Rap2-JNK Removes Synaptic AMPA Receptors during Depotentiation

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Introduction

The sustained interest in synaptic plasticity stems from the belief that synaptic plasticity underlies key aspects of cognitive function such as neural development, adaptation, and learning and memory. In current models, brief periods of repetitive synaptic activity lead to long-lasting changes in synaptic transmission; NMDA-sensitive glutamate receptor (NMDA-R) opening, rise in postsynaptic calcium concentration, activation of intracellular signaling pathways, and synaptic delivery and removal of AMPA-sensitive glutamate receptors (AMPA-Rs) are critical events underlying different forms of synaptic plasticity (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Thomas and Huganir, 2004). There is now compelling evidence that AMPA-Rs with long cytoplasmic tails (e.g., GluR1 or GluR2L) are normally delivered to synapses during activity-induced synaptic enhancement. On the other hand, AMPA-Rs with only short cytoplasmic tails (i.e., GluR2/3) cycle continuously from nonsynaptic to synaptic sites and replace synaptic receptors without changing the synaptic efficacy; their number at synapses can be reduced after activity-induced synaptic depression. Our recent study has demonstrated that small GTPase Ras, dependent on extracellular-regulated kinase 1 and 2 (Erk1/2), controls synaptic delivery of GluR1 and GluR2L-containing AMPA-Rs during long-term potentiation (LTP), whereas small GTPase Rap1, dependent on 38 kDa mitogen-activated protein kinase (p38 MAPK), controls synaptic removal of GluR2/3 during long-term depression (LTD) (Zhu et al., 2002). However, it is still unclear what signaling pathway controls synaptic removal of GluR1- and GluR2L-containing AMPA-Rs.

The small GTPase Rap2 is a member of the Ras GTPase family that exhibits 60% identity to Rap1 (Takai et al., 2001). Although Rap2 has been cloned for more than 15 years (Pizon et al., 1988), its functional role remains elusive. One possibility is that, like Rap1 and Ras, Rap2 may function in MAP kinase signaling, since a recent report suggests that Rap2 activity stimulates c-Jun NH2-terminal kinase (JNK) activity in nonneuronal cells (Machida et al., 2004). As with Erk1/2 and p38 MAPK, JNK is another major MAPK that controls essential cellular processes (Weston and Davis, 2002). However, several pivotal questions, such as whether Rap2-JNK constitutes a functional signaling pathway, what controls Rap2-JNK activity, and which cellular processes depend on Rap2-JNK signaling, remain unknown, in neuronal and nonneuronal cells.

Rap2 and JNK, as well as their regulators, are expressed at excitatory synapses (Husi et al., 2000; Pak et al., 2001; Peng et al., 2004). In this study, we have investigated the role of Rap2 in activity-dependent synaptic plasticity. Using several mutants that permit dissection of the role of Rap2 signaling, we identified a functional Rap2 signaling pathway in hippocampal CA1 neurons. Rap2-JNK signaling pathway receives its inputs from synaptic and NMDA-R activity; synaptic activity and activation of NR2A-containing NMDA-Rs activate Rap2, which stimulates JNK activity. Rap2-JNK signaling pathway sends its outputs to AMPA-Rs: Rap2-JNK activity mediates synaptic removal of GluR1- and GluR2L-containing AMPA-Rs and is essential for depotentiation. It is notable that Rap2 activity (depot-
Husi et al., 2000). To examine possible postsynaptic activity activates Rap2, we measured the present in hippocampal CA1 cells (Figure 1A; see also 30 min (Figure 2C). To further confirm that spontaneous responding wild-type Rap2 or their mutants in hippocampal CA1 regions expressing Rap2-GFP. (Right) Relative amounts of endogenous Rap2 in whole hippocampi (n = 11; p < 0.05) or recombinant Rap2 in hippocampal CA1 regions expressing Rap2-GFP (n = 11; p < 0.005). The relative values and standard errors were normalized to average amounts of endogenous Rap2 from hippocampal CA1 regions. To determine if Rap2 is present in hippocampal tissue, NMDA-Rs stimulates Rap2 activity, we measured di- expression level of Rap2-GFP ranged from two to activate Rap2, producing a tonic depression of trans-

Western blots on these extracts show bands corre-

sponding to Rap2 indicating that these proteins are Rap2-GTP levels that returned to baseline within hippocampal neurons caused a transient increase in synaptic transmission. To test if endogenous Rap2 activity depresses transmission, we expressed a dominant-negative mutant Rap2(dn)-GFP and syn-

pressing in cultured slices in normal culture media for 14 hr. (Lower left) AMPA responses in cells expressing Rap2(ca)-GFP (n = 14; p < 0.01) or Rap2(dd)-GFP (n = 16; p = 0.96) relative to neighboring nonexpressing control cells. (Lower right) NMDA responses in cells expressing Rap2-GFP (n = 14; p = 0.98) or Rap2(dd)-GFP (n = 16; p = 0.57) relative to neighboring nonexpressing control cells. *p < 0.05. See Supplemental Data for the values. 

Figure 1. Expression of Rap2 in Hippocampal CA1 Cells

(A) (Left) Expression of endogenous and recombinant Rap2 proteins in hippocampal CA1 regions, whole hippocampi, and hippocam-

pal CA1 regions expressing Rap2-GFP. (Right) Relative amounts of endogenous Rap2 in whole hippocampi (n = 11; p < 0.05) or recombinant Rap2 in hippocampal CA1 regions expressing Rap2-GFP (n = 11; p < 0.005). The relative values and standard errors were normalized to average amounts of endogenous Rap2 from hippocampal CA1 regions. (B) Two-photon images of GFP fluorescence indicate that Rap2-GFP proteins are present in the dendrites and spines of CA1 pyramidal neurons. The dendritic spines are demarcated by RFP fluorescence. (C) (Upper) Evoked AMPA-R-mediated (~60 mV) and NMDA-R-mediated (+40 mV) responses recorded from nonexpressing (Ctrl) and Rap2(ca)-GFP-expressing cells. Rap2-GFP mutants were expressed in cultured slices in normal culture media for ~14 hr. (Lower left) AMPA responses in cells expressing Rap2(ca)-GFP (n = 14; p < 0.01) or Rap2(dd)-GFP (n = 16; p = 0.96) relative to neighboring nonexpressing control cells. (Lower right) NMDA responses in cells expressing Rap2-GFP (n = 14; p = 0.98) or Rap2(dd)-GFP (n = 16; p = 0.57) relative to neighboring nonexpressing control cells. *p < 0.05. See Supplemental Data for the values. 

Recent studies have shown that NR2A- and NR2B- 

uted and infiltrated in dendritic spines, the sites of excita-
tory synapses (Figure 1B). To determine whether Rap2 affects synaptic function, electrophysiological recordings were obtained simultaneously from nearby expressing and nonexpressing neurons. Afferent fibers were stimulated, and excitatory postsynaptic currents were recorded. Neurons expressing a constitutively active mutant Rap2(V12), Rap2(ca)-GFP, showed ~50% depressed AMPA-R-mediated responses. There was no significant difference in NMDA-R-mediated responses between Rap2(ca)-GFP-expressing and nonexpressing neurons (Figure 1C), consistent with the previous finding that synaptic NMDA-Rs and AMPA-Rs are differentially regulated (Rao and Craig, 1997), and suggesting that the Rap2(ca)-GFP-stimulated depression was not mediated by nonspecific mechanisms. As an additional control, neurons expressing an inactive mutant Rap2(S38), Rap2(dd)-GFP, showed no change in transmission (Figure 1C). These results indicate that Rap2 activity selectively downmodulates AMPA-R-mediated synaptic transmission. 

To determine the effects of Rap2 signaling on synaptic transmission, we included 12 mM MgCl2, which depresses synaptic transmission (Zhu et al., 2000), or APV, a pharmacological blocker of NMDA-Rs, in culture media during expression of Rap2(dn)-GFP or Rap2(wt)-GFP. High Mg2+ and APV blocked the effects of Rap2(dn)-GFP and Rap2(wt)-GFP on AMPA synaptic transmission (Figure 2B), indicating that Rap2-mediated effects required synaptic activity and activation of NMDA-Rs. As a control, we found that the depression of Rap2(ca)-GFP on AMPA-R-mediated transmission was not blocked by high Mg2+ (Figure 3E). To confirm that activation of NMDA-Rs stimulates Rap2 activity, we measured directly the active form of Rap2 (or GTP bound Rap2) by a GST pull-down assay. Application of NMDA to cultured hippocampal neurons caused a transient increase in Rap2-GTP levels that returned to baseline within ~15–30 min (Figure 2C). To further confirm that spontaneous synaptic activity activates Rap2, we measured the levels of Rap2-GTP in slices cultured in media containing either high Mg2+ or APV. Both high Mg2+ and APV reduced the active form of Rap2-GTP in CA1 cells (Figure 2D). Collectively, these results indicate that spontaneous synaptic activity activates NMDA-Rs that in turn activate Rap2, producing a tonic depression of transmission.

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Figure 2. NR2A-Containing NMDA-R-Stimulated Rap2 Activity Depresses Synaptic Transmission

(A) Evoked AMPA-R- and NMDA-R-mediated responses recorded from nonexpressing (Ctrl) and Rap2(wt)-GFP cells or Rap2(wt)-GFP-expressing cells cultured in normal or 12 mM Mg2+ media.

(B) (Left) AMPA responses in Rap2(dn)-GFP- and Rap2(wt)-GFP-expressing cells cultured in normal media [n = 16, p < 0.01 for Rap2(wt)-GFP; n = 16, p < 0.05 for Rap2(wt)-GFP], media with 12 mM Mg2+ [n = 16, p = 0.92 for Rap2(dn)-GFP; n = 16, p = 0.84 for Rap2(wt)-GFP], or media with 100 μM DL-APV [n = 16, p = 0.50 for Rap2(dn)-GFP; n = 16, p = 0.94 for Rap2(wt)-GFP]. (Right) NMDA responses in Rap2(dn)-GFP- and Rap2(wt)-GFP-expressing cells cultured in normal media [n = 16, p = 0.66 for Rap2(dn)-GFP; n = 16, p = 0.76 for Rap2(wt)-GFP], media with 12 mM Mg2+ [n = 16, p = 0.54 for Rap2(dn)-GFP; n = 16, p = 0.50 for Rap2(wt)-GFP], or media with 100 μM DL-APV [n = 16, p = 0.54 for Rap2(dn)-GFP; n = 16, p = 0.41 for Rap2(wt)-GFP]. AMPA-R- and NMDA-R-mediated current amplitude and standard errors were normalized to average amounts of Rap2-GTP at 3 min (n = 4; paired Student’s t test; p = 0.09) after NMDA application.

(E) Relative levels of GTP bound Rap2 and total Rap2 in hippocampal CA1 regions prepared from slices cultured in 12 mM Mg2+ (n = 11; p < 0.01) or 100 μM DL-APV (n = 11; p < 0.005). Relative amounts of total Rap2 in hippocampal CA1 regions prepared from slices cultured in 12 mM Mg2+ (n = 11; p < 0.01) or 100 μM DL-APV (n = 11; p < 0.005). Relative amounts of total Rap and Rap2 from control untreated cells.

containing NMDA-Rs differ significantly in biophysical properties and may thus be designed for controlling de-potentiation/LTP and LTD, respectively (Erreger et al., 2005; Liu et al., 2004; Massey et al., 2004). We wished to know whether NR2A- or NR2B-containing NMDA-Rs or both control Rap2 activity, and we therefore examined the effects of two selective blockers for NR2A- and NR2B-containing NMDA-Rs on the Rap2(dn)-GFP-stimulated synaptic potentiation. We included Ro25-6981, which selectively blocks NR2B-containing NMDA-Rs and LTD (Liu et al., 2004; Massey et al., 2004), or NVP-AAM077, which selectively blocks NR2A-containing NMDA-Rs and depotentiation/LTP (Liu et al., 2004; Massey et al., 2004), in culture media during expression of Rap2(dn)-GFP. We found that NVP-AAM077, but not Ro25-6981, blocked the potentiation stimulated by Rap2(dn)-GFP (Figures 2E and 2F). These results indicate that stimulation of Rap2 signaling requires activation of NR2A-containing NMDA-Rs, but not NR2B-containing NMDA-Rs. As controls, we also examined effects of these two NMDA-R blockers on Rap1 and Ras activities, which mediate LTD and LTP, respectively (Zhu et al., 2002). We found that Ro25-6981, but not NVP-AAM077, blocked the Rap1(dn)-GFP-stimulated potentiation, whereas NVP-AAM077, but not Ro25-6981, blocked the Rap2(dn)-GFP-stimulated synaptic depression (Figures 2E and 2F). These results indicate that stimulation of Rap1 signaling requires activation of NR2B-containing NMDA-Rs, whereas stimulation of Ras signaling requires activation of NR2A-containing NMDA-Rs. Collectively, these results indicate that different subtypes of NMDA-Rs are functionally coupled to different Ras family GTPases (i.e., Ras, Rap1, and Rap2) and that activations of NR2A- and NR2B-containing NMDA-Rs stimulate Rap2 and Rap1 activity, respectively.

Rap2 Activity Stimulates JNK Signaling

To identify potential effectors of Rap2 that might be involved in signaling synaptic depression, we purified binding partners of Rap2-GTP by affinity chromatogra-
Figure 3. Rap2 Signals Synaptic Depression via Activation of JNK

(A) A prominent band specifically associated with GST-Rap2(ca), indicated by the arrow, was excised and subjected to tandem mass spectrometry for protein identification, yielding 21 peptides matching to Traf2- and NCK-interacting kinase (TNIK) and 1 peptide matching to MINK2 (a TNIK-related kinase). (B) Evoked AMPA-R- and NMDA-R-mediated responses recorded from nonsignaling neurons (Ctrl) and Rap2(ca)-GFP-expressing cells cultured in normal media or media containing 12 mM Mg^2+.

(C) (Left) AMPA responses in TNIK(dn)-GFP-expressing cells cultured in normal media or media containing 12 mM Mg^2+. (Right) NMDA responses in Rap2(ca)-GFP-expressing cells cultured in normal media (n = 15; p = 0.88), or media containing 12 mM Mg^2+ (n = 16; p = 0.16), or TNIK(dn)-GFP and Rap2(ca)-RFP-coexpressing cells cultured in normal media (n = 16; p = 0.31).

(D) Evoked AMPA-R- and NMDA-R-mediated current amplitudes and standard errors were normalized to average values from control cells.

(E) (Left) AMPA responses in Rap2(ca)-GFP-expressing cells cultured in normal media containing 5 μM SP600125, 12 mM Mg^2+, or FK506. (Right) AMPA-R- and NMDA-R-mediated current amplitudes and standard errors were normalized to average values from control cells.

21 peptides matching to Traf2- and NCK-interacting kinase (TNIK) and 1 peptide matching to MINK2 (a TNIK-related kinase). These highly related kinases are members of the STE20 family of protein kinases that initiate MAP kinase cascades—TNIK and MINK2 have been shown to specifically act upstream of the JNK pathway (Dan et al., 2001). To test whether TNIK is involved in the Rap2-stimulated synaptic depression, we expressed a GFP-tagged dominant-negative form of TNIK containing only the CNH domain, TNIK(dn)-GFP, alone or with the RFP-tagged constitutively active Rap2(ca), Rap2(ca)-RFP. Neurons expressing TNIK(dn)-GFP had enhanced AMPA responses in comparison to nearby nonexpressing neurons, while NMDA responses were not significantly changed (Figures 3B and 3C). In addition, including high Mg^2+ in culture media blocked the TNIK(dn)-GFP-stimulated synaptic potentiation. These results suggested that synaptic activity stimulates endogenous TNIK signaling that depresses synaptic transmission. Moreover, coexpressing TNIK(dn)-GFP with Rap2(ca)-RFP blocked the Rap2-ca-stimulated synaptic depression in coexpressing neurons (Figure 3C), suggesting that TNIK may be a downstream effector of Rap2 in CA1 neurons.

Since TNIK specifically stimulates JNK signaling (Dan et al., 2001), we wished to know whether the Rap2-stimulated synaptic depression might be mediated by JNK. To test this idea, we included SP600125, an inhibitor of JNK (Bennett et al., 2001), in culture media during expression of Rap2(ca)-GFP. We found that SP600125 blocked the inhibitory effect of Rap2(ca)-GFP on AMPA responses (Figures 3D and 3E). Moreover, we initially cultured hippocampal tissues in media containing SP600125 for 12 hr, then in normal media for another 4–6 hr. We found that Rap2-ca-GFP-expressing neurons treated this way had depressed AMPA responses in comparison to nonexpressing neurons (Figure 3E), indicating that the inhibitory effect of blocking JNK was reversible. This result suggests that the Rap2-stimulated synaptic depression was unlikely mediated by nonspecific mechanisms (cf. McCarthy et al., 1995).

Our recent study has demonstrated that small GTPase Ras, dependent on Erk1/2, controls synaptic potentiation during LTD, whereas small GTPase Rap1, dependent on p38 MAPK, controls synaptic depression during LTD (Zhu et al., 2002). Neither SB203580, which inhibits p38 MAPK (Lee et al., 1999), or PD98059, which inhibits MEK (an upstream kinase of the ERK pathway) (Dudley et al., 1995), blocked the depressive effect of Rap2(ca)-GFP (Figures 3D and 3E). These results indicate that the Rap2-stimulated synaptic depression requires JNK activity, but not Erk1/2 or p38 MAPK activity.

Previous studies have indicated that synaptic depression requires phosphatase 2B (also known as calcineurin) (Mallet et al., 2001; Mulkey et al., 1993; Zhuo et al., 1995). We found that the depressive effect of Rap2-ca-GFP was blocked by FK-506 or cyclosporin A (CsA; Figures 3D and 3E), inhibitors of protein phosphatase 2B (Winder and Sweatt, 2001), indicating that phosphatase 2B is required for Rap2-JNK-mediated synaptic depression. However, it is unclear whether phosphatase 2B relays or is merely required for the Rap2 effect.
Rap2 activity stimulates JNK signaling

(A) Western blots of phospho-p46/54 JNK in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 19, p = 0.88 for phospho-p46 JNK; n = 19, p = 0.87 for phospho-p54 JNK). Rap2 activity stimulates JNK signaling.

(B) Levels of phosphorylated (active) JNK increased in CA1 cells expressing Rap2(ca)-GFP and decreased in CA1 cells expressing Rap2(dn)-GFP. Each lane was loaded with the same amount of protein (40 μg).

(C) Relative amounts of total p42/44 MAPK in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 20, p = 0.71 for p42 MAPK; n = 20, p = 0.32 for p44 MAPK).

To examine whether Rap2 signaling stimulates JNK activity, we immunoblotted hippocampal slices expressing Rap2(ca)-GFP or Rap2(dn)-GFP. Levels of phosphorylated (active) JNK increased in CA1 cells expressing Rap2(ca)-GFP and decreased in CA1 cells expressing Rap2(dn)-GFP (Figure 4A). Phospho-JNK levels were unchanged by Ras(ca)-GFP, Ras(dn)-GFP, Rap1(ca)-GFP, or Rap1(dn)-GFP (Figure 4A). In addition, phosphorylated Erk1/2 and p38 MAPK were unchanged in CA1 cells expressing Rap2(ca)-GFP or Rap2(dn)-GFP (Figures 4B and 4C). The above results indicate that Rap2 acts through JNK to depress AMPA-R-mediated synaptic transmission.

Figure 4. Rap2 Activity Stimulates JNK Signaling

Rap2-JNK Activity Controls Long-Term Depotentiation

We have recently shown that Rap1 controls LTD via activation of p38 MAPK (Zhu et al., 2002). Since the depressive effect of Rap2 requires JNK but not p38 MAPK, we speculated that Rap2-JNK signaling controls depotentiation. To test this idea, we studied pairing-induced depotentiation after first applying a LTP-inducing pairing protocol in neurons expressing either Rap2(dn)-GFP (to block endogenous Rap2 activity) or Rap2(ca)-GFP (to occlude Rap2 activity). As with a previous report (Lee et al., 2000), we found that the pairing protocol using 300 pulses at 1 Hz at −45 mV, which is optimal to induce LTD (Zhu et al., 2002), was also an optimal protocol to induce depotentiation after LTP (Figure 5). To determine whether Rap2 activity is necessary and sufficient for depotentiation, we isolated depotentiation-sensitive neurons expressing Rap2(ca)-GFP, and hippocampal CA1 regions expressing Rap2(dn)-GFP. Each lane was loaded with the same amount of protein (40 μg). Relative amounts of phospho-p38 MAPK in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 20, p = 0.27) or Rap2(dn)-GFP (n = 20, p = 0.49). The relative values and standard errors were normalized to average amounts of phospho-JNK, phospho-Erk1/2, phospho-p38 MAPK, total JNK, total Erk1/2, or total p38 MAPK from control hippocampal CA1 regions. *p < 0.05 (Wilcoxon test). See Supplemental Data for the values.

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Meanwhile, nearby nonexpressing neurons showed pathway-specific LTP and depotentiation (Figures 5D–5F). These results suggest that constitutively active Rap2 continuously depotentiates synaptic transmission, which prevents induction of LTP and occludes further depotentiation. Together, these results indicate that Rap2 activity controls depotentiation.

To test whether Rap2 controls depotentiation via JNK, we incubated slices in the bath solution containing SP600125, an inhibitor of JNK, before (for at least 1 hr) and during depotentiation experiments. We found that SP600125 blocked depotentiation in CA1 neurons (Figures 5G and 5H). In addition, as with Rap2(dn)-GFP, expressing TNIK(dn)-GFP also blocked depotentiation. Collectively, these results suggest that Rap2 controls depotentiation via TNIK-JNK signaling.

Rap2 Removes Synaptic GluR1- and GluR2L-Containing AMPA-Rs

We have previously shown that Rap1 mediates LTD by removing synaptic AMPA-Rs with only short cytoplasmic termini, such as GluR2/3 (Zhu et al., 2002). Since Rap2 activity controls depotentiation, we asked if Rap2 acts to remove the pool of AMPA-Rs with long cytoplasmic termini (e.g., GluR1 and GluR2L), which require activity to be driven into synapses. Previous studies have shown that overexpressions of the cytoplasmic tail constructs of GluR2Lct-GFP and GluR2ct-GFP depress the transmission mediated by AMPA-Rs with long and only short cytoplasmic termini, respectively (Kolleker et al., 2003; Shi et al., 2001). We first examined the effects of coexpression of Rap2(ca)-RFP with either GluR2Lct-GFP or GluR2ct-GFP. Coexpression of Rap2(ca)-RFP with GluR2Lct-GFP showed no more depression than expression of Rap2(ca)-GFP alone, while coexpression of Rap2(ca)-RFP with GluR2ct-GFP showed additive effects (Figures 6A and 6B), indicating that Rap2 activity removed AMPA-Rs with long cytoplasmic termini, but not GluR2/3 AMPA-Rs. We then directly examined the effects of Rap2(ca)-RFP on AMPA-Rs with only short cytoplasmic termini (Figures 6C and 6D). Expressing GluR2(R→Q)-GFP in CA1 neurons enhanced rectification but had no effect on the amplitude of AMPA responses in comparison to nearby control nonexpressing neurons (Figures 6C and 6D), due to replacement of synaptic AMPA-Rs with the "electrophysiologically tagged" GluR2(R→Q)-GFP (Shi et al., 2001). We found that neurons coexpressing Rap2(ca)-RFP with GluR2(R→Q)-GFP had depressed AMPA-R-mediated transmission and further enhanced rectification in comparison to neighboring neurons expressing GluR2 (R→Q)-GFP alone, suggesting that Rap2(ca)-RFP re-

Figure 5. Rap2 Activity Controls Long-Term Depotentiation

(A) Average AMPA-R-mediated synaptic responses obtained before (−60 mV; thick trace) and after (−60 mV; thin trace) LTP-depotentiation-inducing pairing from a pair of Rap2(dn)-GFP-expressing and neighboring nonexpressing cells in LTP-depotentiation double paired (upper left) and control pathway (upper right). (Lower plot) Simultaneously evoked responses recorded from the cell pairs expressing or not expressing Rap2(dn)-GFP against time.

(B) Normalized simultaneously evoked responses recorded from all cell pairs expressing or nonexpressing Rap2(dn)-GFP against time.

(C) Steady-state synaptic AMPA-R-mediated response amplitudes in paired (n = 10; p < 0.01) and control pathways (n = 10; p = 0.28) in cells expressing Rap2(dn)-GFP and neighboring nonexpressing control cells before and after pairing.

(D) Average AMPA-R-mediated synaptic responses obtained before (−60 mV; thick trace) and after (−60 mV; thin trace) LTP-depotentiation-inducing pairing from a pair of Rap2(ca)-GFP-expressing and neighboring nonexpressing cells in LTP-depotentiation double paired (upper left) and control pathway (upper right). (Lower plot) Simultaneously evoked responses recorded from the cell pairs expressing or not expressing Rap2(ca)-GFP against time.

(E) Normalized simultaneously evoked responses recorded from cells expressing or nonexpressing Rap2(ca)-GFP against time.

(F) Steady-state synaptic AMPA-R-mediated response amplitudes in paired (n = 9; p = 0.86) and control (n = 9; p = 0.24) pathways in cells expressing Rap2(ca)-GFP and neighboring nonexpressing control cells before and after pairing. AMPA-R-mediated current amplitude and standard errors were normalized to average initial values from control cells.

(G) Normalized simultaneously evoked responses recorded from cells recorded with or without 5 μM SP600125 included in the bath solution, and cells expressing TNIK(dn)-GFP recorded in the normal bath solution against time.

(H) Steady-state synaptic AMPA-R-mediated response amplitudes before and after LTP-depotentiation-inducing pairing in cells recorded in the normal bath solution (n = 12; p < 0.001) or in the bath solution with 5 μM SP600125 (n = 12; p = 0.54), or cells expressing TNIK(dn)-GFP recorded in the normal bath solution (n = 8; p < 0.005). AMPA-R-mediated current amplitude and standard errors were normalized to average initial values from cells recorded in the normal bath solution. *p < 0.05 (Wilcoxon test). See Supplemental Data for the values.
moves AMPA-Rs other than the newly delivered, rectified GluR2L(R\→Q)-GFP. We also directly examined the effects of Rap2(ca)-RFP on AMPA-Rs with long cytoplasmic termini (Figure 6D). Neurons expressing GluR2L(R\→Q)-GFP or neurons coexpressing GluR1-GFP and Ras(ca)-GFP show enhanced synaptic transmission and rectification, due to synaptic insertion of "electrophysiologically tagged" GluR2L(R\→Q)-GFP by spontaneous synaptic activity (Kolleker et al., 2003; Zhu et al., 2002), or "electrophysiologically tagged" GluR1-GFP by constitutively active Ras (Zhu et al., 2002). Neurons coexpressing Rap2(ca)-RFP with GluR2L(R\→Q)-GFP and neurons coexpressing Rap2(ca)-RFP with GluR1-GFP and Ras(ca)-GFP showed depressed AMPA-R-mediated transmission, and the rectification of the responses was not different from control nonexpressing neurons, suggesting that Rap2 activity removed the newly delivered, rectified GluR2L(R\→Q)-GFP and GluR1-GFP. Together, these results indicate that Rap2 activity removes synaptic AMPA-Rs with long cytoplasmic termini.

Previous studies have demonstrated that pep2m/G10 selectively blocks GluR2/3 from cycling back to synapses (Lee et al., 2002; Luscher et al., 1999; Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Zhu et al., 2000). To further confirm that Rap2 activity removes synaptic AMPA-Rs with long cytoplasmic termini, we used an independent experimental approach, loading CA1 neurons with the peptide pep2m/G10. We found that intracellular loading of pep2m/G10 depressed AMPA-R-mediated synaptic transmission in both Rap2(ca)-GFP-expressing cells and neighboring control nonexpressing cells (Figures 6E–6G). The depression was significantly larger in Rap2(ca)-GFP-expressing cells (~85%) than control cells (~50%). As a control, intracellular loading of S10, an ineffective peptide with a scrambled amino acid order, had no effect on synaptic transmission in neighboring Rap2(ca)-GFP-expressing cells (Figures 6E–6G). Collectively, these results indicate that Rap2 activity removes synaptic AMPA-Rs with long cytoplasmic termini but not those with only short cytoplasmic termini.

Previous studies have shown that dephosphorylation of AMPA-Rs with long cytoplasmic termini correlates with the synaptic removal of these receptors during synaptic depression (Song and Huganir, 2002). We first examined the levels of phosphorylated GluR2L at S841, the sites homologous to GluR1 at S845 (Song and Huganir, 2002), in extracts from slices expressing Rap2(ca)-GFP or Rap2(dn)-GFP. Western blot analysis showed that CA1 cells expressing Rap2(ca)-GFP had decreased phosphorylation of GluR2L at S841, whereas CA1 cells expressing Rap2(dn)-GFP had increased phosphorylation of GluR2L at S841 (Figure 7A). However, CA1 cells expressing Rap2(ca)-GFP or Rap2(dn)-GFP had no significant change in phosphorylation of GluR1 at S831 and S845 (Figure 7B). Given that Rap2-JNK signaling pathway controls depotentiation, which occurs only in recently potentiated synapses (Bear and Linden, 2000; Zhou and Poo, 2004), it is possible that this signaling pathway dephosphorylates only newly delivered AMPA-Rs. Because LTP-inducing stimuli or constitutively active Ras, but not spontaneous activity, drives synaptic delivery of GluR1 in vitro (Shi et al., 2001; Zamanillo et al., 1999; Zhu et al., 2002), we examined the effects of Rap2(ca)-RFP or Rap2(dn)-RFP on GluR1 in CA1 cells that coexpress active Ras(ca)-GFP, which...
drives synaptic delivery of GluR1 (Zhu et al., 2002). A number of studies indicate that the regulated trafficking of postsynaptic AMPA-Rs controls synaptic strength and plasticity at excitatory synapses (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002). Four major synaptic AMPA-R trafficking processes have so far been identified: first, activity-dependent synaptic delivery of GluR1- and GluR2L-, and GluR4-containing AMPA-Rs; second, activity-independent synaptic exchange of GluR1- and GluR2L-, or GluR4-containing AMPA-Rs with GluR2/3 AMPA-Rs; third, activity-independent synaptic cycling of GluR2/3 AMPA-Rs; and fourth, activity-dependent synaptic removal of GluR2/3 AMPA-Rs. We have recently shown that Ras and Rap1 signaling pathways control activity-dependent synaptic AMPA-R delivery and removal, respectively (Zhu et al., 2002). Whether activity-independent synaptic AMPA-R exchange and synaptic AMPA-R cycling are regulated remains unclear. What is missing from this trafficking picture is synaptic removal of AMPA-Rs with long cytoplasmic termini (e.g., GluR1- and GluR2L-containing AMPA-Rs); the obvious questions are how AMPA-Rs

Discussion

In this study, we provide evidence that Rap2 activity stimulates JNK signaling in hippocampal cells. More importantly, our results indicate that Rap2-JNK constitutes a functional signaling pathway that regulates excitatory synapses. This pathway receives inputs from NR2A-containing NMDA-Rs during synaptic activation and sends outputs that result in synaptic removal of AMPA-Rs with long cytoplasmic termini during synaptic depotentiation (Figure 8).

A number of studies indicate that the regulated trafficking of postsynaptic AMPA-Rs controls synaptic strength and plasticity at excitatory synapses (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002). Four major synaptic AMPA-R trafficking processes have so far been identified: first, activity-dependent synaptic delivery of GluR1- and GluR2L-, and GluR4-containing AMPA-Rs; second, activity-independent synaptic exchange of GluR1- and GluR2L-, or GluR4-containing AMPA-Rs with GluR2/3 AMPA-Rs; third, activity-independent synaptic cycling of GluR2/3 AMPA-Rs; and fourth, activity-dependent synaptic removal of GluR2/3 AMPA-Rs. We have recently shown that Ras and Rap1 signaling pathways control activity-dependent synaptic AMPA-R delivery and removal, respectively (Zhu et al., 2002). Whether activity-independent synaptic AMPA-R exchange and synaptic AMPA-R cycling are regulated remains unclear. What is missing from this trafficking picture is synaptic removal of AMPA-Rs with long cytoplasmic termini (e.g., GluR1- and GluR2L-containing AMPA-Rs); the obvious questions are how AMPA-Rs

Figure 7. Rap2 Activity Decreases Phosphorylation of GluR1 and GluR2L Receptors

(A) (Left) Western blots of phospho-p841-GluR2L in control hippocampal CA1 regions and hippocampal CA1 regions expressing Rap2(ca)-GFP and Rap2(dn)-GFP. Each lane was loaded with the same amount of protein (40 μg). (Right) Relative amounts of phospho-p841-GluR2L in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 16; p = 0.005) or Rap2(dn)-GFP (n = 16; p = 0.01). Relative amounts of total GluR2L in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 17; p = 0.74) or Rap2(dn)-GFP (n = 17; p = 0.44).

(B) (Upper left) Western blots of phospho-p831-GluR1 in hippocampal CA1 regions and hippocampal CA1 regions expressing Rap2(ca)-GFP and Rap2(dn)-GFP. Each lane was loaded with the same amount of protein (30 μg). (Upper right) Relative amounts of phospho-p831-GluR1 in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 16; p = 0.13) or Rap2(dn)-GFP (n = 16; p = 0.33). Relative amounts of total GluR1 in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 16; p = 0.11) or Rap2(dn)-GFP (n = 16; p = 0.15). (Lower left) Western blots of phospho-p845-GluR1 in control hippocampal CA1 regions and hippocampal CA1 regions expressing Rap2(ca)-GFP and Rap2(dn)-GFP. Each lane was loaded with the same amount of protein (40 μg). (Lower right) Relative amounts of phospho-p845-GluR1 in hippocampal CA1 regions expressing Rap2(ca)-GFP (n = 16; p = 0.15). Western blots of phospho-p841-GluR2L in control hippocampal CA1 regions and hippocampal CA1 regions expressing Rap2(ca)-GFP and Rap2(dn)-GFP. Each lane was loaded with the same amount of protein (40 μg). (Left) Western blots of phospho-p831-GluR1 in hippocampal CA1 regions expressing Ras(ca)-GFP, Ras(ca)-GFP-IRES-Rap2(ca)-RFP, and Ras(ca)-GFP-IRES-Rap2(dn)-RFP. Each lane was loaded with the same amount of protein (30 μg). (Right) Relative amounts of phospho-p831-GluR1 in hippocampal CA1 regions expressing Ras(ca)-GFP-IRES-Rap2(ca)-RFP (n = 13; p < 0.01) or Ras(ca)-GFP-IRES-Rap2(dn)-RFP (n = 13; p < 0.01) to neurons expressing Ras(ca)-GFP. Relative amounts of total GluR1 in hippocampal CA1 regions expressing Ras(ca)-GFP-IRES-Rap2(ca)-RFP (n = 13; p = 0.42) or Ras(ca)-GFP-IRES-Rap2(dn)-RFP (n = 13; p = 0.86) to neurons expressing Ras(ca)-GFP. (Lower left) Western blots of phospho-p845-GluR1 in control hippocampal CA1 regions expressing Ras(ca)-GFP, Ras(ca)-GFP-IRES-Rap2(ca)-RFP, and Ras(ca)-GFP-IRES-Rap2(dn)-RFP. Each lane was loaded with the same amount of protein (40 μg). (Lower right) Relative amounts of phospho-p845-GluR1 in hippocampal CA1 regions expressing Ras(ca)-GFP-IRES-Rap2(ca)-RFP (n = 15; p = 0.05) or Ras(ca)-GFP-IRES-Rap2(dn)-RFP (n = 15; p = 0.01) to neurons expressing Ras(ca)-GFP. Relative amounts of total GluR1 in hippocampal CA1 regions expressing Ras(ca)-GFP-IRES-Rap2(ca)-RFP (n = 15; p = 0.87) or Ras(ca)-GFP-IRES-Rap2(dn)-RFP (n = 15; p = 0.65) to neurons expressing Ras(ca)-GFP.
Rap2-JNK Signaling and Depotentiation

913

The functional roles of TNIK/MINK2 and phosphatase 2B in Rap2-JNK signaling pathway should address whether TNIK and MINK2 relay the signaling from Rap2 to JNK during synaptic depression and whether phosphatase 2B responds to Rap2-JNK signaling and dephosphorylates AMPA-Rs during depotentiation.

We reveal in this study that Rap2 removes synaptic AMPA-Rs with long cytoplasmic termini during depotentiation (Figure 8). In particular, Rap2-JNK signaling stimulates synaptic removal of GluR2L- and GluR1-containing AMPA-Rs and dephosphorylation of GluR2L at S841 and GluR1 at S831 and S845. These results are largely congruent with previous findings that dephosphorylation of AMPA-Rs with long cytoplasmic termini correlates with synaptic depression (Song and Huganir, 2002). We noted that one of our observations is at odds with a previous study, which reported that depotentiation was accompanied by dephosphorylation of GluR1 at only S845 (Lee et al., 2000). The discrepancy may be explained by a number of differences between our study and the previous report. For example, changes of phosphorylation of GluR1 are dependent on both the basal phosphorylation level of the receptor (presumably regulated by spontaneous synaptic activity) and activity patterns (Blackstone et al., 1994; Mammen et al., 1997).

In our study, culture slices, in which spontaneous synaptic activity is largely preserved (Zhu et al., 2000), were used, and most synapses of almost all CA1 neurons were affected by expression of recombinant Rap2 proteins. In contrast, the previous study examined a small number of synapses of CA1 neurons activated by electric stimuli in acute slices. Consistent with our observation, a recent study reported that the Rab5-driven synaptic removal of GluR1 is accompanied by dephosphorylation of GluR1 at both S831 and S845 in CA1 neurons (Brown et al., 2005). Further experiments are needed to determine whether the differences in experimental conditions, as well as other factors (e.g., sensitivity of anti-phospho-GluR1 antibodies), may underlie the difference between these observations.

Whether MAPK pathways cross talk is somewhat controversial. Recent evidence suggests that MAPK pathways may function independently in subcellular compartments in nonneuronal cells (Enserink et al., 2002; Mochizuki et al., 2001; Xia et al., 1995). Consistent with this idea, we have previously found that Ras-Erk1/2 and Rap1-p38 MAPK pathways independently signal synaptic delivery of GluR1- and GluR2L-containing AMPA-Rs during LTP and synaptic removal of GluR2/3 AMPA-Rs during LTD in CA1 neurons, respectively (Zhu et al., 2002). Here, we further show that Rap2-JNK controls synaptic removal of AMPA-Rs with long cytoplasmic termini (i.e., GluR1 and GluR2L) during depotentiation. Our results also suggest that activation of NR2A-containing NMDA-Rs stimulates Rap2-JNK and Ras-Erk1/2 signaling, while activation of NR2B-containing NMDA-Rs stimulates Rap1-p38 MAPK signaling. These results are consistent with recent findings that depotentiation/LTP and LTD require activation of NR2A- and NR2B-containing NMDA-Rs, respectively (Liu et al., 2004; Massey et al., 2004). Since the signaling input receptors, molecular links, targets of signaling, and functional effects are different, our data suggest that three major MAPK signaling pathways, Rap2-JNK,
Rap1-p38 MAPK, and Ras-Erk1/2, serve as independent signaling pathways (Figure 8).

The most extensively studied examples of synaptic plasticity are NMDA-R-dependent LTP, LTD, and depotentiation. Our results, together with several recent findings, are now compelling an intriguing notion that independent molecular and cellular processes control distinct forms of synaptic plasticity (Figure 8). In particular, these results indicate that depotentiation, LTP, and LTD are triggered by activation of different NMDA-Rs (Liu et al., 2004; Massey et al., 2004), mediated by separate MAPK signaling pathways and distinct aspects of AMPA-R trafficking (Zhu et al., 2000; and this study), and they employ special pools of endosomes (Brown et al., 2005; Ehlers, 2000; Lee et al., 2004; Luischer et al., 1999; Park et al., 2004). Obvious puzzles that still remain are how these three MAPK signaling pathways are linked to different NMDA-R subunits, and how they regulate cycling of distinct endosomes. Functional characterization of the guanine nucleotide exchange factors and GTPase activating proteins for Ras and Rap, a variety of which are prominent at excitatory synapses (Husi et al., 2000; Pak et al., 2001; Peng et al., 2004), as well as examination of the effects of Rap and Rap on cycling of endosomes, should provide useful answers to these important questions.

Experimental Procedures

Biochemical Analyses

Hippocampal extracts were prepared by homogenizing hippocampal CA1 regions isolated from cultured slices (Zhu et al., 2000; Zhu et al., 2002), or hippocampal culture cells (Pak et al., 2001). Viral expression efficacy of recombinant proteins in these experiments was high (>95% of CA1 neurons). Homogenization solution contained the following: 10 mM HEPES, 150 mM NaCl, 10 mM EDTA, 4 mM EGTA, 0.2 mM PMSF, 0.1 mM Na3P04, 0.5 mM NaF, 1 mM Na3VO4, 0.0001% Chymostatin, 0.0001% Leupeptin, 0.0001% Antipain, 0.0001% Pepstatin, and 1% Triton. Membranes were blotted with anti-phospho-JNK (1:2000; Cell Signaling Technology, Beverly, MA), anti-phospho-Erk1/2 (1:10000; Cell Signaling), anti-phospho-p38 MAPK antibody (1:2000; Cell Signaling), anti-phospho-p44/42 MAPK (gift from Dr. R. Malinow), anti-phospho-p845-GluR1 (1:800; Chemicon, Temecula, CA), or anti-phospho-p831-GluR1 (1:1000; Chemicon), stripped, and reblotted with anti-JNK (1:2000; Cell Signaling), anti-Erk1/2 (1:2000; Cell Signaling), anti-p38 MAPK antibody (1:1000; Cell Signaling), anti-GluR2L (Kolleker et al., 2003), or anti-GluR1 (1:8000; Chemicon). Active Rap2 was detected by affinity precipitation of Rap2-GTP with GST-linked Rap binding domain of Raf-1 or RafGDS (GST-RBD) as described in a previous report (de Rooij et al., 1999). The bound Rap2-GTP was then eluted and Western blotted with anti-Rap2 antibodies (1:1000, Santa Cruz Bio-technology, Santa Cruz, CA; or 1:2500, Transduction Laboratories, Lexington, KY). Rap effectors were purified by incubating GST-Rap1(V12), GST-Rap2(V12) (gifts of Dr. J. de Gunzburg), or GST alone with membrane extracts (crude synaptosomes from 40 adult rat brains solubilized in 1% Triton X-100). Bound material was washed, concentrated, extracted, and then separated by SDS-PAGE. A 150 kDa band specifically associated with active Rap2 was excised and subjected to microcapillary LC/MS/MS tandem mass spectrometry for protein identification. Western blots were quantified by chemiluminescence and densitometry scanning of the films under linear exposure conditions.

Constructs of Recombinant Proteins and Expression

Rap2 mutant constructs, including the constitutively active mutant (G12 – E), dominant-negative mutant (S17 – N), and null/dead mutant (F39 – S; Machida et al., 2004), were generated using Quick Change Site-Directed mutagenesis kit (Strategene, La Jolla, CA). These mutants were then subcloned into pEGFP-C1 (enhanced GFP, Clontech Laboratories, Palo Alto, CA) or PRF-P-C1 (generated by replacing EGFP in pEGFP-C1 with dsRed-MST-B), GFP-tagged dominant-negative TNK, TNK(dn)-GFP, was made by ligating the C-terminal regulatory domain of TNK sequence into pEGFP-C1 (Taira et al., 2004). To coexpress Ras(ca)-GFP with Rap2(ca)-RFP or Rap2(ca)-RFP virally, Ras(ca)-GFP and Rap2(ca)-RFP or Rap2(cn)-RFP were subcloned before and after an internal ribosomal entry site (IRES) sequence (from pIRES2OG) to produce Rap2(ca)- Rap2-GFP-IRES-Rap2(ca)-RFP or Rap2(cn)-GFP-IRES-Rap2(cn)-RFP. Other constructs were made as previously described (Kolleker et al., 2003; Zhu et al., 2000; Zhu et al., 2002). Constructs were expressed in CA1 neurons in hippocampal slices using Sindbis virus or biosilics transfection. Slices were prepared from postnatal 6- to 7-day-old rats, infected with virus or transfected using gene gun after 6-8 days in vitro, and incubated on culture media and 5% CO2 for 14 ± 2 h before experiments. By monitoring GFP or RFP fluorescence, we observed that recombinant Rap2 proteins first appeared in CA1 neurons after 6-8 h of infection or transfection. Western blot analysis showed that recombinant Rap2 expression level reached 6-8 times the endogenous Rap2 level after 14 ± 2 h of infection or transfection (see Figure 1A). Thus, all experiments were performed within 14 ± 2 h after infection or transfection to minimize activation of multiple or nonspecific pathways due to excessive overexpression of recombinant proteins and/or prolonged activation of Rap2 signaling pathway (e.g., autocrine/paracrine effects; see McCarthy et al., 1995).

Electrophysiology

Simultaneous whole-cell recordings were obtained from pairs or triples of neighboring infected/transfected and noninfected/nontransfected CA1 neurons, under visual guidance using fluorescence and transmitted light illumination (Larkum and Zhu, 2002; Zhu et al., 2000). Synaptic currents were recorded using up to three Axopatch-200B and/or Axoclamp-1D amplifiers (Axon Instruments, Foster City, CA). Bath solution (29°C ± 1.5°C), unless otherwise stated, contained the following: 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 4 mM MgCl2, 26 mM NaHCO3, 1 mM Na2HPO4, 11 mM glucose, 0.1 mM picrotoxin, 0.01 mM bicuculline, and 0.002 mM 2-chloroadenosine (pH 7.4 and gassed with 5% CO2/95% O2). These drugs were included during recordings. NR2A- and NR2B-containing NMDA-R inhibitors Ro25-6981 (Sigma-Aldrich, St. Louis, MO) and NVP-AAM077 (Novartis Pharma AG, Basel, Switzerland), which were included in culture media, were not included in the bath solution during recordings. Patch recording pipettes (3–6 MΩ) contained the following: 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl2, 4 mM Na2ATP, 0.4 mM Na3GTP, 10 mM sodium phosphocreatine, 0.6 mM EGTA, and 0.1 mM spermine (pH 7.25). The access resistance (10–25 MΩ) and input resistance (300–500 MΩ) were continuously monitored during experiments and varied less than 20% during any experimental series. Occasionally, recordings had higher access resistance or lower input resistance, or displayed changes in access and input resistance more than 20%; these recordings were not included in analysis. Synaptic responses were evoked by bipolar electrical stimuli with single voltage pulses (200 μs, up to 20 V) placed in s. radiatum up to 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 NR2A 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 to 5 V within 5 min after formation of whole-cell configuration. Depotentiation is induced by a pairing protocol using 300 pulses at 1 Hz at ~45 mV 30 min after induction of LTP in the presence of 100 μM CNQX, 100 μM AP5, and 0.5 mM D-AP5.
Scotch Plains, NJ) were dissolved (1 mM) in Cs-based internal solu-
tion. All results are reported as mean ± SEM, and statistical differ-
ces of the means were determined using Wilcoxon nonparamet-
ric test unless otherwise stated. Significance was set at p < 0.05.

Supplemental Data
The Supplemental Data for this article can be found online at http://
www.neuron.org/cgi/content/full/46/6/905/DC1/.

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Erratum

Rap2-JNK Removes Synaptic AMPA Receptors during Depotentiation

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The authors wish to correct two errors in the paper. The sentence that begins on page 913, right column, line 16 should read as follows (change is underlined): “We noted that one of our observations is at odds with a previous study, which reported that depotentiation was accompanied by dephosphorylation of GluR1 at only S831 (Lee et al., 2000).”

In Figure 8, GluR2/3 and GluR1/2L were mistakenly switched in Rap1 and Rap2 signaling pathways. The correct figure is printed here.

Figure 8. Model for Ras-Erk1/2, Rap1-38 MAPK, and Rap2-JNK Pathway-Mediated Synaptic Plasticity

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