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Ras and Rap Control AMPA Receptor Trafficking during Synaptic Plasticity

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Summary

Recent studies show that AMPA receptor (-R) trafficking is important in synaptic plasticity. However, the signaling controlling this trafficking is poorly understood. Small GTPases have diverse neuronal functions and their perturbation is responsible for several mental disorders. Here, we examine the small GTPases Ras and Rap in the postsynaptic signaling underlying synaptic plasticity. We show that Ras relays the NMDA-R and CaMKII signaling that drives synaptic delivery of AMPA-Rs during long-term potentiation. In contrast, Rap mediates NMDA-R-dependent removal of synaptic AMPA-Rs that occurs during long-term depression. Ras and Rap exert their effects on AMPA-Rs that contain different subunit composition. Thus, Ras and Rap, whose activity can be controlled by postsynaptic enzymes, serve as independent regulators for potentiating and depressing central synapses.

Introduction

Synaptic plasticity is believed to underlie key aspects of brain development, learning, and memory. The most widely studied examples of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus. In these models, brief periods of repetitive synaptic activity lead to sustained changes in synaptic transmission. NMDA receptor opening and rise in postsynaptic calcium concentration during repetitive synaptic activity are critical events to these forms of plasticity. A number of studies indicate that these inducing stimuli lead to regulated trafficking of postsynaptic AMPA-sensitive glutamate receptors (AMPA-Rs) into (for LTP) and out of (for LTD) excitatory synapses (Luscher et al., 2000; Malinow et al., 2000; Scannevin and Huganir, 2000; Sheng and Lee, 2001). However, the biochemical pathways linking NMDA receptor activity with receptor trafficking are poorly understood.

Recent studies have identified Ras and Rap, members of the Ras superfamily of small GTPases, as well as their activators (GEFs) and inactivators (GAPs) at synapses (Chen et al., 1998; Husi et al., 2000; Kim et al., 1998; Pak et al., 2001; Ye et al., 2000). These small GTPases function as molecular switches that cycle between a GDP bound inactive and a GTP bound active state that can trigger distinct cellular responses (reviewed in Takai et al., 2001; Zwartkruis and Bos, 1999). In several nonneuronal contexts, active Ras is proneoplastic while active Rap antagonizes this effect. The mechanism of antogonism, whether it is due to mutual inhibition of the signaling pathways or their end targets, is still in debate (reviewed in Bos et al., 2001). In the nervous system, members of the Ras superfamily have been shown to control important neuronal functions (Dolmetsch et al., 2001; Pak et al., 2001; Wu et al., 2001; Ye et al., 2000) and have significant effects on behavior (Brambilla et al., 1997; Costa et al., 2002). Moreover, several diseases causing cognitive impairment (e.g., autism, neurofibromatosis, and X-linked mental retardation) are associated with mutations in Ras superfamily members or enzymes controlling their activity (Antonarakis and Van Aelst, 1998; Chelly and Mandel, 2001; Comings et al., 1996). However, it is not known if or how these signaling molecules control synaptic function.

Fast excitatory synaptic transmission is mediated by AMPA-Rs, multimeric proteins composed of the subunits GluR1 to GluR4 (GluRA to GluRD; Hollmann and Heinemann, 1994; Seeburg, 1993). The cytoplasmic carboxyl tails of the constituent subunits, which show either long or short forms (Kohler et al., 1994), controls the trafficking characteristics of AMPA-Rs (Passafaro et al., 2001; Shi et al., 2001). AMPA-Rs with long cytoplasmic tails (e.g., GluR1 or GluR4) are restricted from synapses and delivered to synapses during activity-induced synaptic enhancement (Hayashi et al., 2000; Zhu et al., 2000). AMPA-Rs with only short cytoplasmic tails (e.g., GluR2 or GluR3) cycle continuously from nonsynaptic to synaptic sites in an activity independent manner; their number at synapses can be reduced after activity-induced synaptic depression (Kim et al., 2001; Lin et al., 2000; Luscher et al., 1999; Luthi et al., 1999; Shi et al., 2001; Snyder et al., 2001).

In this study, we have investigated the role of Ras and Rap in activity-dependent synaptic plasticity. Since the structural basis of small GTPase function is well characterized (Boriack-Sjodin et al., 1998; Feig, 1999; Scheffzek et al., 1997; White et al., 1995), we used several mutants that permit dissection of the role of Ras and Rap molecules in signaling pathways. We found that Ras and Rap signal two independent pathways at synapses. Ras mediates activity-induced synaptic enhancement by driving synaptic delivery of AMPA-Rs containing long cytoplasmic tails requiring p42/44 MAPK activation. In contrast, Rap mediates activity-induced synaptic depression by removing synaptic AMPA-Rs containing short cytoplasmic tails requiring p38 MAPK activation. It is notable that Ras and Rap can have opposite effects in carcinogenesis as well as synaptic plasticity.

Results

Ras Enhances while Rap Depresses AMPA-R-Mediated Synaptic Transmission

To determine if Ras and Rap are present in hippocampal tissue, we prepared extracts from whole hippocampus

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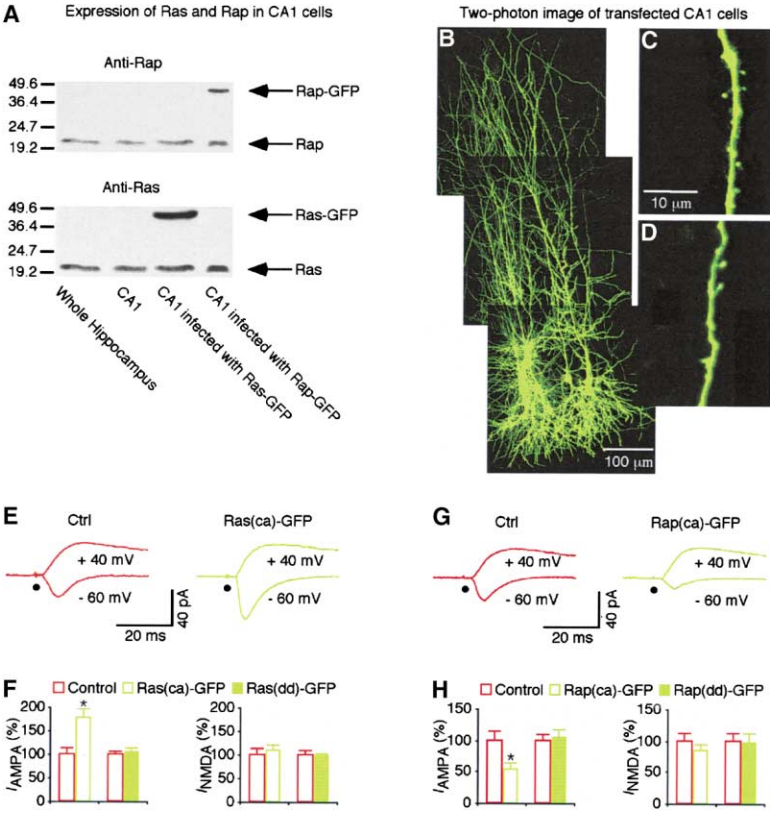

Figure 1. Expression of Endogenous and Recombinant Rap and Ras in Hippocampus

(A) Expression of endogenous and recombinant Rap and Ras proteins in whole hippocampus ($n = 8$), hippocampal CA1 region ($n = 8$), hippocampal CA1 region infected with Ras-GFP ($n = 7$), and hippocampal CA1 region infected with Rap-GFP ($n = 7$).

(B–D) Two-photon images of GFP fluorescence indicate that Rap-GFP (B–C) and Ras-GFP (D) proteins are present in the dendrites and spines of CA1 pyramidal neurons.

(E) Evoked AMPA-R- (−60 mV) and NMDA-R- (+40 mV) mediated responses recorded from uninfected (Ctrl) and Ras(ca)-GFP infected cells.

(F) Left, AMPA responses in cells expressing Ras(ca)-GFP ($n = 18$; $p < 0.005$) and Ras(dd)-GFP ($n = 22$; $p = 0.95$) relative to uninfected neighboring control cells. Right, NMDA responses in cells expressing Ras(ca)-GFP ($n = 18$; $p = 0.27$) and Ras(dd)-GFP ($n = 22$; $p = 0.60$) relative to uninfected neighboring control cells.

(G) Evoked AMPA-R- (−60 mV) and NMDA-R- (+40 mV) mediated responses recorded from uninfected (Ctrl) and Rap(ca)-GFP infected cells.

(H) Left, AMPA responses in cells expressing Rap(ca)-GFP ($n = 20$; $p < 0.005$) and Rap(dd)-GFP ($n = 20$; $p = 0.91$) relative to uninfected neighboring control cells. Right, NMDA responses in cells expressing Rap(ca)-GFP ($n = 20$; $p = 0.12$) and Rap(dd)-GFP ($n = 20$; $p = 0.81$) relative to uninfected neighboring control cells. AMPA-R- and NMDA-R-mediated current amplitude and standard errors were normalized to average values from control cells. See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values. Asterisk indicates $p < 0.05$ (same in following figures).

and also a microdissected CA1 region of hippocampus (see Experimental Procedures). Western blots on these extracts show bands corresponding to Ras and Rap indicating that these proteins are present in hippocampal CA1 cells (see also Kim et al., 1990). To examine possible postsynaptic functions of these molecules, we acutely overexpressed Ras, Rap, or their mutants (see Experimental Procedures) in hippocampal CA1 pyramidal neurons. These constructs were tagged with GFP, allowing unambiguous identification of expressing cells. About ~15 hr after infection, Ras-GFP and Rap-GFP could be detected by Western blots (Figure 1). In neurons, these proteins showed a homogeneous expression pattern, including expression in dendritic spines, sites of excitatory synapses (Figures 1B–1D). To determine whether Ras and Rap affect synaptic function, electrophysiological recordings were obtained simultaneously from nearby expressing and nonexpressing neurons. Afferent fibers were stimulated and excitatory postsynaptic currents (epscs) were recorded. Neurons expressing a constitutively active form of Ras, Ras(ca)-GFP, showed ~80% potentiated transmission mediated by AMPA type glutamate receptors (AMPA-Rs), but no significant change in transmission mediated by NMDA-Rs (Figures 1E–1F). As a control, neurons expressing Ras(dd), an inactive form of Ras mutant, showed no change in transmission (Figure 1F). In contrast to Ras, neurons expressing a constitutively active form of Rap, Rap(ca)-GFP, showed ~50% depressed AMPA-R-mediated transmission, and no significant change in NMDA-R-mediated transmission (Figures 1G–1H). Again, neurons expressing Rap(dd)-GFP, an inactive mutant form of Rap, showed no change in transmission (Figure 1H). The results indicate that Ras and Rap activities selectively up- and down-modulate, respectively, AMPA-R-mediated synaptic transmission.

Ras Mediates Activity-Driven Synaptic Addition of AMPA Receptors

To determine if endogenous Ras activity potentiates AMPA-R-mediated transmission, we expressed a dominant-negative form of Ras, Ras(dn)-GFP. Neurons expressing Ras(dn)-GFP depressed AMPA synaptic transmission by ~35% (Figure 2A). Furthermore, neurons expressing the wild-type form of Ras, Ras(wt)-GFP, enhanced AMPA-R-mediated transmission by ~35% (Figure 2B). These results support the view that endogenous Ras activity contributes to a tonic potentiation of transmission. The effects of Ras(dn)-GFP or Ras(wt)-GFP on AMPA synaptic transmission were blocked if APV, an NMDA-R antagonist, or high concentration of $MgCl_2$, which depresses neural activity (Zhu et al., 2000), was included in culture and recording media (Figure 2C). These results indicate that spontaneous synaptic activity activates NMDA-Rs that in turn activate endogenous or recombinant Ras producing a tonic potentiation of transmission. As a control, we found that the potentiation by Ras(ca)-GFP of AMPA-R-mediated transmission

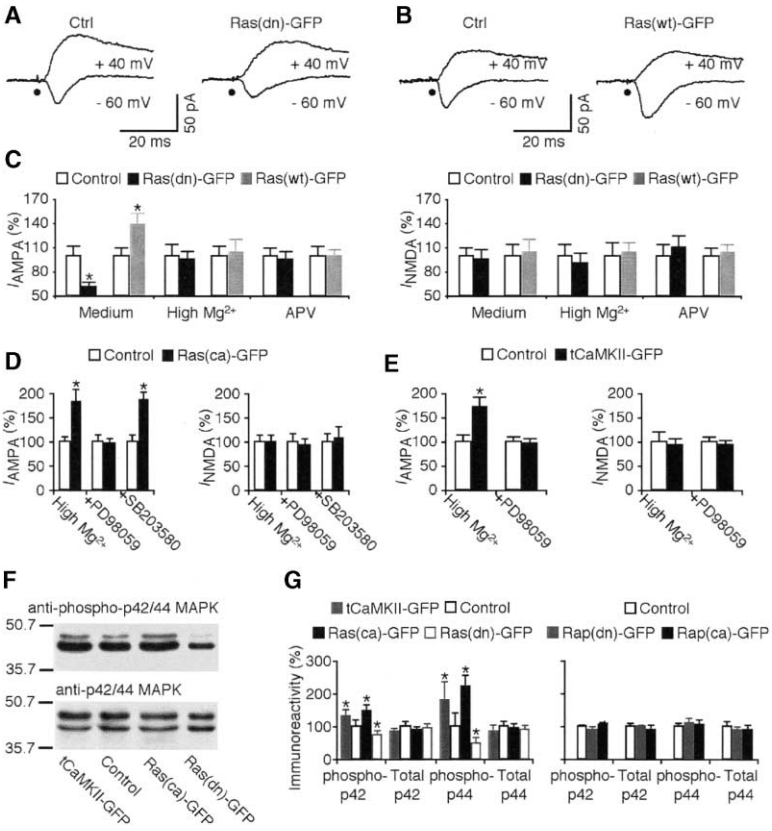

Figure 2. Activation of Endogenous Ras Signaling Pathway by Spontaneous Activity and CaMKII

(A) Evoked AMPA-R- and NMDA-R-mediated responses recorded from uninfected (Ctrl) and infected Ras(dn)-GFP cells.

(B) Evoked AMPA-R- and NMDA-R-mediated responses recorded from uninfected (Ctrl) and Ras(wt)-GFP infected cells.

(C) Left, AMPA responses in cells expressing Ras(dn)-GFP and Ras(wt)-GFP relative to uninfected neighboring control cells. Slices were maintained in normal medium ($n = 20$; $p < 0.005$ for Ras(dn)-GFP; $n = 18$; $p < 0.005$ for Ras(wt)-GFP) or with 12 mM Mg^{2+} ($n = 20$; $p = 0.99$ for Ras(dn)-GFP; $n = 20$; $p = 0.77$ for Ras(wt)-GFP), or 100 μM DL-APV ($n = 20$; $p = 0.55$ for Ras(dn)-GFP; $n = 20$; $p = 0.82$ for Ras(wt)-GFP). Right, NMDA responses in cells expressing Ras(dn)-GFP and Ras(wt)-GFP relative to uninfected neighboring control cells. Slices were maintained in normal medium ($n = 20$; $p = 0.33$ for Ras(dn)-GFP; $n = 18$; $p = 0.91$ for Ras(wt)-GFP) or with 12 mM Mg^{2+} ($n = 20$; $p = 0.55$ for Ras(dn)-GFP; $n = 20$; $p = 0.97$ for Ras(wt)-GFP), or 100 μM DL-APV ($n = 20$; $p = 0.33$ for Rap(dn)-GFP; $n = 20$; $p = 0.74$ for Ras(wt)-GFP).

(D) Left, AMPA responses in cells expressing Ras(ca)-GFP relative to uninfected neighboring control cells. Slices were maintained in high Mg^{2+} (12 mM) medium ($n = 20$; $p < 0.05$), 25 μM PD98059 ($n = 19$; $p = 0.49$), and 2 μM SB203580 ($n = 14$; $p < 0.005$). Right, NMDA responses in cells expressing Ras(ca)-GFP relative to uninfected neighboring control cells. Slices were maintained in high Mg^{2+} (12 mM) medium ($n = 20$; $p = 0.88$), 25 μM PD98059 ($n = 19$; $p = 0.40$), and 2 μM SB203580 ($n = 14$; $p = 0.68$).

(E) Left, AMPA responses in cells expressing tCaMKII-GFP relative to uninfected neighboring control cells. Slices were maintained in high Mg^{2+} (12 mM) medium ($n = 20$; $p < 0.01$), 25 μM PD98059 ($n = 22$; $p = 0.66$). Right, NMDA responses in cells expressing tCaMKII-GFP relative to uninfected neighboring control cells. Slices were maintained in high Mg^{2+} (12 mM) medium ($n = 20$; $p = 0.60$), 25 μM PD98059 ($n = 22$; $p = 0.36$). AMPA-R and NMDA-R mediated current amplitude and standard errors were normalized to average values from control cells for (C–E).

(F) Western blots of phospho-p42/44 MAPK in control hippocampal CA1 region, hippocampal CA1 region infected with Ras(ca)-GFP, hippocampal CA1 region infected with Ras(dn)-GFP, and hippocampal CA1 region infected with tCaMKII-GFP. Each lane was loaded with the same amount of protein (15 μg).

(G) Left, relative amounts of phospho-p42/44 MAPK in hippocampal CA1 region infected with tCaMKII-GFP ($n = 8$; $p < 0.05$ for phospho-p42 MAPK; $n = 8$; $p < 0.05$ for phospho-p44 MAPK), Ras(ca)-GFP ($n = 8$; $p < 0.05$ for phospho-p42 MAPK; $n = 8$; $p < 0.05$ for phospho-p44 MAPK), or Ras(dn)-GFP ($n = 8$; $p < 0.05$ for phospho-p42 MAPK; $n = 8$; $p < 0.05$ for phospho-p44 MAPK). Relative amounts of total p42/44 MAPK in hippocampal CA1 region infected with tCaMKII-GFP ($n = 6$; $p = 0.92$ for phospho-p42 MAPK; $n = 6$; $p = 0.12$ for phospho-p44 MAPK), Ras(ca)-GFP ($n = 6$; $p = 0.46$ for phospho-p42 MAPK; $n = 6$; $p = 0.46$ for phospho-p44 MAPK), or Ras(dn)-GFP ($n = 6$; $p = 0.60$ for phospho-p42 MAPK; $n = 6$; $p = 0.92$ for phospho-p44 MAPK). Right, relative amounts of phospho-p42/44 MAPK in hippocampal CA1 region infected with Rap(dn)-GFP ($n = 8$; $p = 0.16$ for phospho-p42 MAPK; $n = 8$; $p = 0.67$ for phospho-p44 MAPK) or Rap(ca)-GFP ($n = 8$; $p = 0.78$ for phospho-p42 MAPK; $n = 8$; $p = 0.48$ for phospho-p44 MAPK). Relative amounts of total p42/44 MAPK in hippocampal CA1 region infected with Rap(dn)-GFP ($n = 8$; $p = 0.67$ for phospho-p42 MAPK; $n = 8$; $p = 0.21$ for phospho-p44 MAPK) or Rap(ca)-GFP ($n = 8$; $p = 0.40$ for phospho-p42 MAPK; $n = 6$; $p = 0.16$ for phospho-p44 MAPK). The relative values and standard errors were normalized to average amounts of phospho-p42/44 MAPK or total p42/44 MAPK from control hippocampal CA1 region. See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values.

was not blocked by high concentration of $MgCl_2$, or 2 μM SB203580, which inhibits p38 MAPK (Lee et al., 1999) and blocks Rap-activity induced depression and LTD (see below). The potentiation by Ras(ca)-GFP of AMPA-R-mediated transmission was blocked by PD98059 which inhibits MEK, the p42/44 MAPK activating enzyme (Dudley et al., 1995) and a downstream mediator of Ras signaling (Van Aelst et al., 1993; Figure 2D). To examine if Ras activates p42/44 MAPK, we made extracts from slices expressing Ras(ca)-GFP or Ras(dn)-GFP. Western blot analysis indicates that the phosphorylated (active) form of p42/44 MAPK is enhanced or decreased in tissue expressing Ras(ca)-GFP or Ras(dn)-GFP, respectively (Figures 2F–2G). As a control, we found that the phosphorylated form of p42/44 MAPK was not changed in tissue expressing Rap(ca)-GFP or Rap(dn)-GFP (Figure 2G). These results indicate that Ras, acting through the p42/44 MAPK signaling pathway, enhances AMPA-R-mediated synaptic transmission.

Previous studies have shown that calcium/calmodulin-dependent protein kinase II (CaMKII) can enhance AMPA-R-mediated transmission by delivering GluR1 receptors to synapses. We wished to know whether this effect is mediated by Ras signaling pathway. We tested

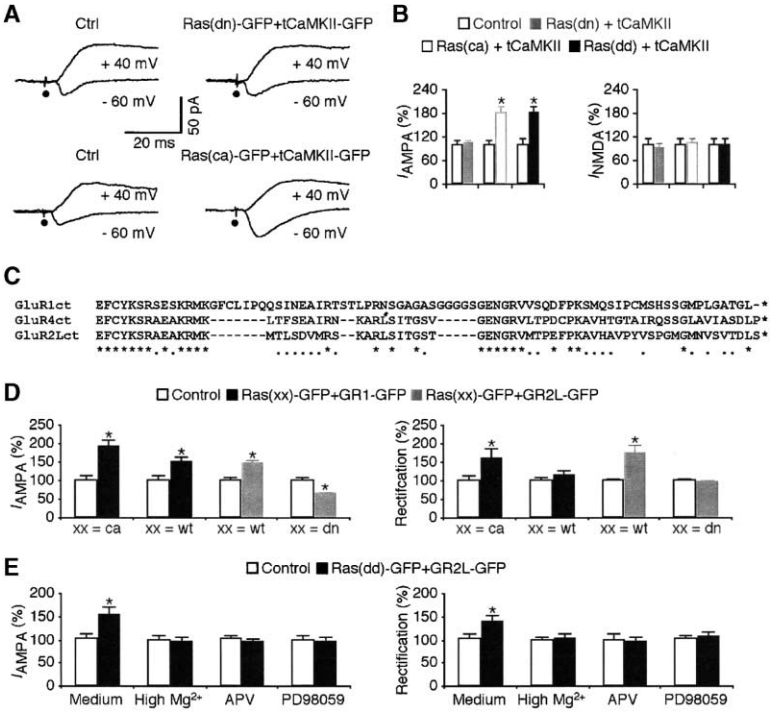

Figure 3. Relay of CaMKII Signaling and Delivery of GluR1 and GluR2L Receptors by Ras Activity

(A) Evoked AMPA-R- (−60 mV) and NMDA-R- (+40 mV) mediated responses recorded from untransfected (Ctrl) and cotransfected Ras(dn)-GFP and tCaMKII-GFP cells, and from untransfected (Ctrl) and cotransfected Ras(ca)-GFP and tCaMKII-GFP cells. Ras(dn)-GFP and tCaMKII-GFP or Ras(ca)-GFP and tCaMKII-GFP were coexpressed in cultured slices maintained in high Mg^{2+} culture medium for ~15 hr.

(B) Left, AMPA responses in cells coexpressing Ras(dn)-GFP and tCaMKII-GFP ($n = 22$; $p = 0.73$), cells coexpressing Ras(ca)-GFP and tCaMKII-GFP ($n = 20$; $p < 0.005$), or cells coexpressing Ras(dd)-GFP and tCaMKII-GFP ($n = 16$; $p < 0.005$). Right, NMDA responses in cells coexpressing Ras(dn)-GFP and tCaMKII-GFP ($n = 22$; $p = 0.83$), or cells coexpressing Ras(ca)-GFP and tCaMKII-GFP ($n = 20$; $p = 0.72$), or cells coexpressing Ras(dd)-GFP and tCaMKII-GFP ($n = 16$; $p = 0.92$). AMPA-R and NMDA-R mediated current amplitude and standard errors were normalized to average values from control cells.

(C) Alignments of the cytoplasmic carboxyl termini of GluR1, GluR4, and GluR2L. Identical and homologous residues of these three AMPA-R subunits are indicated by * and • respectively. Note that GluR2L shares more homologous sequence with GluR4 than with GluR1.

(D) Left, AMPA responses in cells coexpressing Ras(ca)-GFP and GluR1-GFP ($n = 16$; $p < 0.001$), or cells coexpressing Ras(wt)-GFP and GluR1-GFP ($n = 20$; $p < 0.005$), or cells coexpressing Ras(wt)-GFP and GluR2L-GFP ($n = 16$; $p < 0.005$), or cells coexpressing Ras(dn)-GFP and GluR2L-GFP ($n = 14$; $p < 0.01$) relative to untransfected neighboring control cells. Right, rectification of the cells coexpressing Ras(ca)-GFP and GluR1-GFP ($n = 16$; $p < 0.005$), or cells coexpressing Ras(wt)-GFP and GluR1-GFP ($n = 20$; $p = 0.11$), or cells coexpressing Ras(wt)-GFP and GluR2L-GFP ($n = 16$; $p < 0.005$), or cells coexpressing Ras(dn)-GFP and GluR2L-GFP ($n = 14$; $p = 0.59$).

(E) Left, AMPA responses in cells coexpressing Ras(dd)-GFP and GluR2L-GFP relative to untransfected neighboring control cells. Slices were maintained in normal medium ($n = 14$; $p < 0.05$), or with 12 mM Mg^{2+} ($n = 16$; $p = 0.64$), or 100 μM DL-APV ($n = 14$; $p = 0.59$), or 25 μM PD98059 ($n = 16$; $p = 0.61$). Right, rectification of the same cells. Slices were maintained in normal medium ($n = 14$; $p < 0.005$), or with 12 mM Mg^{2+} ($n = 16$; $p = 0.88$), or 100 μM DL-APV ($n = 14$; $p = 0.93$), or 25 μM PD98059 ($n = 16$; $p = 0.68$). AMPA-R mediated current amplitude, rectification, and standard errors were normalized to average values from control cells. See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values.

this by manipulating the Ras signaling pathway in neurons expressing a truncated, constitutively active form of CaMKII (Hayashi et al., 2000), tCaMKII-GFP. Neurons expressing tCaMKII-GFP showed ~80% increase in AMPA-R-mediated transmission, which was blocked by an inhibitor of MEK, PD98059 (Figure 2E). Furthermore, neurons cotransfected with Ras(dn)-GFP and tCaMKII-GFP showed no significant potentiation, indicating that dominant-negative Ras blocked the synaptic potentiation induced by CaMKII (Figure 3A; note that for this experiment, slices were maintained in high Mg^{2+} medium to prevent the activity-dependent synaptic depression induced by expression of Ras(dn)-GFP). Moreover, neurons cotransfected with Ras(ca)-GFP and tCaMKII-GFP showed ~80% potentiation in AMPA-R-mediated transmission (Figure 3A), similar to neurons cotransfected with the inactive Ras(dd)-GFP and tCaMKII-GFP (Figure 3B). With Western blot analysis, we detected increased phosphorylated p42/44 MAPK in slices expressing tCaMKII-GFP (Figures 2F–2G). These results indicate that Ras activity is increased by CaMKII activity and that Ras activity is necessary and sufficient to relay the CaMKII signaling that produces synaptic potentiation.

To examine if AMPA receptors are driven into synapses by Ras we used electrophysiologically tagged receptors. When acutely expressed, AMPA-R subunits form receptors that show rectification and display distinct trafficking properties (Hayashi et al., 2000; Shi et al., 2001; Zhu et al., 2000). Receptors containing GluR1-GFP are not normally delivered to synapses by spontaneous activity, but are delivered to synapses by CaMKII activity or LTP (Hayashi et al., 2000). We cotransfected Ras(ca)-GFP and GluR1-GFP, and noted an increased rectification, indicating delivery of recombinant GluR1-containing receptors to synapses (Figure 3D). Thus, Ras(ca)-GFP mimics tCaMKII in driving GluR1 into synapses.

As noted above, the dominant-negative Ras blocked a tonic potentiation of transmission driven by spontaneous activity. Since spontaneous activity does not drive GluR1 into synapses (Hayashi et al., 2000; Shi et al., 2001), we asked if some other AMPA-R is driven into synapses by spontaneous activity. GluR4 is similar in sequence to GluR1 at the critical cytoplasmic terminus and is driven by spontaneous activity in immature tissue (Zhu et al., 2000). However, GluR4 shows very little expression and it is not delivered to synapses at this age in hippocampus (Zhu et al., 2000), and thus seems an unlikely candidate. A previous study detected an alter-

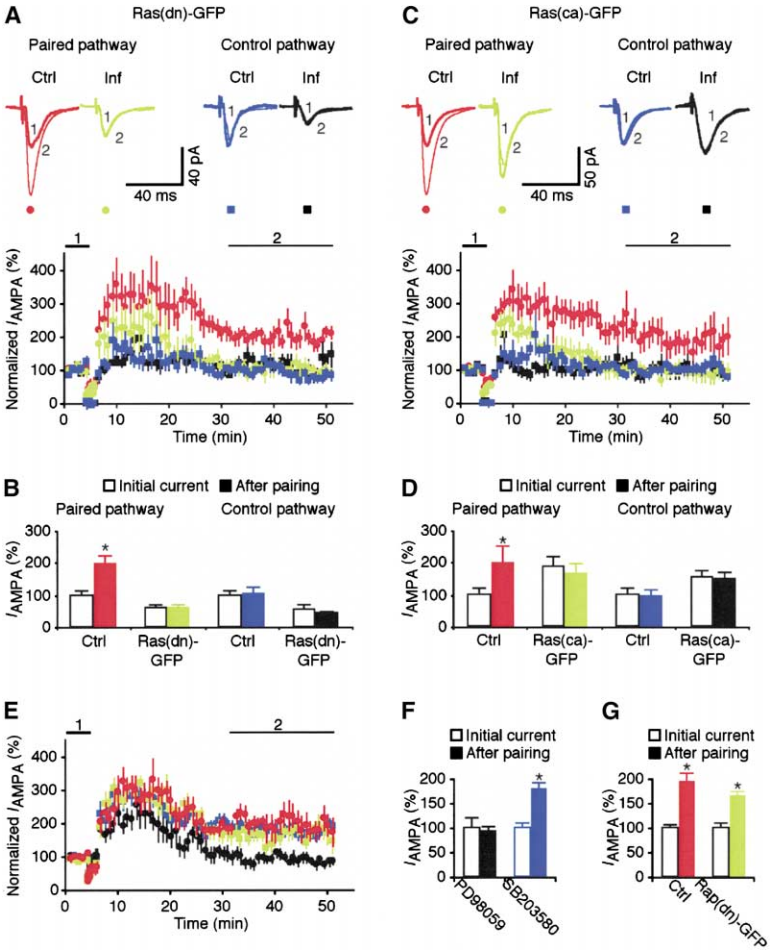

Figure 4. Signaling Long-Term Potentiation by Ras Activity

(A) Average AMPA-R-mediated synaptic responses obtained before (−60 mV, thick trace) and after (−60 mV, thin trace) pairing from a pair of infected (Ras(dn)-GFP) and uninfected cells in paired (upper left) and control pathway (upper right). Lower plot, normalized simultaneously evoked responses recorded from cells expressing or not expressing Ras(dn)-GFP against the time.

(B) Steady state synaptic AMPA-R-mediated response amplitudes in paired ($n = 9$; $p < 0.05$) and control pathways ($n = 9$; $p = 0.40$) in cells expressing Ras(dn)-GFP and uninfected neighboring control cells before and after pairing.

(C) Average AMPA-R-mediated synaptic responses obtained before (−60 mV, thick trace) and after (−60 mV, thin trace) pairing from a pair of infected (Ras(ca)-GFP) and uninfected cells in paired (upper left) and control pathway (upper right). Lower plot, normalized simultaneously evoked responses recorded from cells expressing or not expressing Ras(ca)-GFP against the time.

(D) Steady-state synaptic AMPA-R-mediated response amplitudes in paired ($n = 8$; $p < 0.05$) and control pathways ($n = 8$; $p = 0.78$) in cells expressing Ras(ca)-GFP and uninfected neighboring control cells before and after pairing.

(E) Normalized responses from cells recorded with either PD98059 or SB203580 included in the bath solution and from pairs of Rap(dn)-GFP expressing and nonexpressing cells.

(F) Steady-state synaptic AMPA-R-mediated response (PD98059: $n = 10$; SB203580: $n = 12$; t test; $p < 0.001$).

(G) Steady-state synaptic AMPA-R-mediated response amplitudes in cells expressing Rap(dn)-GFP and uninfected neighboring control cells before and after pairing ($n = 10$; $p = 0.20$). AMPA-R mediated current amplitude and standard errors were normalized to average values from control cells in (B) and (D). See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values.

natively spliced transcript of GluR2 (GluR2L) in the rat brain (Kohler et al., 1994), which codes for an AMPA-R subunit with a long cytoplasmic tail like that of GluR1 and GluR4. The higher homology to GluR4 at the cytoplasmic terminus suggests that it may be responsible for spontaneous Ras activity-mediated potentiation (Figure 3C). We thus coexpressed various Ras mutants with GluR2L-GFP. Neurons expressing Ras(wt)-GFP with GluR2L-GFP or Ras(dd)-GFP with GluR2L-GFP showed increased rectification, indicating synaptic delivery of recombinant GluR2L receptors (Figures 3D and 3E). This increased rectification was blocked if slices were maintained in elevated concentration of $MgCl_2$, APV or PD98059 (Figure 3E). Furthermore, neurons expressing Ras(dn)-GFP with GluR2L-GFP showed no change in rectification (Figure 3D). These results indicate that spontaneous synaptic activity can deliver GluR2L receptors to synapses via activation of the Ras pathway. Stronger or longer activation of the Ras pathway, as by Ras(ca)-GFP expression, is required to deliver GluR1 receptors to synapses.

To test whether Ras mediates the signaling that induces long-term potentiation (LTP), we studied pairing-induced LTP in neurons infected by either Ras(dn)-GFP or Ras(ca)-GFP. Transmission onto neurons expressing Ras(dn)-GFP was initially depressed compared to nearby control neurons (Figure 4A) and pairing stimuli produced no persistent potentiation. As controls, nearby nonexpressing neurons did show pathway specific LTP (Figures 4A–4B). This indicates that activation of Ras signaling pathway is necessary for producing LTP. Above, we showed that Ras signaling potentiates transmission and mimics LTP by driving GluR1 to synapses. To examine whether Ras activity occludes LTP, we also examined LTP in neurons expressing Ras(ca)-GFP. Transmission onto neurons expressing Ras(ca)-GFP was initially larger compared to nearby control neurons (Figure 4C). Again, pairing stimuli produced no persistent potentiation in these infected neurons but did induce pathway specific LTP in nearby noninfected neurons (Figures 4C–4D). As a control, we infected neurons with the inactive Ras(dd)-GFP. Pairing stimuli induced the same amount of LTP in neurons infected with Ras(dd)-GFP and in nearby noninfected neurons (ctrl: 193.5 ± 23.2% from initial −26.5 ± 4.1 pA; inf: 183.7 ± 24.7% from initial –24.2 ± 4.6 pA; $n = 10$; $p = 0.58$).

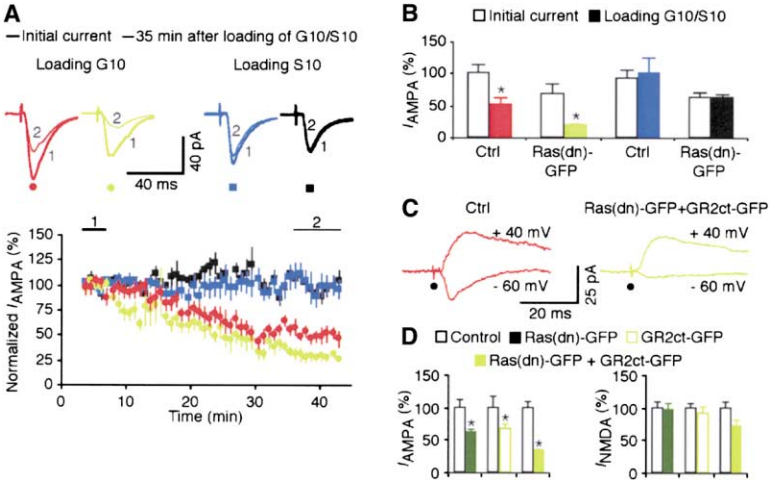

Figure 5. Ras Does Not Control GluR2-Dependent Trafficking

(A) Upper left, average AMPA-R-mediated synaptic responses obtained immediately (−60 mV, thick trace) and 35 min after whole-cell infusion of pep2m/G10 (−60 mV, thin trace) from a pair of infected (Ras(dn)-GFP) and uninfected cells. Upper right, average AMPA-R-mediated synaptic responses obtained immediately (−60 mV, thick trace) and 35 min after whole-cell infusion of S10 (−60 mV, thin trace) from a nearby pair of infected (Ras(dn)-GFP) and uninfected cells in the same slice. Lower plot, normalized simultaneously evoked responses recorded from cells expressing or not expressing Ras(dn)-GFP against the time after forming whole-cell configuration, loaded with either pep2m/G10 or S10.

(B) Residual synaptic MPA-R-mediated response amplitudes (at −60 mV) in cells expressing Ras(dn)-GFP and uninfected neighboring control cells after loading pep2m/G10 ($n = 10$; $p < 0.01$) or S10 ($n = 10$; $p = 0.80$) for 35 min. AMPA-R mediated current amplitude and standard errors were normalized to average value from control cells loading with pep2m/G10.

(C) Evoked AMPA-R- (−60 mV) and NMDA-R- (+40 mV) mediated responses recorded from untransfected (Ctrl) and cotransfected Ras(dn)-GFP and GluR2ct-GFP cells.

(D) Left, AMPA responses in cells expressing Ras(dn)-GFP alone (see Figure 1E), cells expressing GluR2ct-GFP alone ($n = 16$; $p < 0.05$) or cells coexpressing Ras(dn)-GFP and GluR2ct-GFP ($n = 14$; $p < 0.005$) relative to uninfected neighboring control cells. Right, NMDA responses in cells expressing Ras(dn)-GFP alone, cells expressing GluR2ct-GFP alone ($n = 16$; $p = 0.96$) or cells coexpressing Ras(dn)-GFP and GluR2ct-GFP ($n = 14$; $p = 0.06$) relative to uninfected neighboring control cells. AMPA-R and NMDA-R mediated current amplitude and standard errors were normalized to average values from control cells. See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values.

As an additional control, we examined LTP in neurons expressing Rap(dn)-GFP. LTP-inducing stimuli induced a prominent potentiation in these cells (Figure 4E; $n = 10$; $p < 0.01$). Furthermore, we confirmed that, as with tetanus-induced LTP, our pairing-induced LTP was blocked by a MEK inhibitor and not blocked by the p38 MAPK inhibitor (Figures 4E–4F). These results indicate that Ras activity is both necessary and sufficient to produce LTP. In addition to the sustained potentiation, the pairing protocol also induced a transient potentiation in both control neurons and neurons expressing Ras(dn)-GFP or Ras(ca)-GFP. This transient potentiation is unlikely dependent on Ras activity or GluR1 trafficking (Shi et al., 2001) and may be due to phosphorylation-dependent modulation of channel conductance of synaptic AMPA receptors (Benke et al., 1998; Derkach et al., 1999) and/or other pre- or postsynaptic mechanisms.

Previous results indicate that LTP drives into synapses AMPA receptors that do not participate in a rapidly recycling pool of GluR2-containing receptors (Hayashi et al., 2000). This rapidly recycling pool can be probed with the peptide pep2m/G10, which interrupts a GluR2-NSF interaction, reduces synaptic delivery of receptors, and depresses transmission (Luscher et al., 1999; Luthi et al., 1999; Nishimune et al., 1998; Noel et al., 1999; Osten et al., 1998; Song et al., 1998). We examined the effects of pep2m/G10 on transmission in neurons expressing GFP-Ras(dn). Transmission onto neurons expressing Ras(dn)-GFP was initially depressed by ~35% compared to nearby control neurons (Figure 5A). Loading pep2m/G10 depressed transmission in both infected neurons and nearby noninfected neurons over the course of 30–40 min. The depression was significantly more in infected neurons (~70%) than in nearby noninfected neurons (~50%; Figure 5B). This supports the notion that Ras(dn)-GFP blocks delivery of receptors that do not incorporate into the rapid cycling pool that is blocked by pep2m/G10. Moreover, coexpression of GFP-Ras(dn) with the GluR2 carboxyl terminus (GFP-GluR2ct, which blocks rapid cycling, Shi et al., 2001) showed additive effects (Figures 5C–5D). These results indicate that the receptors delivered to synapses by Ras activity do not participate in the cycling pool of GluR2-containing AMPA receptors.

Rap Mediates Activity-Dependent Removal of Synaptic AMPA Receptors

As shown above (Figure 1), overexpression of Rap depresses AMPA-R-mediated transmission. To test if endogenous Rap plays a role, we expressed a dominant-negative form of Rap, Rap(dn)-GFP. Neurons expressing Rap(dn)-GFP show potentiated AMPA synaptic transmission (Figure 6A). Furthermore, neurons expressing the wild-type form of Rap, Rap(wt)-GFP, showed depressed AMPA-R-mediated transmission (Figure 6B). These results support the view that endogenous Rap activity contributes to a tonic depression of transmission. The effects of Rap(dn)-GFP or Rap(wt)-GFP on AMPA synaptic transmission were blocked when APV or high concentration of $MgCl_2$ was included in culture media (Figure 6C). These results indicate that spontaneous synaptic activity activates NMDA-Rs that in turn activate endogenous or recombinant Rap producing a tonic depression of transmission. As a control, we found that the depression of Rap(ca)-GFP on AMPA-R-mediated transmission was not blocked by high concentration of $MgCl_2$.

The downstream molecules that relay Rap signaling are less clear. A recent report suggests that Rap signaling may be relayed by p38 MAPK (Sawada et al., 2001),

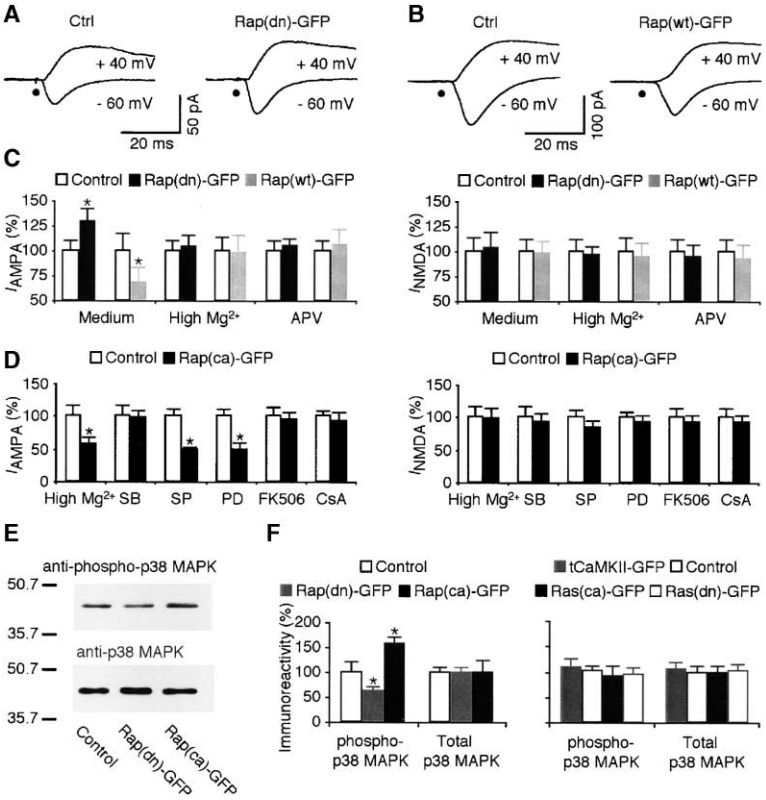

Figure 6. Activation of Endogenous Rap Signaling Pathway by Spontaneous Activity.

(A) Evoked AMPA-R- and NMDA-R-mediated responses recorded from uninfected (Ctrl) and infected Rap(dn)-GFP cells.

(B) Evoked AMPA-R- and NMDA-R-mediated responses recorded from uninfected (Ctrl) and Rap(wt)-GFP infected cells.

(C) Left, AMPA responses in cells expressing Rap(dn)-GFP and Rap(wt)-GFP relative to uninfected neighboring control cells. Slices were maintained in normal medium ($n = 20$; $p < 0.01$ for Rap(dn)-GFP; $n = 14$; $p < 0.005$ for Rap(wt)-GFP) or with 12 mM Mg^{2+} ($n = 22$; $p = 0.78$ for Rap(dn)-GFP; $n = 20$; $p = 0.77$ for Rap(wt)-GFP), or 100 μM DL-APV ($n = 19$; $p = 0.69$ for Rap(dn)-GFP; $n = 20$; $p = 0.71$ for Rap(wt)-GFP). Right, NMDA responses in cells expressing Rap(dn)-GFP and Rap(wt)-GFP relative to uninfected neighboring control cells. Slices were maintained in normal medium ($n = 20$; $p = 0.46$ for Rap(dn)-GFP; $n = 14$; $p = 0.73$ for Rap(wt)-GFP) or with 12 mM Mg^{2+} ($n = 22$; $p = 0.86$ for Rap(dn)-GFP; $n = 20$; $p = 0.68$ for Rap(wt)-GFP), or 100 μM DL-APV ($n = 19$; $p = 0.36$ for Rap(dn)-GFP; $n = 20$; $p = 0.65$ for Rap(wt)-GFP).

(D) Left, AMPA responses in cells expressing Rap(ca)-GFP relative to uninfected neighboring control cells. Slices were maintained in high Mg^{2+} (12 mM) medium ($n = 14$; $p < 0.005$), 2 μM SB203580 ($n = 14$; $p = 0.93$), 5 μM SP600125 ($n = 14$; $p < 0.005$), 25 μM PD98059 ($n = 14$; $p < 0.005$), 50 μM FK-506 ($n = 14$; $p = 0.73$), or 250 μM CsA ($n = 16$; $p = 0.64$). Right, NMDA responses in cells expressing Rap(ca)-GFP relative to uninfected neighboring control cells. Slices were maintained in high Mg^{2+} (12 mM) medium ($n = 14$; $p = 0.83$), 2 μM SB203580 ($n = 14$; $p = 0.55$), 5 μM SP600125 ($n = 14$; $p = 0.12$), PD98059 ($n = 14$; $p = 0.66$), 50 μM FK-506 ($n = 14$; $p = 0.30$), or 250 μM CsA ($n = 16$; $p = 0.84$). AMPA-R and NMDA-R mediated current amplitude and standard errors were normalized to average values from control cells for (C) and (D).

(E) Western blots of phospho-p38 MAPK in control hippocampal CA1 region, hippocampal CA1 region infected with Rap(dn)-GFP and hippocampal CA1 region infected with Rap(ca)-GFP. Each lane was loaded with the same amount of protein (35 μg).

(F) Left, relative amounts of phospho-p38 MAPK in hippocampal CA1 region infected with Rap(dn)-GFP ($n = 8$; $p < 0.05$) or Rap(ca)-GFP ($n = 8$; $p < 0.05$). Relative amounts of total p38 MAPK in hippocampal CA1 region infected with Rap(dn)-GFP ($n = 6$; $p = 0.92$) or Rap(ca)-GFP ($n = 6$; $p = 0.75$). Right, relative amounts of phospho-p38 MAPK in hippocampal CA1 region infected with tCaMKII-GFP ($n = 8$; $p = 0.48$), Rap(dn)-GFP ($n = 8$; $p = 0.48$), or Rap(ca)-GFP ($n = 8$; $p = 0.58$). Relative amounts of total p38 MAPK in hippocampal CA1 region infected with tCaMKII-GFP ($n = 8$; $p = 0.16$), or Rap(dn)-GFP ($n = 6$; $p = 0.78$), or Rap(ca)-GFP ($n = 6$; $p = 0.89$). The relative values and standard errors were normalized to average amount of phospho-p38 MAPK or total p38 MAPK from control hippocampal CA1 region. See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values.

a protein kinase whose phosphorylation increases after induction of LTD produced by activation of metabotropic glutamate receptors (Bolshakov et al., 2000). To test whether the synaptic depression produced by Rap activity is mediated by p38 MAPK, we examined the level of phospharylated p38 MAPK in slices expressing Rap(ca)-GFP or Rap(dn)-GFP. Phospho-p38 MARK increased in slices expressing Rap(ca)-GFP and decreased in slices expressing Rap(dn)-GFP (Figures 6E–6F), indicating that Rap activity controls p38 MAPK activity. As a control, we found that the phosphorylated form of p38 MAPK was not changed in tissue expressing tCaMKII-GFP, Ras(ca)-GFP, or Ras(dn)-GFP (Figure 6F). Consistent with this, the synaptic depression produced by Rap(ca)-GFP was blocked by incubating slices in 2 μM SB203580 which inhibits p38 MAPK (Lee et al., 1999; Figure 6D). Some of the cellular processes regulated by the c-Jun amino-terminal kinase group of MAPK (JNK) and p38 MAPK signaling pathways are similar (Ip and Davis, 1998; Xia et al., 1995). Because 2 μM SB203580 inhibits little JNK activity (Whitmarsh et al., 1997), the above experiment suggests that JNK is unlikely to relay Rap-mediated synaptic depression. Consistent with the view, we found that 5 μM SP600125, an inhibitor of JNK (Bennett et al., 2001) did not block the depression of Rap(ca)-GFP in AMPA-R-mediated transmission (Figure 6D). Furthermore, 25 μM PD98059, which inhibits MEK, blocks LTP and the effects of Ras(ca) and tCaMKII (see above), did not prevent depression of AMPA-R-mediated transmission by Rap(ca). This result indicates that Rap(ca) is unlikely to depress transmission by inhibiting endogenous Ras activity. If this were so, then neurons expressing Rap(ca) should show no depression compared to nearby neurons in which Ras downstream activity is inhibited by PD98059. These data indicate that Rap depresses transmission by activating p38 MAPK.

To examine if Rap activity is necessary for LTD, we delivered LTD-inducing stimuli and recorded from neurons expressing Rap(dn)-GFP as well as from nearby control neurons. Transmission onto neurons expressing Rap(dn)-GFP was initially potentiated compared to nearby control neurons (Figure 7A). LTD stimuli produced no last-

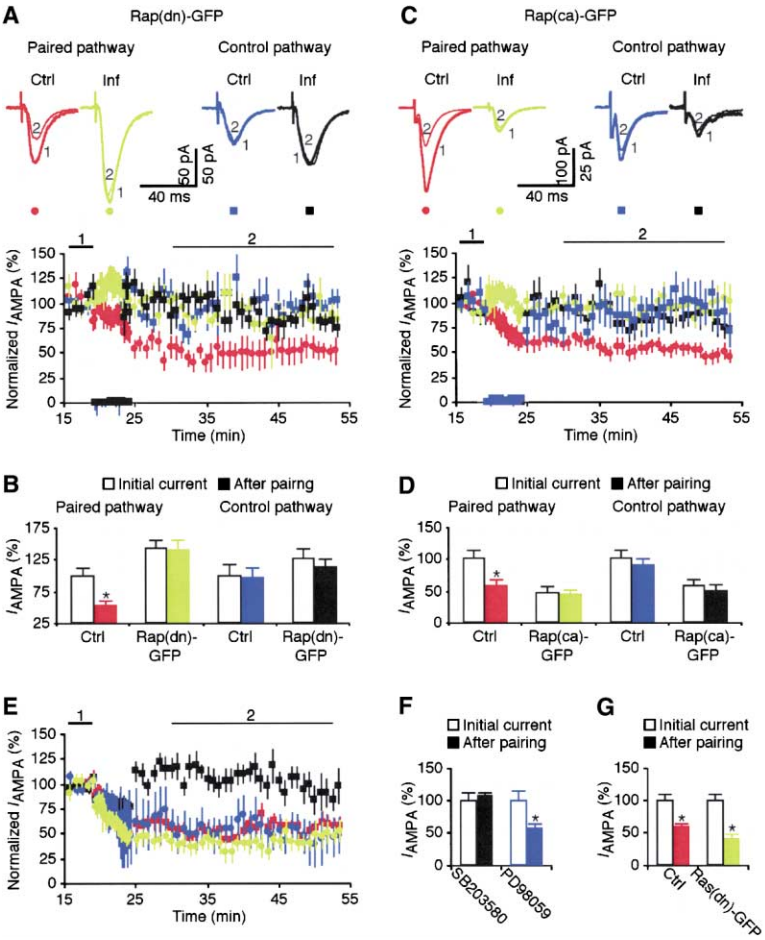

Figure 7. Signaling Long-Term Depression by Rap Activity

(A) Average AMPA-R-mediated synaptic responses obtained before (−60 mV, thick trace) and after (−60 mV, thin trace) pairing from a pair of infected (Rap(dn)-GFP) and uninfected cells in paired (upper left) and control pathway (upper right). Lower plot, normalized simultaneously evoked responses recorded from cells expressing or not expressing Rap(dn)-GFP against the time.

(B) Steady-state synaptic AMPA-R-mediated response amplitudes in paired ($n = 10$; $p < 0.01$) and control pathways ($n = 10$; $p = 0.80$) in cells expressing Rap(dn)-GFP and uninfected neighboring control cells before and after pairing.

(C) Average AMPA-R-mediated synaptic responses obtained before (−60 mV, thick trace) and after (−60 mV, thin trace) pairing from a pair of infected (Rap(ca)-GFP) and uninfected cells in paired (upper left) and control pathway (upper right). Lower plot, normalized simultaneously evoked responses recorded from cells expressing or not expressing Rap(ca)-GFP against the time.

(D) Steady-state synaptic AMPA-R-mediated response amplitudes in paired ($n = 11$; $p < 0.005$) and control pathways ($n = 11$; $p = 0.72$) in cells expressing Rap(ca)-GFP and uninfected neighboring control cells before and after pairing.

(E) Normalized responses from cells recorded with either SB203580 or PD98059 included in the bath solution and from pairs of Ras(dn)-GFP expressing and nonexpressing cells.

(F) Steady-state synaptic AMPA-R-mediated response (SB203580: $n = 12$; PD98059: $n = 8$; t test; $p < 0.001$).

(G) Steady-state synaptic AMPA-R-mediated response amplitudes in cells expressing Ras(dn)-GFP and uninfected neighboring control cells before and after pairing ($n = 10$; $p = 0.05$). AMPA-R mediated current amplitude and standard errors were normalized to average values from control cells in (B) and (D). See Supplemental Data available at http://www.cell.com/cgi/content/full/110/4/443/DC1 for the values.

ing depression in neurons expressing Rap(dn)-GFP. In contrast, nearby nonexpressing neurons did show pathway specific LTD (Figures 7A–7B). This indicates that activation of Rap signaling pathway is necessary for producing LTD. To examine whether Rap activity occludes LTD, we also delivered LTD-inducing stimuli onto neurons expressing Rap(ca)-GFP. Neurons expressing Rap(ca)-GFP had initially smaller transmission compared to nearby control neurons (Figure 7C). LTD stimuli produced no further lasting depression in these neurons, while nearby neurons showed pathway specific LTD (Figures 7C–7D). As a control, we found that LTD-inducing stimuli depressed transmission in neurons expressing Rap(dd)-GFP to the same degree as in nearby noninfected neurons (ctrl: 53.8 ± 5.4% from initial −36.9 ± 7.0 pA; inf: 50.5 ± 6.4% from initial –40.8 ± 5.5 pA; $n = 10$; $p = 0.72$). As an additional control, we examined LTD in neurons expressing Ras(dn)-GFP. LTD-inducing stimuli induced a prominent depression in these cells (Figure 7E; $n = 10$; $p < 0.01$). Furthermore, we confirmed that our NMDA-R-dependent pairing-induced LTD was blocked by inhibition of p38 MAPK and was not affected by inhibition of MEK (Figures 7E–7F). These results indicate that Rap activity produces LTD via phosphorylation of p38 MAPK. Previous studies have shown a role for phosphatase 2B in the production of LTD (Mulkey et al., 1994). It is possible that Rap signaling is upstream of phosphatase function, as the effects of Rap(ca)-GFP on AMPA-R-mediated transmission was blocked by FK-506 or CsA (Figure 6D), inhibitors of protein phosphatase 2B.

LTD appears to involve the removal of AMPA receptors from synapses (reviewed in Carroll et al., 2001). This effect is mimicked and occluded if cycling of GluR2-containing receptors is blocked with the peptide pep2m/G10. To test if Rap-induced depression acts on a similar pool of AMPA-Rs, we examined the effects of pep2m/G10 on transmission in neurons expressing Rap(ca)-GFP. Transmission onto neurons expressing Rap(ca)-GFP was initially depressed by ~50% compared to nearby control neurons (Figure 8A). Loading pep2m/G10 depressed transmission onto control (nontransfected) cells by ~50% over the course of 30–40 min, while cells expressing Rap(ca)-GFP showed no significant depression (Figures 8A–8B). Moreover, coexpression of Rap(ca)-GFP along with the GluR2ct-GFP showed no more depression than expression of Rap(ca)-GFP alone (Figures 8C–8D). Furthermore, we examined the effects of Rap(ca)-GFP expression on cells expressing GluR2(R→Q)-GFP.

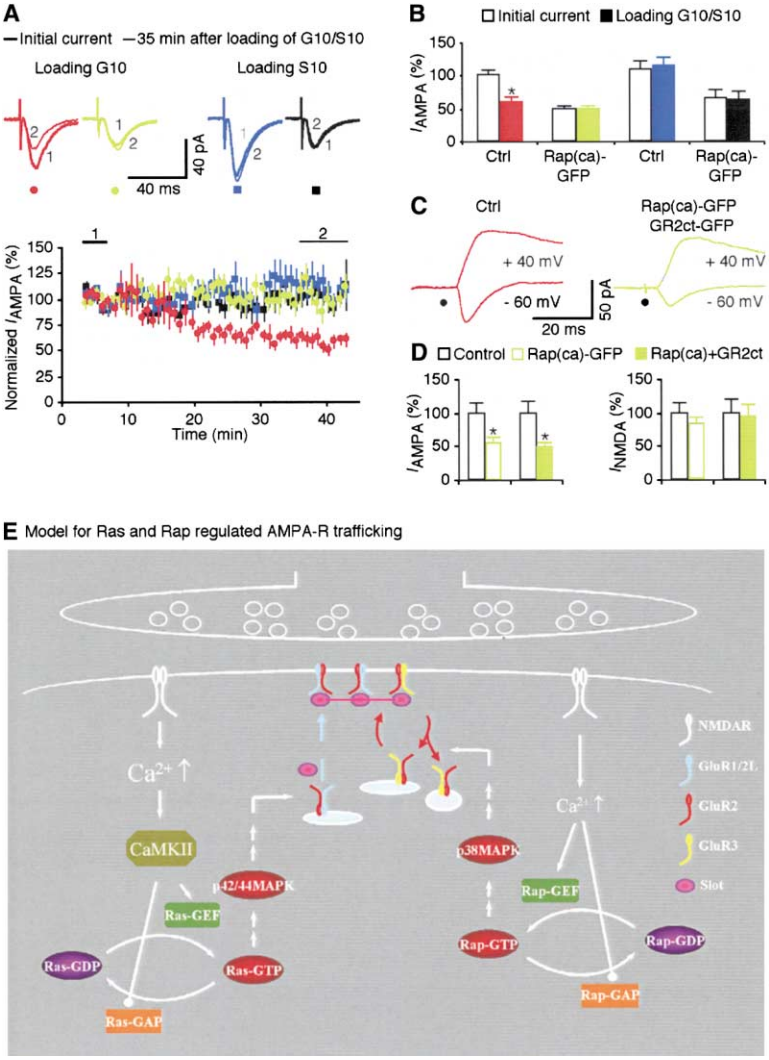

Figure 8. Removal of GluR2-Containing Receptors by Rap Activity

(A) Upper left, average AMPA-R-mediated synaptic responses obtained immediately (−60 mV, thick trace) and 35 min after whole-cell infusion of pep2m/G10 (−60 mV, thin trace) from a pair of infected (Rap(ca)-GFP) and uninfected cells. Upper right, average AMPA-R-mediated synaptic responses obtained immediately (−60 mV, thick trace) and 35 min after whole-cell infusion of S10 (−60 mV, thin trace) from a nearby pair of infected (Rap(ca)-GFP) and uninfected cells in the same slice. Lower plot, normalized simultaneously evoked responses recorded from cells expressing or not expressing Rap(ca)-GFP against the time after forming whole-cell configuration, loaded with either pep2m/G10 or S10.

(B) Residual synaptic AMPA-R-mediated response amplitudes (at −60 mV) in cells expressing Rap(ca)-GFP and uninfected neighboring control cells after loading pep2m/G10 ($n = 10$; $p < 0.01$) or S10 ($n = 10$; $p = 0.58$) for 35 min. AMPA-R mediated current amplitude and standard errors were normalized to average value from control cells loading with pep2m/G10.

(C) Evoked AMPA-R- (−60 mV) and NMDA-R- (+40 mV) mediated responses recorded from untransfected (Ctrl) and cotransfected Rap(ca)-GFP and GluR2ct cells.

(D) Left, AMPA responses in cells expressing Rap(ca)-GFP alone (see Figure 1E) or cells coexpressing both Rap(ca)-GFP and GluR2ct ($n = 15$; $p < 0.01$) relative to uninfected neighboring control cells. Right, NMDA responses in cells expressing Rap(ca)-GFP alone or in cells coexpressing both Rap(ca)-GFP and GluR2ct ($n = 15$; $p = 0.78$) relative to uninfected neighboring control cells. AMPA-R- and NMDA-R-mediated current amplitude and standard errors were normalized to average values from control cells.

(E) Model for small GTPases Ras and Rap regulated AMPA-R trafficking. Left pathway, activation of NMDA-R induces a large amount of Ca^{2+} ion influx, which activates CaMKII-Ras-p42/44MAPK signaling pathway. This signaling pathway signals delivery of AMPA-Rs with long cytoplasmic tails. Right pathway, activation of NMDA-R induces a small amount of Ca^{2+} ion influx, which activates Rap-p38MAPK signaling pathway. This signaling pathway signals removal of AMPA-Rs with only short cytoplasmic tails.

This AMPA receptor shows rectification and can replace the endogenous cycling pool of receptors; thus, the cycling pool of receptors is tagged electrophysiologically (Shi et al., 2001). Cells coexpressing Rap(ca)-GFP and GluR2(R→Q)-GFP, showed depressed AMPA-R-mediated transmission by ~50% but the rectification of the response was not different from control cells (ctrl: −55.8 ± 4.8 pA; tsf: −28.1 ± 3.4 pA; $n = 14$; $p <$ 0.005 for amplitude; ctrl: 2.11 ± 0.26; tsf: 1.92 ± 0.21; $n = 14$; $p = 0.98$ for rectification). These results indicate that Rap removes the same pool of AMPA receptors from synapses as that removed by LTD.

Discussion

Recent studies on synaptic plasticity indicate that receptor trafficking is likely to play a significant role (Luscher et al., 2000; Malinow et al., 2000; Scannevin and Huganir, 2000; Sheng and Lee, 2001). In the case of LTP and LTD, NMDA receptors are activated and the ensuing postsynaptic calcium rise is thought to trigger biochemical reactions that eventually lead to an increase or decrease, respectively, in the number of AMPA receptors at the synapse. For LTP, there is considerable evidence indicating that the calcium-sensitive enzyme, CaMKII is the downstream sensor of NMDA-R activation (reviewed in Lisman et al., 1997). CaMKII is activated during LTP, its activity will enhance transmission by delivering GluR1-containing AMPA receptors to synapses, this enhancement occludes further LTP, and pharmacologic or genetic inhibition of CaMKII activity blocks LTP. However, the signaling downstream of CaMKII is less well established. For instance, a recent report identifies 28 proteins in synapses that can serve as CaMKII substrates (Yoshimura et al., 2000). Here, we examined the potential role of Ras as a downstream effector for CaMKII activity for several reasons. A synaptic RasGAP has been identified at synapses (Chen et

al., 1998; Kim et al., 1998), which can be phosphorylated by CaMKII. Furthermore, Ras downstream effectors (e.g., MAP kinase) have been implicated in LTP (English and Sweatt, 1997) and activity-dependent structural plasticity (Wu et al., 2001). And lastly, a large fraction of individuals with mutations in NF1, a neuronal RasGAP, suffer from mental retardation (Gutmann, 1999; Silva et al., 1997). We find that a dominant-negative form of Ras blocks the potentiating effects of CaMKII, and active forms of Ras mimic the effects of CaMKII activity. Active CaMKII increases levels of phosphorylated p42/44 MAPK. Dominant-negative Ras blocks LTP while active forms of Ras mimic and occlude LTP. We conclude that Ras mediates the signaling activated by CaMKII that is responsible for LTP (Figure 8E). Of interest, we find that spontaneous activity can also stimulate Ras and this enhances transmission, possibly by delivering AMPA receptors that contain GluR2L. Presumably, during LTP induction, Ras is activated more strongly, and this is sufficient to deliver GluR1-containing AMPA receptors. It is possible that activity-dependent delivery of GluR2L is responsible for the LTP observed in juvenile mice lacking GluR1 (Zamanillo et al., 1999). Future studies will be required to determine the role of GluR2L in synaptic transmission and plasticity.

We have also examined the electrophysiological effects of Rap, a closely related family member of Ras whose function is less well defined (Zwartkruis and Bos, 1999; Bos et al., 2001). Recent studies have identified a RapGAP (SPAR) at synapses (Pak et al., 2001). Overexpression of SPAR, which would be expected to decrease levels of Rap activity, leads to structural changes in spine morphology (Pak et al., 2001). However, the effects on synaptic transmission were not examined. Here, we find that active forms of Rap depress AMPA-R-mediated transmission in a manner that mimics and occludes LTD. Furthermore, a dominant-negative form of Rap blocks LTD. Active Rap enhances levels of active p38 MAPK, consistent with recent findings indicating p38 MAPK as a downstream target of Rap (Sawada et al., 2001) and able to control some forms of LTD (Bolshakov et al., 2000). These results indicate that an increase in Rap activity is likely to be one of the steps mediating the signal initiated by LTD-inducing stimuli and eventually leading to the removal of synaptic AMPA receptors (Figure 8E). As with Ras, spontaneous activity appears to activate Rap sufficient to produce synaptic effects. In the case of Rap, this produces tonic depression. It is not clear how NMDA-R activity produced by spontaneous activity or during LTD-inducing stimuli leads to an increase in Rap activity. One possibility is that the influx of Ca^{2+} ions produced by NMDA-R activation can control a RapGEF and activate Rap (Grewal et al., 2000; Kawasaki et al., 1998; M'Rabet et al., 1998).

Our results indicate that spontaneous neural activity continuously adds to synapses AMPA receptors containing long cytoplasmic tails via Ras activity and continuously removes AMPA receptors from synapses containing only short cytoplasmic tails via Rap activity. Similarly, this and previous studies argue that LTP adds to synapses AMPA receptors containing long cytoplasmic tails while LTD removes receptors containing only short cytoplasmic tails. These results indicate the existence of a replacement mechanism at synapses that can exchange AMPA receptors containing long cytoplasmic tails with those containing only short cytoplasmic tails, which explains the observation that LTP and LTD can reverse each other (Dudek and Bear, 1993; Mulkey and Malenka, 1992). In fact, this replacement has previously been detected (Zhu et al., 2000) and may itself be under some form of regulation. For example, a more robust replacement appears to occur in dissociated neuronal preparations where LTD stimuli lead to rapid removal of AMPA receptors with long cytoplasmic tails (Carroll et al., 2001). Thus, the rate of receptor replacement and relative number of receptors with long or short cytoplasmic tails at a synapse may control the amount of LTP or LTD available at that synapse.

Studies in nonneuronal cells have indicated that Ras and Rap can play antagonistic roles. In fibroblasts, Rap was originally identified in a screen for revertants of the morphology displayed by K-Ras-transformed cells (Kitayama et al., 1989). Some subsequent studies have suggested that Rap competes with Ras by sequestering Ras effectors into an inactive complex (Boussiotis et al., 1997; Cook et al., 1993; Mochizuki et al., 1999). However, more recent studies suggest that the signaling pathways controlled by Ras and Rap may be functionally and spatially separated (reviewed in Bos et al., 2001). Consistent with the latter view is the finding that Rap activation and inactivation appears to be regulated by GEFs and GAPs that do not act on the prototypic oncogenic Ras GTPases (Zwartkruis and Bos, 1999). Here, we provide evidence that the signaling pathways controlled by Ras and Rap can exert their effects on distinct targets (Figure 8E). The Ras pathway enhances transmission by controlling the synaptic delivery of receptors containing long cytoplasmic termini (e.g., GluR1 or GluR2L). In contrast, Rap depresses transmission by controlling the synaptic removal of AMPA receptors containing only short cytoplasmic termini (e.g., GluR2 or GluR3). Moreover, Ras activity-mediated synaptic potentiation and LTP are selectively blocked by inhibition of p42/44 MAPK but not by inhibition of p38 MAPK, while Rap activity-mediated synaptic depression and LTD are selectively blocked by inhibition of p38 MAPK but not by inhibition of p42/44 MAPK. Finally, Ras activity enhances only phosphorylation of p42/44 MAPK but not p38 MAPK, while Rap activity enhances only phosphorylation of p38 MAPK but not p42/44 MAPK. Since the effects, molecular mediators and targets of signaling are distinct, our data are consistent with the view that the actions of Ras and Rap at synapses are due to effects other than mutual inhibition.

Experimental Procedures

Biochemical Analyses

Hippocampal extracts were prepared by homogenizing whole hippocampi from two-week-old rats or CA1 regions isolated from cultured slices (Hayashi et al., 2000; Zhu et al., 2000). Expression efficacy in these experiments was high (>95% of CA1 neurons). Homogenizing solution contained (in mM or percentage): HEPES 10, NaCl 150, EDTA 10, EGTA 4, PMSF 0.2, Chymostatin 0.0001%, Leupeptin 0.0001%, Antipain 0.0001%, Pepstatin 0.0001%, and Triton 1%. In Western blots, membranes were probed with anti-Rap1 antibody (1:500; Transduction Laboratories, Lexington, KY), stripped and reblotted with anti-Ras antibody (1:1000; Transduction Laboratories, Lexington, KY), restripped and blotted with anti-GFP antibody

(1:1000; Boehringer Mannheim, Roche Molecular Biochemicals). To detect phospho-p42/44 MAPK and phospho-p38 MAPK, 0.1 M NaPPi, 0.5 M NaF, and 1 mM Na_3VO_4 were added in the standard homogenizing solution. Membranes were blotted with either anti-phospho-p42/44 MAPK (1:3000) or anti-phospho-p38 MAPK antibody (1:800), stripped and reblotted with anti-p42/44 (1:500) or anti-p38 antibody (1:1000) (Cell Signaling Technology, Beverly, MA). Western blots were quantified by chemiluminescence and densitometric scanning of the films under linear exposure conditions.

Constructs of Recombinant Receptors and Expression

Constructs of Rap-GFP, Ras-GFP, and GluR2ct-GFP were made by in-frame ligation of Rap1, H-Ras sequences, and GluR2 coding sequence from amino acid 813–862 into pEGFP-C1 (enhanced GFP, Clontech Laboratories, Palo Alto, CA). tCaMKII-GFP, GluR1-GFP, and GluR2L-GFP were made as previously described (Hayashi et al., 2000; Zhu et al., 2000). Ras mutant constructs [(Feig, 1999), constitutively active mutant (G12→V), dominant-negative mutant (S17→N) and null/dead mutant (T35→A, D38→A)] were generated from wild-type Ras sequence using Quick Change Site-Directed mutagenesis kit (Stratagene, La Jolla, CA). Rap mutant constructs, [constitutively active mutant (Q63→E) (Maly et al., 1994), dominant-negative mutant (S17→N), and null/dead mutant (T35→A, D38→A)], were generated in a similar manner. Constructs were expressed in CA1 neurons in rat hippocampal slices, using Sindbis virus or biolistics transfection. Slices were prepared from postnatal 6–7 day old animals, infected with virus or transfected using gene gun after 6–8 days in vitro, and incubated on culture medium and 5% CO_2. For pharmacological experiments, slices were maintained in culture medium containing drugs from the time they were infected or transfected. Expressing recombinant Ras and Rap proteins longer than ~24 hr induced changes in dendritic spine morphology and in basic membrane electrophysiological properties (see also Tonini et al., 1999; Wu et al., 2001). Thus, to minimize activation of multiple or nonspecific pathways, all experiments reported in this study were performed within 15 ± 3 hr after infection or transfection.

Electrophysiology

Simultaneous whole-cell recordings were obtained from pairs of nearby infected/transfected and noninfected/nontransfected CA1 neurons, under visual guidance using fluorescence and transmitted light illumination. Bath solution (29 ± 1.5°C), unless otherwise stated, contained (in mM): NaCl 119, KCl 2.5, $CaCl_2$ 4, $MgCl_2$ 4, $NaHCO_3$ 26, NaH_2PO_4 1, glucose 11, picrotoxin 0.1, bicuculline 0.01, and 2-chloroadenosine 0.002, at [pH 7.4] and gassed with 5% CO_2/95% O_2. 2-chloroadenosine was included to prevent bursting. For experiments in which slices were maintained in culture with PD98059, SB203580, FK-506, or cyclosorine A (Calbiochem, La Jolla, CA), or SP600125 (gift from Signal Pharmaceutical Inc, San Diego, CA), these drugs were included during recordings. Patch recording pipettes (3-6 MΩ) contained (in mM): cesium methanesulfonate 115, CsCl 20, HEPES 10, $MgCl_2$ 2.5, Na_2ATP 4, Na_3GTP 0.4, sodium phosphocreatine 10, EGTA 0.6, and spermine 0.1, at [pH 7.25] or potassium gluconate 115, HEPES 10, $MgCl_2$ 2, MgATP 2, Na_2ATP 2, Na_3GTP 0.3 and KCl 20, at [pH 7.25] for current (voltage-clamp) and voltage (current-clamp) recordings, respectively. Whole-cell recordings were made with Axopatch-1D or Axoclamp 2A amplifiers (Axon Instruments, Foster City, CA). The resting membrane potentials (ctrl: −57.9 ± 0.6 mV; inf: −57.2 ± 0.7 mV; $n = 12$; $p = 0.34$ for Ras(ca)-GFP; ctrl: −57.9 ± 1.3 mV; inf: −58.0 ± 1.0 mV; $n = 12$; $p = 0.99$ for Rap(ca)-GFP) and input resistances (ctrl: 130.7 ± 5.6 MΩ; inf: 120.0 ± 7.0 MΩ; $n = 12$; $p = 0.10$ for Ras(ca)-GFP; ctrl: 120.1 ± 4.4 MΩ; inf: 117.2 ± 4.3 MΩ; $n = 12$; $p = 0.70$ for Rap(ca)-GFP) and time constant (ctrl: 23.7 ± 0.9 ms; inf: 23.2 ± 1.3 ms; $n = 12$; $p = 0.66$ for Ras(ca)-GFP; ctrl: 20.7 ± 1.4 ms; inf: 20.8 ± 1.2 ms; $n = 12$; $p = 0.94$ for Rap(ca)-GFP), measured in current clamp mode, were similar for noninfected and infected cells. Junction potentials were not corrected. Synaptic responses were evoked by bipolar electrodes with single voltage pulses (200 μs, up to 20 V) placed in s. radiatum ~300–500 μm from the CA1 cells. Synaptic AMPA responses at −60 mV and +40 mV were averaged over 90 trials and their ratio was used as an index of rectification. To minimize the effect from AMPA responses, the peak NMDA responses at +40 mV were measured after digital subtraction of estimated AMPA responses at +40 mV. LTP was induced by a pairing protocol using 200 pulses at 2 Hz at −5 mV within 5 min after formation of whole-cell configuration. LTD was induced by pairing 300 pulses at 1 Hz at −45 mV, 15 min after formation of whole-cell configuration. Slices were incubated in solution containing 25 μM PD98059 or 2 μM SB203580 before (for at least 1 hr) and during LTP and LTD experiments. pep2m/G10 (KRMKVAKNAQ) and S10 (VRKKNMAKQA) (Research Genetics, Huntsville, AL) were dissolved (2 mM) in Cs-based internal solution. Experiments were done at 22°–23°C. All results are reported as mean ± SEM and statistical differences of the means were determined using Wilcoxon nonparametric test unless otherwise stated. Significance was set at $p < 0.05$.

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