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Arc/Arg3.1 Mediates Homeostatic Synaptic Scaling of AMPA Receptors

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Summary

Homeostatic plasticity may compensate for Hebbian forms of synaptic plasticity, such as long-term potentiation (LTP) and depression (LTD), by scaling neuronal output without changing the relative strength of individual synapses. This delicate balance between neuronal output and distributed synaptic weight may be necessary for maintaining efficient encoding of information across neuronal networks. Here, we demonstrate that Arc/Arg3.1, an immediate-early gene (IEG) that is rapidly induced by neuronal activity associated with information encoding in the brain, mediates homeostatic synaptic scaling of AMPA type glutamate receptors (AMPARs) via its ability to activate a novel and selective AMPAR endocytic pathway. High levels of Arc/Arg3.1 block the homeostatic increases in AMPAR function induced by chronic neuronal inactivity. Conversely, loss of Arc/Arg3.1 results in increased AMPAR function and abolishes homeostatic scaling of AMPARs. These observations, together with evidence that Arc/Arg3.1 is required for memory consolidation, reveal the importance of Arc/Arg3.1's dynamic expression as it exerts continuous and precise control over synaptic strength and cellular excitability.

Introduction

Arc/Arg3.1 is an immediate-early gene (IEG) that is dynamically regulated by neuronal activity and is tightly coupled to behavioral encoding of information in neuronal circuits (Guzowski et al., 2005). Arc/Arg3.1 mRNA traffics to distal dendrites where it accumulates at sites of synaptic activity and is locally translated (Steward et al., 1998). In vivo, Arc/Arg3.1 is coordinately induced in populations of neurons that mediate learning, such as place cells of the hippocampus (Ramirez-Amaya et al., 2005) and behavior-specific neural networks in parietal (Burke et al., 2005), visual (Tagawa et al., 2005; Wang et al., 2006), and olfactory (Zou and Buck, 2006) cortices. For example, 5 min of spatial exploration elicits transcriptional induction of Arc/Arg3.1 in ~40% of CA1 neurons (Guzowski et al., 2005). Moreover, Arc/Arg3.1 is repeatedly induced in the same network during exploration of the same space (Guzowski et al., 2006) and during memory consolidation (Ramirez-Amaya et al., 2005), indicating that in vivo expression can be maintained at elevated steady-state levels in specific neuronal networks. Consistent with the notion that Arc/Arg3.1 protein is required for learning and memory (Guzowski et al., 2000), Arc/Arg3.1 knockout (KO) mice demonstrate impaired maintenance of LTP and consolidation of long-term memory but exhibit normal short-term memory (Plath et al., 2006 [this issue of *Neuron*]). Molecular studies indicate that Arc/Arg3.1 modulates AMPAR trafficking by interacting with two proteins intricately involved in endocytosis, endophilin 2/3 and dynamin (Chowdhury et al., 2006 [this issue of *Neuron*]).

Changes in synaptic strength are proposed to underlie memory storage in neuronal circuits (Malenka and Nicoll, 1999; Martin et al., 2000). Hebbian forms of plasticity such as LTP and LTD can modify the strength of individual synapses. However, unrestrained potentiation or depression can result in saturation of a neuron's ability to encode information (Moser et al., 1998). Homeostatic compensation for these acute changes in synaptic strength is required to maintain neuronal output in the normal range but must be accomplished without erasing information encoded by the distributed synaptic strengths (Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004). For example, chronic blockade of network activity for several days results in an increase in surface and synaptic AMPARs, while a chronic increase in activity reduces surface and synaptic AMPARs (O'Brien et al., 1998; Turrigiano et al., 1998).

Here, we present evidence that the Arc/Arg3.1-endocytic pathway mediates homeostatic scaling of AMPARs. Arc/Arg3.1 protein is dynamically regulated by chronic changes in neuronal activity that normally evokes synaptic scaling. Overexpression of Arc/Arg3.1 blocks the upregulation of surface AMPARs and mEPSCs induced by chronic neuronal inactivity. Conversely, Arc/Arg3.1 KO neurons exhibit a scaled increase in surface AMPARs and AMPAR-mediated mEPSCs that mimics the upregulation of synaptic function induced by chronic inactivity. Strikingly, Arc/Arg3.1 KO neurons exhibit gross deficits in homeostatic scaling of surface AMPARs and AMPAR-mediated mEPSCs. These findings support a simple model in which Arc/Arg3.1 regulates an endocytic pathway whose activity is continuously coupled to neuronal excitability by the transcription of Arc/Arg3.1. This process regulates steady-state AMPARs and permits the homeostatic scaling of synaptic strength.

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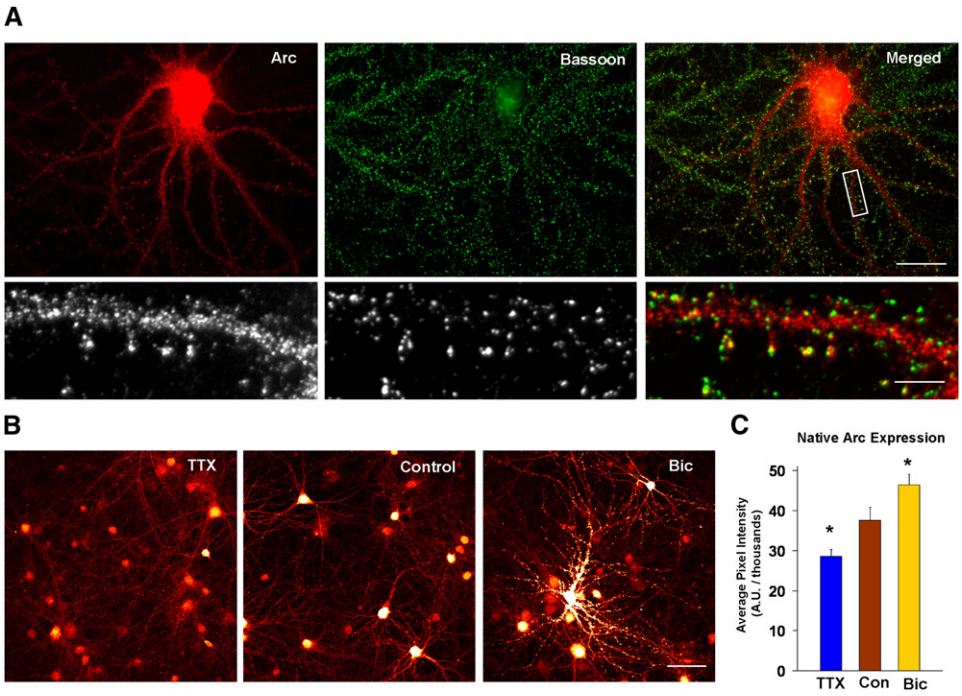

Figure 1. Arc/Arg3.1 Protein Is Dynamically Regulated by Neuronal Activity in Primary Culture

(A) Endogenous Arc/Arg3.1 is expressed in the cell body and dendrites of DIV 28 primary hippocampal neurons. There is enrichment of protein at synapses, as shown by colocalization with the presynaptic marker bassoon. (Scale bars represent 30 μm and 8 μm in magnified dendrites.)

(B) Representative pictures of Arc/Arg3.1 protein in DIV 28 primary cortical neurons. Arc/Arg3.1 protein is downregulated after 2 days of TTX treatment and is conversely upregulated with 2 days of bicuculline treatment. (Pictures are shown using a Glow scale, with white as the highest pixel intensity and red as the lowest intensity. Scale bar, 80 μm.)

(C) Quantification of protein levels in cortical neurons shows a significant downregulation with TTX treatment (n = 41 cells) as compared with untreated neurons (n = 38) and a significant upregulation with bicculline treatment (n = 48). (*$p < 0.05$). Error bars in all figures represent the standard error of the mean.

Results

Arc/Arg3.1 Protein Expression Is Regulated by Neuronal Activity in Primary Culture

Arc/Arg3.1 mRNA induction and protein synthesis are regulated by neuronal activity in vivo (Steward et al., 1998; Steward and Worley, 2001). To elucidate the synaptic function of Arc/Arg3.1, we examined Arc/Arg3.1 expression in primary neuronal cultures. Many features of Arc/Arg3.1 that are observed in vivo are also recapitulated in culture (Rao et al., 2006). Arc/Arg3.1 expression is relatively low in preadolescent brain but reaches peak levels by postnatal day 28 (Lyford et al., 1995). Similarly, in primary hippocampal or cortical cultures, Arc/Arg3.1 expression is relatively low prior to 12 days in vitro (DIV) but increases markedly in older neurons (data not shown) and correlates with the appearance of mature synapses. In these older cultures, Arc/Arg3.1 protein is present throughout distal dendrites and spines, and many of these puncta colocalize with the synaptic marker bassoon (Figure 1A). Arc/Arg3.1 expression in primary culture is also dependent on spontaneous neuronal activity, which is consistent with its role as an IEG (Figures 1B and 1C). Long-term TTX treatment of high-density cortical neurons, which blocks all evoked neuronal activity, significantly reduces Arc/Arg3.1 expression. Bicuculline, which blocks inhibitory neurotransmission mediated by $GABA_A$ receptors and increases neuronal firing, significantly increases Arc/Arg3.1 expression (Figures 1B and 1C). Similar results are seen in low-density hippocampal neurons (data not shown).

Arc/Arg3.1 Blocks Homeostatic Scaling of AMPARs Induced by Chronic Blockade of Neuronal Activity

Since Arc/Arg3.1 expression is reciprocally regulated by activity and facilitates AMPAR endocytosis (Chowdhury et al., 2006), we hypothesized that Arc/Arg3.1 may be involved in mechanisms that permit homeostatic AMPAR scaling. Consistent with this idea, overexpression of Arc/Arg3.1 results in a cell-wide decrease in the surface expression of GluR1-containing AMPARs, mimicking the effect observed with chronic bicuculline treatment (Figures 2A and 2B). Arc/Arg3.1 overexpression also blocked the homeostatic upregulation of GluR1 induced by chronic TTX treatment (Figures 2A and 2B). This indicates that low levels of Arc/Arg3.1 protein are required for optimal homeostatic upregulation of AMPARs induced by chronic inactivity. Indeed, since TTX treatment also reduces endogenous Arc/Arg3.1 levels, we observed greater relative downregulation of AMPARs when Arc/Arg3.1 is overexpressed in the presence of TTX. We next examined AMPAR expression in high-density cortical neurons, a preparation optimized for biochemical assays of surface proteins and for electrophysiological assays of synaptic events (Rumbaugh et al., 2003, 2006). In contrast to hippocampal cultures, Arc/Arg3.1 expression driven by a Sindbis viral vector had little

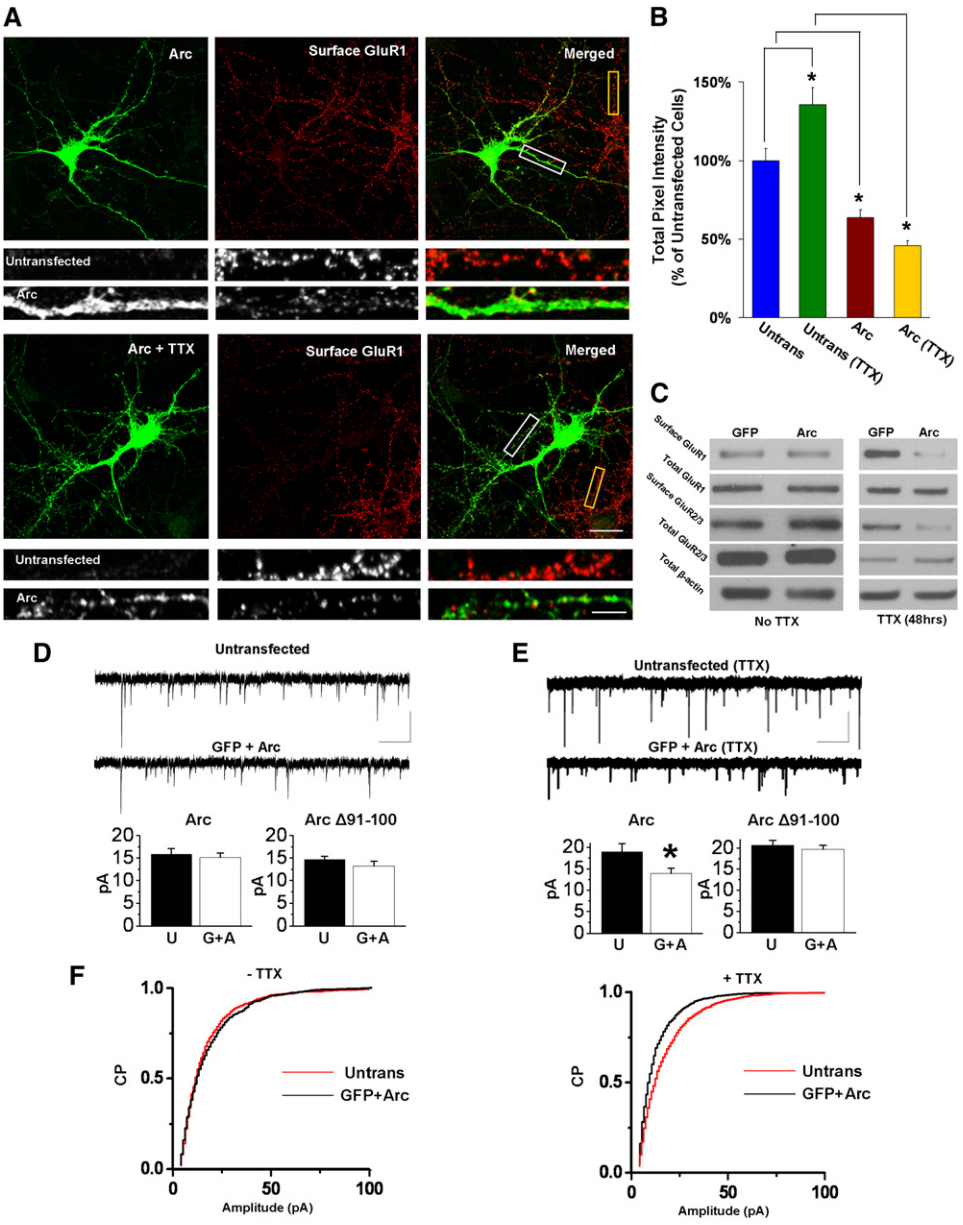

Figure 2. Arc/Arg3.1 Overexpression Blocks TTX-Induced Synaptic Scaling

(A) Representative images of Arc/Arg3.1 transgene expression in low-density hippocampal neurons, showing a reduction of surface GluR1 as compared to neighboring untransfected cells, 16 hr posttransfection. Treatment with TTX-enhanced surface GluR1, which was blocked by expression of Arc/Arg3.1 transgene. (White boxes show magnified Arc/Arg3.1 transfected dendrites and yellow boxes highlight untransfected dendrites. (Scale bars, 30 μm and 8 μm in magnified dendrites).

(B) Quantification of surface GluR1 experiments. Results from one experiment are shown and are representative of data collected from at least two other experiments. Arc/Arg3.1 expression causes a significant decrease in the total intensity of surface GluR1 puncta (64% ± 5% of untransfected cells, n = 42 dendritic regions from 14 cells) compared with neighboring untransfected cells (n = 42/14). TTX treatment significantly enhanced GluR1 intensity (136% ± 10%, n = 42/14). Arc/Arg3.1 expression blocked the TTX-induced increase in surface GluR1 (46% ± 3%, n = 42/14). (*$p \ll 0.001$).

(C) Western blots of primary high-density cortical cultures show that Arc/Arg3.1 Sindbis virus expression has no affect on surface GluR1 or 2 with no treatment but dramatically reduced surface levels when treated with TTX, blocking the upregulation observed in cells transfected with GFP virus.

(D) Traces selected from recordings of untransfected, GFP + WT-Arc/Arg3.1-, or GFP + Δ91–100Arc/Arg3.1-transfected neurons. Histograms represent the average mEPSC amplitude of each population. No change in amplitude or frequency was observed. (Scale = 20 pA, 400 ms).

(E) Traces selected from whole-cell recordings of untransfected GFP + WT-Arc/Arg3.1 or GFP + Δ91–100Arc/Arg3.1-expressing neurons. In each case, neurons were treated with 1 μM TTX for 48 hr. mEPSCs were only downregulated in WT-Arc/Arg3.1-expressing neurons. (*$p < 0.05$).

(F) Left: Cumulative probability distribution of mEPSC amplitudes from all events in either untransfected (U; n = 2597) or GFP + Arc/Arg3.1 (G+A; n = 2450)-transfected neurons. Right: Cumulative probability distribution of mEPSC amplitudes from all events in either untransfected (U; n = 1540) or GFP + Arc/Arg3.1 (G+U; n = 1476)-transfected neurons. These neurons were treated with 1 μM TTX for 48 hr.

effect on AMPAR surface expression as detected by surface biotinylation (Figure 2C). We asked whether this difference might be due to high levels of neuronal network activity and endogenous Arc/Arg3.1 expression common to high-density cortical cultures. Consistent with this hypothesis, treatment with TTX, which reduces the endogenous level of Arc/Arg3.1, revealed a striking effect of Arc/Arg3.1 transgene expression on surface AMPARs. Arc/Arg3.1-expressing neurons treated with TTX exhibited an ~50% reduction of surface AMPARs compared to control neurons expressing only GFP. Similarly, electrophysiological recordings from these neurons demonstrated that, in the absence of TTX, Arc/Arg3.1 transgene expression had no effect on the amplitude of AMPAR-mediated mEPSCs (Figure 2D). By contrast, in the presence of TTX, Arc/Arg3.1 transgene reduced the amplitude of AMPAR-mediated mEPSCs (Figure 2E) and blocked the TTX-induced scaling effect. Importantly, cumulative probability distributions of mEPSC amplitudes were uniformly reduced by Arc/Arg3.1 transgene expression (Figure 2F). This suggests that Arc/Arg3.1 scales AMPAR responses in a manner similar to activity-dependent homeostatic synaptic scaling. Strikingly, we found that a deletion mutant of Arc/Arg3.1 (aaΔ91–100) that does not interact with endophilin (Chowdhury et al., 2006) had no effect on mEPSCs and did not block the TTX-induced increase in mEPSCs (Figures 2D and 2E). Since this region is also required for downregulation of AMPARs (Chowdhury et al., 2006), these data provide compelling evidence that Arc/Arg3.1 regulates homeostatic scaling of AMPARs though a molecular interaction with the endocytic machinery.

Loss of Arc/Arg3.1 Results in Increased AMPAR Function that Mimics the Homeostatic State Induced by Chronic Inactivity

To assess the function of endogenous Arc/Arg3.1, we performed various experiments on Arc/Arg3.1 KO mice. These mice are viable and do not exhibit any gross changes in neural architecture (Plath et al., 2006). We isolated primary neurons from these mice and compared them with neurons derived from wild-type (WT) animals. To further explore the contribution of Arc/Arg3.1 in homeostasis, we examined surface AMPAR levels in Arc/Arg3.1 KO neurons using DIV 21–28 low-density hippocampal neurons. If endogenous Arc/Arg3.1 is involved in promoting the removal of AMPARs from synapses, then neurons lacking Arc/Arg3.1 might exhibit an increase in steady-state levels of these receptors. In support of this hypothesis, we found that Arc/Arg3.1 KO neurons exhibit a dramatic increase in surface GluR1 when compared with WT neurons (Figure 3A). Puncta from Arc/Arg3.1 KO neurons exhibit increased size, number, and intensity compared with WT neurons (see Figure S1 in the Supplemental Data available with this article online), suggesting a global upregulation of surface GluR1 levels. Importantly, cultured Arc/Arg3.1 KO neurons form similar numbers of synapses and have a normal compliment of synaptic proteins when compared to WT neurons (Figures 3C and S2). In contrast to GluR1 surface levels, surface GluR2 levels were unchanged in Arc/Arg3.1 KO neurons (Figure 3B), suggesting that the removal of endogenous Arc/Arg3.1 from synapses changes the subunit composition of AMPARs to favor GluR1-containing receptors.

The cell biological observations made in Arc/Arg3.1 KO neurons cannot definitively show that increases in surface AMPARs lead to changes in synaptic strength. To directly examine this issue, AMPAR-mediated mEPSCs were recorded from WT and Arc/Arg3.1 KO neurons. Arc/Arg3.1 KO neurons possessed larger AMPAR-mediated mEPSC amplitudes relative to WT neurons (Figure 3D). Notably, the increase of mEPSC amplitudes was distributed over the entire range of recorded events, as shown by cumulative probability distributions (Figure 3D). This is similar to the homeostatic synaptic scaling that occurs in WT neurons treated with TTX. Thus, reduction of Arc/Arg3.1, by either genetic deletion or TTX, results in a similar state of enhanced surface AMPAR expression and function.

Arc/Arg3.1 Regulates Homeostatic Synaptic Scaling of AMPARs

To directly assess the effect of Arc/Arg3.1 in homeostatic scaling of AMPARs, we treated both WT and Arc/Arg3.1 KO low-density hippocampal neurons with TTX or bicuculline for 48 hr and examined surface AMPAR levels. WT mouse neurons exhibited a robust increase in surface GluR1 after TTX treatment and significantly lower surface levels after bicuculline treatment (Figure 4A). Strikingly, Arc/Arg3.1 KO neurons exhibited no changes in surface GluR1 levels after either treatment indicating a complete absence of activity-dependent homeostatic scaling of AMPARs in hippocampal neurons (Figure 4A). In electrophysiological assays, TTX treatment of WT neurons resulted in a significant increase in mEPSC amplitudes and a multiplicative shift in the cumulative probability distribution, indicative of synaptic scaling of AMPARs (Figure 4B). By contrast, Arc/Arg3.1 KO neurons treated with TTX failed to exhibit an enhancement of mEPSC amplitudes (Figure 4C), possibly because KO neurons are already homeostatically upregulated. In response to bicuculline, both WT and Arc/Arg3.1 KO neurons exhibited a modest downregulation of AMPAR (Figures 4B and 4C). This result contrasts with histochemical assays in hippocampal neurons and suggests that mechanisms in addition to Arc/Arg3.1 may contribute to AMPAR downregulation in highly active cortical neurons.

We next asked whether the absence of homeostatic increases of AMPARs in Arc/Arg3.1 KO neurons following TTX treatment might be due to saturation of mechanisms that can deliver AMPARs to the cell surface. To address this issue, we briefly stimulated WT and Arc/Arg3.1 KO neurons with glycine, a treatment that results in a rapid and persistent change in synaptic function though the insertion of AMPARs. This process is believed to mimic LTP (Liao et al., 2001; Lu et al., 2001). Glycine treatment produced an equivalent increase of surface AMPAR in WT and Arc/Arg3.1 KO neurons (Figure 5). Thus, AMPAR insertion in response to transient synaptic activation is intact in Arc/Arg3.1 KO neurons.

Discussion

The present study provides evidence that Arc/Arg3.1 mediates synaptic scaling of AMPARs over a broad range of synaptic activity in mature neurons. Arc/Arg3.1 directly

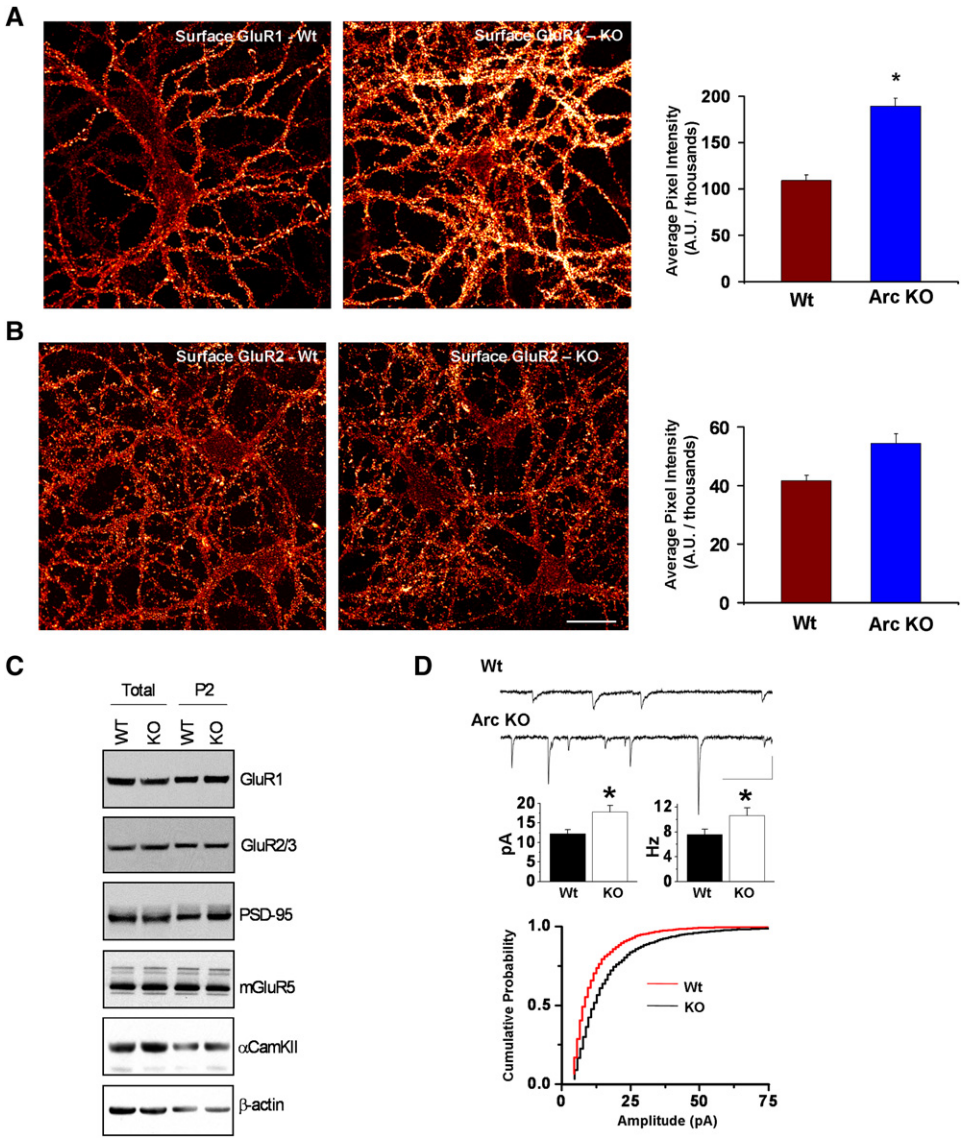

Figure 3. Surface GluR1 and Synaptic Strength Are Increased in Arc/Arg3.1 KO Mice

(A) Representative pictures of surface GluR1 in WT and KO primary hippocampal neurons. Arc/Arg3.1 KO neurons (n = 60/20) have significantly increased surface levels compared to Arc/Arg3.1 WT neurons (n = 54/18). Similar results are reported in Figure 8A of Chowdhury et al. (2006), although the results reported reflect two different experiments. (* $p < 0.001$).

(B) Representative pictures of surface GluR2 in WT and KO primary hippocampal neurons. No significant difference in surface GluR2 levels was observed ($p = 0.2$). (Scale bar, 30 μm).

(C) Western blots of synaptic proteins in WT and Arc/Arg3.1 KO neurons obtained from total and P2 fractionated brain lysates, showing no dramatic changes in protein expression.

(D) Traces selected from recordings of either WT (n = 23) or Arc/Arg3.1 KO (n = 21) neurons. Histograms represent the average mEPSC amplitude or frequency of each population. Arc/Arg3.1 KO neurons have significantly higher mEPSC amplitudes (*$p < 0.01$). (Scale = 30 pA, 100 ms). Cumulative probability distribution of mEPSC amplitudes from all events in either WT (n = 2454) or Arc/Arg3.1 KO (n = 2222) neurons are shown.

interacts with components of the endocytic pathway, including dynamin and endophilin, and selectively increases the rate of AMPAR endocytosis (Chowdhury et al., 2006). These data suggest the following model: in the absence of Arc/Arg3.1 or in conditions of persistent low activity where Arc/Arg3.1 expression is dramatically reduced, Arc/Arg3.1-dependent endocytosis is minimized, causing a shift in the steady-state AMPAR distribution toward membrane insertion. In conditions of persistent high activity, high levels of Arc/Arg3.1 are available to facilitate endocytosis of AMPARs, with consequent downregulation of synaptic AMPARs. Thus, Arc/Arg3.1 acts to titrate surface AMPARs, and this allows optimal synaptic plasticity by maintaining distributed synaptic weights. Periods of sustained Arc/Arg3.1 expression act to regulate synaptic scaling and thus counteract saturation of synaptic strength inherent in Hebbian positive-feedback loops.

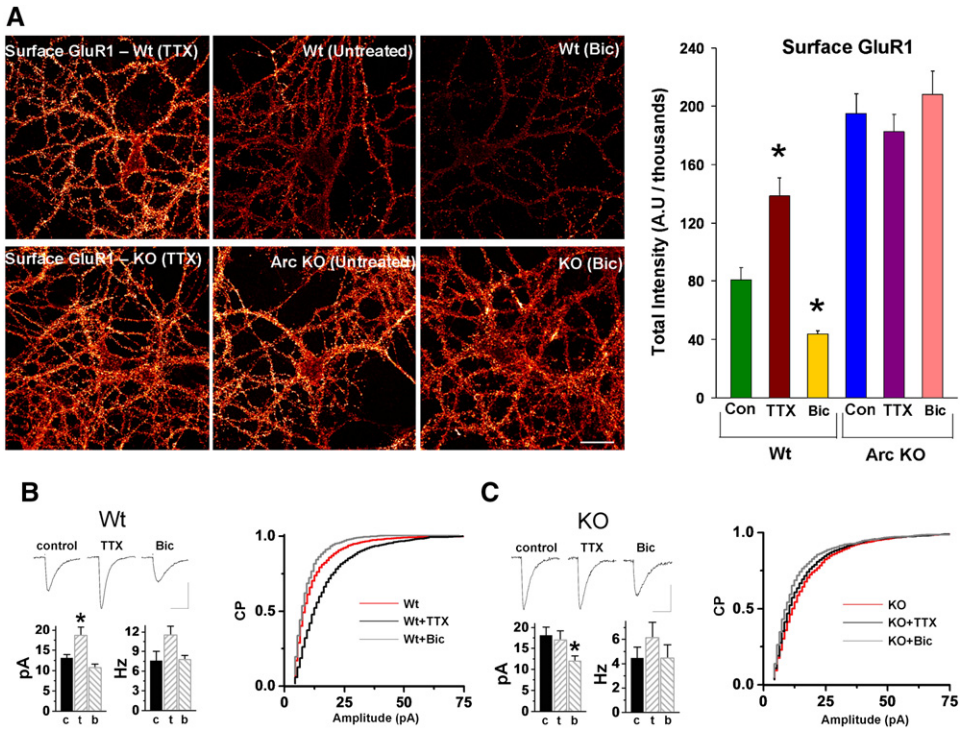

Figure 4. Synaptic Scaling of AMPARs Is Abolished in Arc/Arg3.1 KO Neurons

(A) Representative images of surface GluR1 in WT and KO neurons treated for 2 days with TTX or bicuculline. WT neurons undergo significant scaling, with an increase in surface levels with TTX and a decrease in surface levels with bicuculline treatment. In contrast, KO neurons do not exhibit any changes in surface levels. (Scale bar, 30 μm). Quantification of surface levels show that WT neurons have a significant decrease in total intensity (n = 30/11 for all) with TTX treatment and a significant increase in intensity with bicuculline treatment. In contrast, no significant difference in surface GluR1 intensity was observed in KO neurons treated with TTX ($p = 0.5$) or bicuculline ($p = 0.5$). (*$p < 0.001$).

(B) Top: Representative mEPSC (averaged; ~100 events) from a control (n = 9), TTX (1 μM; 48 hr; n = 9)-, or bicuculline (20 μM; 48 hr; n = 9)-treated WT mouse neuron. Bottom: Histograms represent the average mEPSC amplitude or frequency of each population. mEPSC amplitudes are significantly higher after TTX treatment (*$p < 0.05$). (Scale = 10 pA, 5 ms). Right: Cumulative probability distribution of mEPSC amplitudes from all events in control, TTX-, and bicuculline-treated WT neurons.

(C) Top: Representative mEPSC (averaged; ~100 events) from a control (n = 15), TTX (1 mM; 48 hr; n = 15)-, or bicuculline (20 mM; 48 hr; n = 10)-treated Arc/Arg3.1 KO mouse neuron. Histograms represent the average mEPSC amplitude or frequency of each population. In contrast to WT neurons, TTX treatment does not result in an increase in mEPSC amplitude. (Scale = 10 pA, 5 ms). Right: Cumulative probability distribution of mEPSC amplitudes from all events in control, TTX-, and bicuculline-treated KO neurons. Virtually no scaling of amplitudes can be observed in KO neurons.

Molecular Mechanisms Underlying Homeostatic Scaling of AMPA Receptors

Homeostatic synaptic scaling of AMPARs has been elegantly described in physiological terms in both cortical and hippocampal neurons (O'Brien et al., 1998; Thiagarajan et al., 2005; Turrigiano et al., 1998). Cortical neurons consistently exhibit changes in mEPSC amplitude without any changes in frequency, suggesting

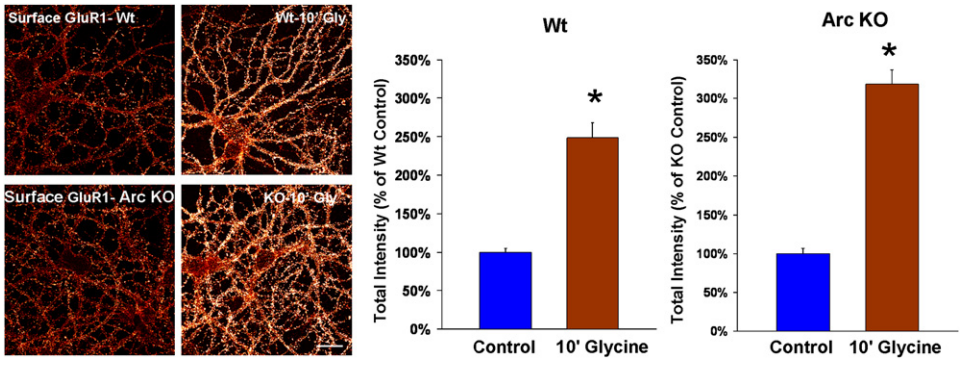

Figure 5. Arc/Arg3.1 KO Neurons Exhibit Normal Insertion of GluR1 Immediately after Chemical LTP

Representative pictures of surface GluR1 in cultures treated with Glycine. Although Arc/Arg3.1 KO neurons have higher basal surface GluR1, pictures were obtained to equalize WT and KO basal levels so as to directly compare changes induced with glycine treatment. Quantitation of surface GluR1 shows that both WT and KO neurons showed robust and significant increases in surface levels 10 min after glycine treatment (*$p \ll 0.001$).

a postsynaptic mechanism. In contrast, hippocampal neurons have been reported to exhibit increases in mEPSC amplitude and frequency (Thiagarajan et al., 2002). Presynaptic changes such as enlargement of presynaptic terminals and their vesicle pools have also been observed in hippocampal neurons (Murthy et al., 2001). Further complicating these results, a recent study showed that the expression locus of homeostasis is governed by how long the cultures are incubated in vitro and not by cell type. In both cortical and hippocampal neurons, 2 days of TTX treatment induced an increase in mEPSC amplitude in cells that were less than 14 DIV, without affecting mEPSC frequency. However, in cultures older than 18 DIV, the same treatment induced a large increase in mEPSC frequency and a reduced effect on amplitude (Wierenga et al., 2006). The molecular mechanisms underlying these changes are unclear. Our results implicate Arc/Arg3.1 as one member of a molecular pathway that permits AMPAR scaling, but these data do not address other homeostatic mechanisms of regulating neuronal output. We find differences in the action of Arc/Arg3.1 in hippocampal versus cortical neurons. Arc/Arg3.1 is more effective in downregulating AMPARs in low-density hippocampal cultures than in high-density cortical neurons. This is probably due to lower basal Arc/Arg3.1 in low-density neurons, but we cannot rule out region specific differences in cofunctional molecules.

In hippocampal neurons, homeostatic increases in surface AMPARs induced by prolonged inactivity have been shown to be mainly due to increases in GluR2-lacking receptors (Thiagarajan et al., 2005). In our studies of Arc/Arg3.1 KO neurons, Arc/Arg3.1 effects appear to be preferential for GluR1, as KO neurons show no overt changes in surface GluR2, suggesting an increase in GluR2-lacking receptors at the plasma membrane. Arc/Arg3.1 expression in pyramidal neurons of hippocampal slice cultures produces a selective downregulation of AMPARs that requires Arc/Arg3.1's ability to bind endophilin and is blocked by agents that inhibit NMDA receptor-dependent LTD, including the calcineurin inhibitor FK506 and peptides that mimic the C terminus of GluR2 (Rial Verde et al., 2006 [this issue of *Neuron*]). However, when Arc/Arg3.1 is expressed in WT neurons, we see downregulation of both GluR1 and GluR2 (Figure 2C). These results confirm that Arc/Arg3.1 can endocytose GluR2 in an acute manner. It is possible that the apparent GluR1 selectivity in the Arc/Arg3.1 KO is a consequence of long-term depletion of the protein. This may promote a shift in the subunit composition of AMPAR pools toward more GluR2-lacking AMPARs, similar to what is seen with prolonged inactivity. Further work is needed to clarify Arc/Arg3.1's precise role in determining AMPAR subunit specificity.

A recent report indicates that TNF-α, which is derived from glia, is required for homeostatic upregulation of AMPARs in neurons induced by chronic activity blockade, but not downregulation due to increased activity (Stellwagen and Malenka, 2006). The molecular mechanism of TNF-α regulation remains to be determined in both homeostasis and synaptic maturation. In addition, it is unclear if TNF-α plays a direct role in information storage or is just a permissive factor needed for correct neuronal network development.

Arc/Arg3.1 Mediates Homeostasis and Hebbian Synaptic Plasticity

AMPARs are highly dynamic and undergo rapid shuttling between the plasma membrane and internal recycling pools (Luscher et al., 1999; Park et al., 2004). Both LTD and LTP involve modulation of endocytosis and exocytosis of AMPA receptors and numerous AMPA-interacting molecules such as PICK1 and NSF have been shown to be important for regulating AMPAR trafficking and synaptic plasticity (Song and Huganir, 2002). Long-term maintenance of LTP and LTD requires new protein synthesis (Huber et al., 2000; Nguyen and Kandel, 1996; Otani and Abraham, 1989), and both LTP- and LTD-inducing stimuli enhance the production of an overlapping set of proteins through shared biochemical pathways such as the MAPK cascade and the mTOR pathway. It has been hypothesized that synapses are "tagged" by plasticity-inducing stimuli and capture proteins that subserve LTP and LTD (Frey and Morris, 1997). The molecular nature of the tag or the precise proteins that are captured are unknown. Both LTP and LTD are disrupted in Arc/Arg3.1 KO mice (Plath et al., 2006), and Arc/Arg3.1-induced synaptic depression mimics LTD in slices (Rial Verde et al., 2006). This evidence suggests that Arc/Arg3.1 may be a critical component for bidirectional plasticity. The direction may be governed by the nature of the tag or the context/prior history of the neuron (i.e., Arc/Arg3.1 could critically regulate the metaplasticity of the cell). Basal synaptic transmission and mEPSCs are normal in slices from Arc/Arg3.1 KO mice (Plath et al., 2006). However, slices have much lower spontaneous network activity than primary culture (see Plath et al. [2006]), and thus Arc/Arg3.1-dependent homeostasis is more apparent in our culture model. In addition, there are numerous homeostatic mechanisms that control basal transmission, including intrinsic excitability and modulation of ion channel properties (Marder and Goaillard, 2006). Since Arc/Arg3.1 is deleted from birth, other homeostatic processes could compensate and keep basal transmission normal. Further experiments that manipulate Arc/Arg3.1 for acute periods in vivo are needed to directly look at whether Arc/Arg3.1 is required for the maintenance of normal basal transmission in a large population of cells.

It is intriguing that Arc/Arg3.1 is induced in the genomic response to both homeostatic synaptic plasticity and protein synthesis-dependent Hebbian plasticity, as it has been proposed that they share underlying molecular mechanisms (Yeung et al., 2004). In vivo evidence for the importance of Arc/Arg3.1's role in homeostatic plasticity comes from recent work showing that Arc/Arg3.1 KO mice have deficits in orientation tuning in the visual cortex (Wang et al., 2006). Arc/Arg3.1 KO mice exhibit activation of a larger neuronal ensemble with reduced orientation specificity. As a consequence, the orientation selectivity of neuronal spiking activities is significantly reduced. Suppression of responses at nonpreferred orientations was critically dependent on Arc/Arg3.1 protein, whereas responses at preferred orientations were not significantly affected, suggesting Arc/Arg3.1 plays a more critical role in less-active neurons and thus may increase the signal-to-noise of the system.

Exactly how homeostasis affects Hebbian plasticity remains unclear. It is possible that Arc/Arg3.1's effects

on LTP/LTD are secondary to poor homeostatic scaling in neurons or that these two processes may be independent of each other. Our manipulations of Arc/Arg3.1 are cell wide, whereas in vivo regulation may only affect specific neuronal circuits. Thus, our data do not rule out a role of Arc/Arg3.1 in metaplasticity of specific synapses.

Arc/Arg3.1 and Consolidation of Memory

Arc/Arg3.1 KO mice exhibit deficits in long-term consolidation of memory (Plath et al., 2006) that could be linked to changes in AMPAR homeostasis. Arc/Arg3.1 is normally expressed at high levels in brain of awake, behaving animals (Lyford et al., 1995). The time course of the mRNA and protein expression is rapid and transient and returns to baseline levels within 2 hr (Ramirez-Amaya et al., 2005). However, Arc/Arg3.1 can be repeatedly induced in the same network with repetition of the same behavioral paradigm (Guzowski et al., 2006) and is reactivated during periods when animals are resting or sleeping, as the hippocampus is "off line" (Ramirez-Amaya et al., 2005). Thus, Arc/Arg3.1 protein can be persistently upregulated in specific neurons as they engage in network-specific learning and consolidation. Our studies predict that homeostatic-scaling is an ongoing process in these neurons and is essential for consolidation.

In summary, our findings suggest that Arc/Arg3.1 controls surface AMPAR levels in a homeostatic manner and acts to keep surface levels and subunit composition optimal for Hebbian plasticity. These processes are required for consolidation of information. Arc/Arg3.1 may also play a key role in cognitive disorders, as disruption of Arc/Arg3.1 expression has been observed in Fragile X mental retardation (Zalfa et al., 2003) and Alzheimer's disease (Dickey et al., 2003; Palop et al., 2005). Further understanding of Arc/Arg3.1's function and regulated expression should help elucidate the molecular mechanisms of memory storage by linking protein-synthesis-dependent synaptic plasticity, AMPAR trafficking, and homeostatic synaptic scaling.

Experimental Procedures

Expression Constructs

All the expression constructs were made by PCR. Internal deletion and point mutant were made either using QuikChange Site-Directed Mutagenesis Kit (Stratagene) or by megaprimer method (Barik, 2002). The sequence of the primers used to generate each mutant will be supplied upon request. PCR products were cloned into expression vectorpRK5 (Genentech). All constructs were verified by sequencing.

Antibodies

All antibodies were previously described or were acquired commercially: Bassoon (Stressgen), GluR1-N (pAb, JH1816 [Rumbaugh et al., 2003]), GluR1-C (JH1710 [Ye et al., 2000]), GluR2-C (pAb, JH1707 [Blackstone et al., 1992]) pAb, Arc/Arg3.1 (pAb [Lyford et al., 1995]), Arc/Arg3.1 (mAb, Santa Cruz), β-actin (mAb, Sigma), α-CaMKII (mAb, Boehringer Mannheim), PSD-95 (mAb, Affinity Bioreagents).

Recombinant Sindbis Virus and Infection

Arc/Arg3.1 ORF was first subcloned into pIRES2-EGFP and transferred into pSinRep5 (Invitrogen). At 14–21 DIV, cultured neurons were infected with virus. Experiments were usually performed 12–16 hr after infection.

Preparation of Crude Synaptosomal Fraction from Brain

The subcellular fractionation procedure was performed according to the technique of Huttner et al. (1983). All procedures were performed at 4°C. In brief, rat brains were homogenized in 10 volumes of buffered sucrose (0.32 M sucrose, 4 mM HEPES/NaOH [pH 7.4], 1 mM EDTA, 1 mM EGTA, and protease inhibitors cocktail) with a glass-Teflon homogenizer. The homogenate was centrifuged at 800 × g for 15 min, and the supernatant was collected. The supernatant was again centrifuged at 9000 × g for 15 min and pellet was collected as crude synaptosomal fraction, P2.

Coimmunoprecipitation and Immunoblotting

Six- to eight-week-old Arc/Arg3.1 WT and KO mice were sacrificed by decapitation and forebrain regions dissected. Protein concentration of an aliquot of total homogenate was measured. The rest of the homogenate was used for P2 fractionation as described above, and protein concentration of an aliquot of P2 was measured. Equal amount of total protein from WT and KO was loaded. For coimmunoprecipitation, crude synaptosomal fraction (P2) was sonicated in PBS with 1% Triton X-100, 1mM EDTA, 1 mM EGTA, and protease inhibitors cocktail (Roche). The homogenate was centrifuged at 100,000 × g for 20 min at 4°C, and supernatants with equal amount of protein were incubated with 2 μg of rabbit polyclonal antibodies for GluR1 and GluR2. After 1.5 hr of mixing at 4°C protein A agarose slurry was added and incubated for another hour. The beads were washed with PBS + 1%Triton X-100 three times and eluted with SDS loading buffer. The samples were than analyzed by SDS-PAGE and western blotting.

Biotinylation Assay

For surface biotinylation, infected or drug-treated cortical neurons were cooled on ice, washed twice with ice-cold PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and then incubated with PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mg/ml Sulfo-NHS-SS-Biotin (Pierce) for 30 min at 4°C. Unreacted biotin was quenched by washing cells three times with ice-cold 100 mM Glycine (pH 7.4). Cultures were harvested in RIPA buffer. Homogenates were centrifuged at 132,000 rpm for 20 min at 4°C. The resulting supernatant volume was measured and 15% of it separated as the total protein. The remaining 85% of the homogenate was rotated overnight at 4°C with Streptavidin beads (Pierce). Precipitates were washed with RIPA buffer and analyzed by immunoblotting with each antibody.

Cell Culture and Neuronal Transfection

Low-density hippocampal neurons were prepared as described previously (Banker and Cowan, 1977). High-density cortical cultures from embryonic day 18 (E18) rat pups were prepared as reported previously (Rumbaugh et al., 2003). Mouse cultures were prepared in a similar manner from E16.5–E17.5 mouse pups. Neuronal transfections were performed with Lipofectamine 2000 (Invitrogen) in DIV 12–14 neurons and were analyzed 16–24 hr after initial incubation.

Immunocytochemistry, Microscopy, and Data Analysis

Cells were fixed in 4% paraformaldehyde, 4% sucrose containing PBS solution for 20 min at 4°C and were subsequently permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were then blocked for 1 hr in 10% normal donkey/goat serum (NGS). Primary antibodies were diluted in 10% NGS and incubated with neurons for 1 hr at room temperature or overnight at 4°C. Alexa 488, Alexa 555, or Alexa 647-conjugated secondary antibodies (1:500; Molecular Probes) to the appropriate species were diluted in 10% NDS and incubated at room temperature for 1 hr. Coverslips were mounted on precleaned slides with PermaFluor and DABCO.

To label surface GluR1-containing AMPA receptors, 2.5 μg of GluR1-N JH1816 pAb was added to neuronal growth media and incubated at 10°C for 20 min. The unbound excess antibody was quickly washed with fresh warmed growth medium and then fixed and mounted according to the methods described above.

Glycine stimulation was carried out as described before (Watt et al., 2004). Neurons were incubated at 37°C and 5% CO_2 for 15 min in ACSF containing the following: NaCl, 126 mM; KCl, 5.5 mM; $MgSO_4$, 0.4 mM; NaH_2PO_4, 1 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2 mM; dextrose, 14 mM; glycine, 0.2 mM; bicuculline, 0.01 mM. Then, we replaced the medium in the culture dishes and returned

them to the tissue culture incubator until immunocytochemistry was performed. Immunofluorescence was viewed and captured using a Zeiss LSM 510 confocal laser scanning microscope. Quantification of surface GluR1 puncta were carried out essentially as described (Rumbaugh et al., 2003), using Metamorph imaging software (Universal Imaging). Images were acquired and saved as multichannel TIFF files with a dynamic range of 65,536 gray levels (16-bit binary; Multi-Track acquisition for confocal). To measure punctate structures, neurons were thresholded by gray value at a level close to 50% of the dynamic range. Background noise from these images was negligible. After a dendrite segment was selected, all puncta were treated as individual objects and the characteristics of each, such as pixel area, average fluorescence intensity, and total fluorescence intensity, were logged to a spreadsheet. In addition, each dendrite length was logged in order to calculate puncta density and total intensity per dendritic length (all values shown are per 10 μm of dendrite). Transfected cells were compared with neighboring untransfected cells in individual coverslips. The average single pixel intensity from each region was calculated and averages from all regions were derived. Significance was determined by a paired Student's t test.

Electrophysiology and mEPSC Analysis

Whole-cell patch-clamp recordings were performed from forebrain cultures at the DIV indicated. To isolate AMPAR-mediated mEPSCs, neurons were continuously perfused with artificial cerebral-spinal fluid (aCSF) at a flow rate of 1 ml/min. The composition of aCSF was as follows: 150 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 0.1 mM DL-APV, 0.005 mM strychnine, 0.1 mM picrotoxin, and 0.001 mM tetrodotoxin. The osmolarity of aCSF was adjusted to 305–310 and pH was 7.3–7.4. Intracellular saline consisted of the following: 135 mM Cs-$MeSO_4$, 10 mM CsCl, 10 mM HEPES, 5 mM EGTA, 2 mM $MgCl_2$, 4 mM Na-ATP, and 0.1 mM Na-GTP. This saline was adjusted to 290–295 mOsm, and pH was 7.2.

Transfected neurons were selected based on fluorescent (eGFP) signal. Once the whole-cell recording configuration was achieved, neurons were voltage clamped and passive properties were monitored throughout. In the event of a change in series resistance (Rs) or input resistance (Ri) >15% during the course of a recording, the data were excluded from the set. mEPSCs were acquired through a MultiClamp 700A amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 5 kHz. Sweeps of 20 s with zero latency (essentially "gap free") were acquired until a sufficient number of events were recorded (a minimum of 5 and no longer than 30 min). Data were recorded continuously only after a period of 2 minutes, during which the cell was allowed to stabilize. mEPSCs were detected manually with MiniAnalysis software (Synaptosoft Inc) by setting the amplitude threshold to $\sqrt{}$ RMS × 3 (usually 4 pA). Once a minimum of 100 events had been collected from a neuron, the amplitude, frequency, rise time (time to peak), decay time (10%–90%), and passive properties were measured. In all electrophysiological experiments, a similar amount of data was acquired from both transfected and untransfected neurons on the same day. We have found that recording transfected neurons followed by recording an untransfected neuron in the immediate vicinity yields remarkably consistent results. This is likely a result of reducing errors that arise from slight changes in neuronal density between preps or changes in the density of neurons from different areas of a coverslip. These parameters were crucial for obtaining reliable, low-variability data between experimental populations. Data from each group were then averaged, and statistical significance was determined by Student's t test (unless noted otherwise). All electrophysiological experiments were performed from at least two individual platings of neurons from three different transfections.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/52/3/475/DC1/.

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