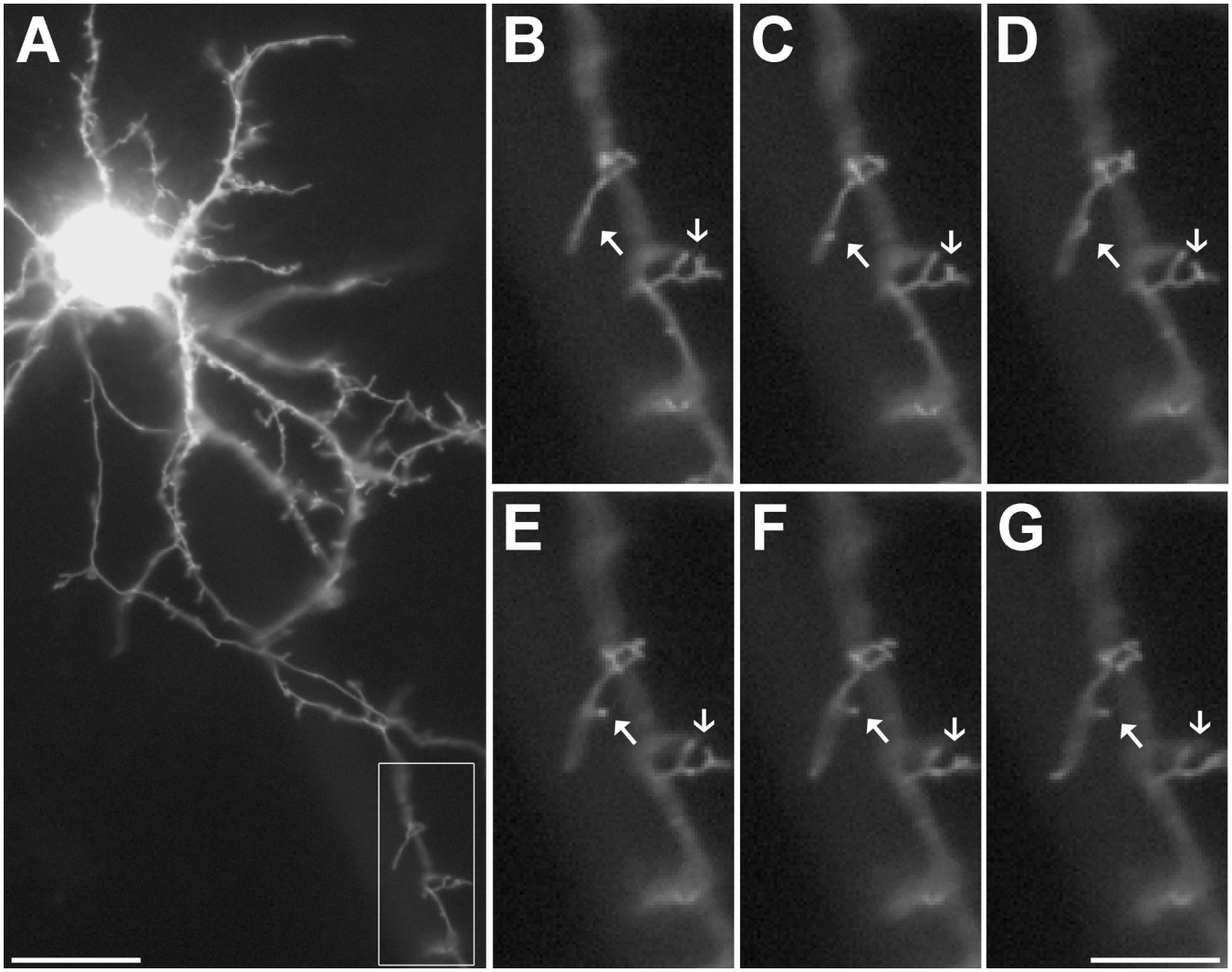


**Figure 2.**

Motility of small filopodia in transfected neurons. Filopodia display a high degree of motility over the imaging period employed, and often emerge or retract within the imaging period. (A) shows a representative neuron, transfected with GluR1, in which dynamic filopodia could be visualized. (B) through (G) represent higher magnification images taken at 20s intervals from the region delineated by the box in (A). Time-lapse sequence represents 2 minutes in total. Scale bars in (A) and (G) represents 20μm and 5μm respectively. Filled arrow (left) indicates a filopodium which was demonstrated emerge over the imaging period; unfilled arrow (right) indicates a filopodium which retracted during the imaging period.

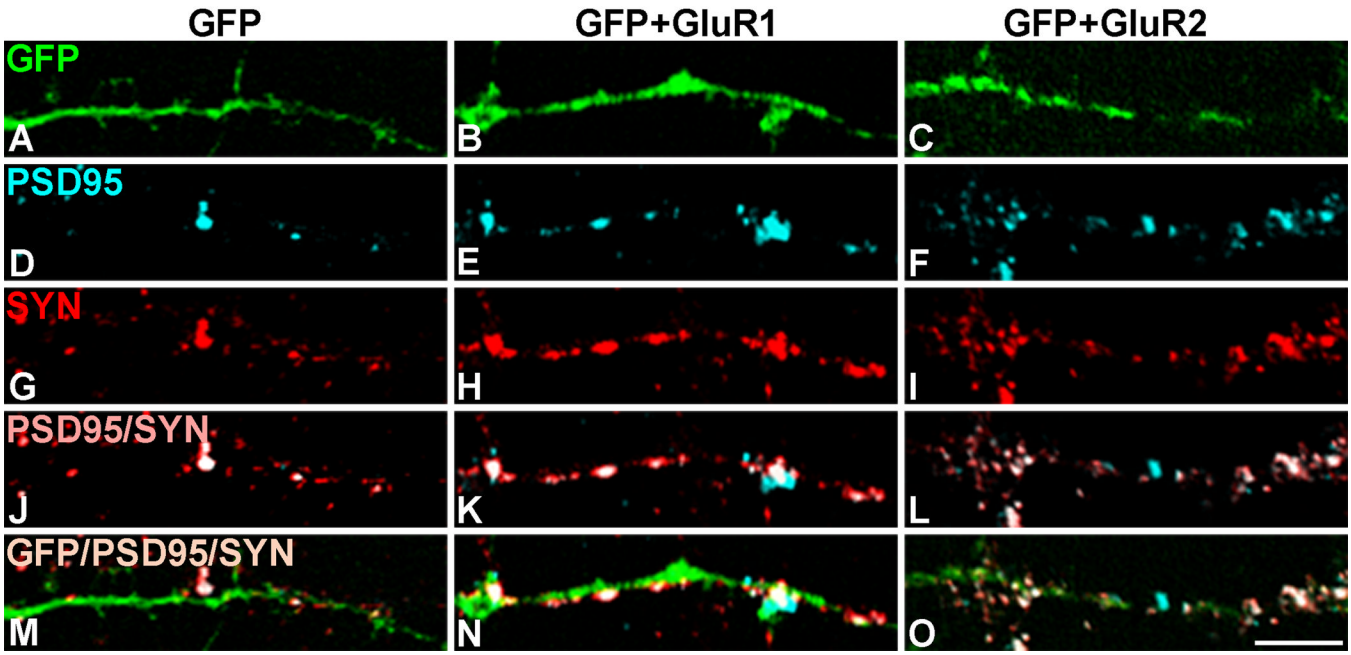


**Figure 3.**

Endogenous PSD95 and synaptophysin staining in response to GluR1 or GluR2 overexpression. Representative region of dendrite from a control neuron expressing GFP (A) or co-expressing GluR1 (B) or GluR2 (C). Neurons were immunolabeled for endogenous PSD95 (D, E, F) and co-labeled for synaptophysin (G, H, I). Overlaid images show that many PSD95 puncta are closely apposed to synaptophysin (J, K, L) and the dendrite (M, N, O), but that many extrasynaptic puncta also exist. Over-expression of both GluR1 and GluR2 caused an increase in the density of PSD95 and synaptophysin puncta. Scale bar represents 10μm.

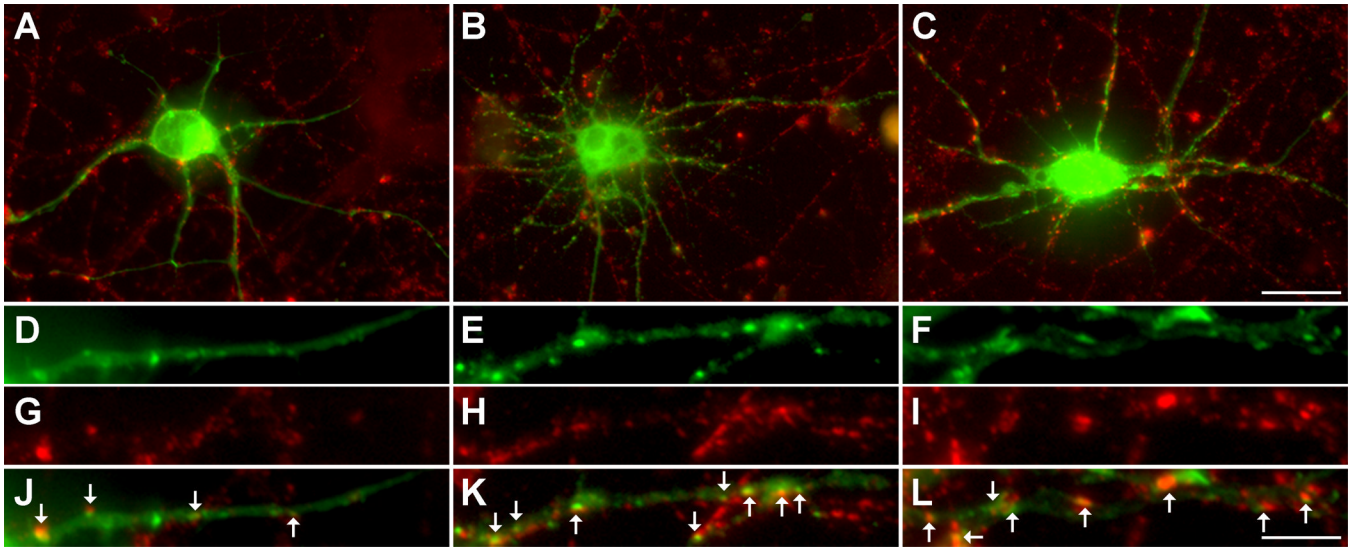


**Figure 4.**

Estimation of the number of GFP-PSD95 puncta in cortical neurons transfected with GluR1 or GluR2 and co-transfected with GFP-PSD95. Upper panel: (A) overlaid image of a representative neuron transfected with GFP-tagged PSD95 and stained for endogenous synaptophysin. (B, C) overlaid images of representative neurons co-transfected with GFP-tagged PSD95 and GluR1 or GluR2, respectively, and stained for endogenous synaptophysin. Scale bar represents 20μm. Lower panels: Higher magnification of representative regions of dendrite from neurons in upper panel, illustrating GFP-PSD95 puncta (D, E, F), endogenous synaptophysin (G, H, I) and overlaid images (J, K, L). Scale bar in (L) represents 10μm. Many GFP-PSD95 puncta were seen to be closely apposed to fluorescent-labeled endogenous synaptophysin (arrows in J, K, L); however, a substantial number of GFP-PSD95 puncta are not opposed to synaptophysin labeling.

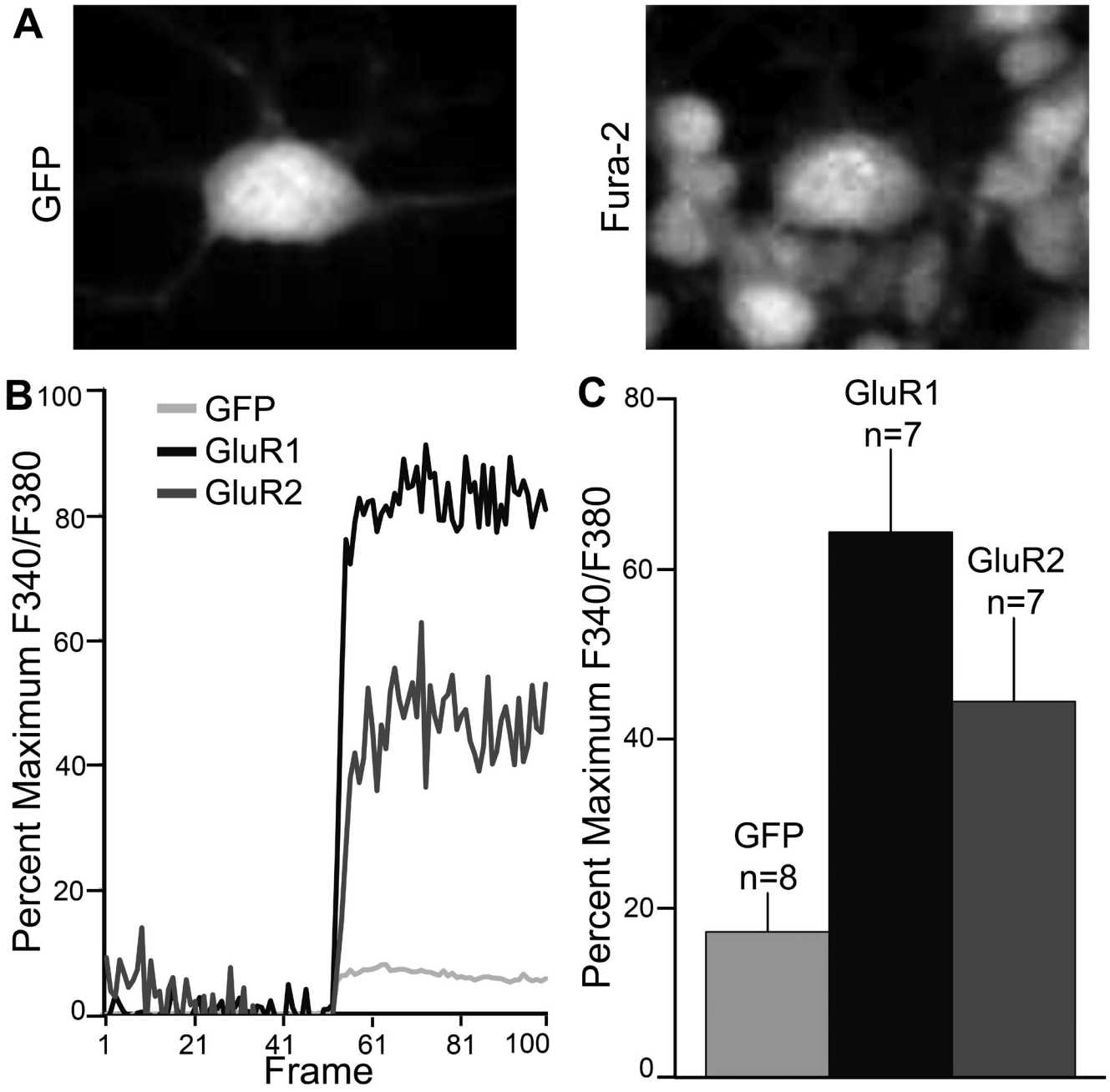


**Figure 5.**

Measurement of elevated calcium levels in transfected neurons in response to glutamate. (A) Representative transfected neuron identified by the presence of GFP (left), and the same cell loaded with fura-2 (right), recorded using a wavelength of 380nm. (B) Representative calcium response profiles for cells from each transfection group. Calcium response was normalized as a percentage of the maximum response. Traces were combined so that the initiation of the response to glutamate is shown at Frame 51; frames were collected at 2s intervals. (C) Average increase in calcium signal seen across all cells in the groups. Values represent mean ± s.e.m. (GFP, n= 8; GluR1, n=7; GluR2, n=7).

**Table 1**

Effects of GluR1 / GluR2 expression on key architectural parameters of cortical neuron dendrites

| Neuron Parameter | Treatment | | | |
|---|---|---|---|---|
| | GFP | GluR1 | GluR2 | $F_{(2,78)}$ ; $p$ |
| Primary dendrites (n) | 6.5 ± 0.4 | 7.5 ± 0.5 | 7.4 ± 0.6 | $F$ = 1.085; $p$ = 0.3430 |
| Branch points (n) | 19.4 ± 1.4 | 29.4 ± 2.1 * | 29.9 ± 3.8 * | $F$ = 5.242; $p$ = 0.0073 |
| Branch tips (n) | 26.8 ± 1.6 | 39.3 ± 2.3 * | 39.6 ± 3.9 * | $F$ = 7.099; $p$ = 0.0015 |
| Total dendrite arbor per cell (μm) | 1559 ± 85 | 2008 ± 108 * | 2013 ± 169 * | $F$ = 4.402; $p$ = 0.0154 |
| Average length of segment (μm) | 34.8 ± 1.9 | 30.6 ± 1.7 | 33.0 ± 1.7 | $F$ = 1.462; $p$ = 0.2379 |

Numbers represent mean ± SEM of GFP (n = 27), GluR1 (n = 28) or GluR2 (n = 26) expressing neurons. $F$ and $p$ values were created with ANOVA.

* Represents significant difference from GFP (Scheffe' post hoc test).

**Table 2**

Effects of AMPA receptor expression on dendritic protrusions in DIV8 cortical neurons.

| | Treatment | | | |
|---|---|---|---|---|
| **Dendritic parameter:** | **GFP** | **GluR1** | **GluR2** | **$F_{2,27}$; p** |
| Filopodia | 206 ± 17 | 176 ± 28 | 253 ± 35 | F=1.974; p=0.158 |
| Thin Spines | 5.4 ± 1.4 | 7.8 ± 1.4 | 5.5 ± 1.3 | F=0.979; p=0.389 |
| Stubby Spines | 3.8 ± 2.0 | 1.4 ± 0.8 | 2.3 ± 0.7 | F=0.837; p=0.444 |
| Mushroom Spines | 0.2 ± 0.1 | 0 | 0.3 ± 0.2 | F=1.703; p=0.316 |
| Filopodial Density (per 100μm length) | 14.1 ± 1.6 | 10.5 ± 1.7 | 13.7 ± 1.8 | F=2.500, p=0.100 |
| Spine Density (per 100μm length) | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.4 ± 0.1 | F=1.169, p=0.326 |

Numbers represent mean ± SEM of GFP (n = 10), GluR1 (n=10) or GluR2 (n=10) expressing neurons. F and p values were created using one-way ANOVA; no significant differences were detected between treatment groups.

**Table 3**

Estimation of the number synaptic and extrasynaptic PSD95 puncta

| Parameter: | GFP-PSD95 | GFP-PSD95 + GluR1 | GFP-PSD95 + GluR2 |
|---|---|---|---|
| PSD95 puncta per 200μm (n) | 30 ± 2 | 38 ± 4 | 49 ± 8 |
| Synaptophysin Puncta per 200μm (n) | 43 ± 6 | 52 ± 6 | 61 ± 9 |
| Colocalized Puncta per 200μm (n) | 19 ± 4 | 30 ± 5 | 37 ± 8 |
| PSD95 puncta colocalized with Synaptophysin (%) | 42 ± 8 | 65 ± 9 | 80 ± 18 |

Data represent mean ± s.e.m. of 5 neurons per treatment group.