Regulation of AMPA receptor subunit GluA1 surface expression by PAK3 phosphorylation

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AMPA receptors (AMPARs) are the major excitatory receptors of the brain and are fundamental to synaptic plasticity, memory, and cognition. Dynamic recycling of AMPARs in neurons is regulated through several types of posttranslational modification, including phosphorylation. Here, we identify a previously unidentified signal transduction cascade that modulates phosphorylation of serine residue 863 (S863) in the GluA1 AMPAR subunit and controls surface trafficking of GluA1 in neurons. Activation of the EphR–Ephrin signal transduction pathway enhances S863 phosphorylation. Further, EphB2 can interact with Zizimin1, a guanine–nucleotide exchange factor that activates Cdc42 and stimulates S863 phosphorylation in neurons. Among the numerous targets downstream of Cdc42, we determined that the p21-activated kinase-3 (PAK3) phosphorylates S863 in vitro. Moreover, specific loss of PAK3 expression and pharmacological inhibition of PAK both disrupt activity-dependent phosphorylation of S863 in cortical neurons. EphB2, Cdc42, and PAKs are broadly capable of controlling dendritic spine formation and synaptic plasticity and are implicated in multiple cognitive disorders. Collectively, these data delineate a novel signal cascade regulating AMPAR trafficking that may contribute to the molecular mechanisms that govern learning and cognition.

Zizimin | AMPA | ephR | synaptic | PSD

AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) are the primary mediators of excitatory synaptic transmission in the brain (1). AMPARs are tetrameric ion channels that display distinct functional properties, based on which combinations of subunits, named GluA1–4, are coassembled to form receptor subtypes. Several studies demonstrate that aberrant AMPAR trafficking results in impaired memory and cognition and is associated with numerous neurological disorders (2). Dynamic control of AMPAR recycling to and from synapses is regulated through posttranslational modifications of receptor subunits and through specific interactions with accessory proteins, which in turn can be regulated by neuronal activity. For instance, patterned increases in neuronal activity can initiate signaling cascades that recruit AMPARs into the synaptic membrane and result in an overall strengthening of the synapse; this form of synaptic plasticity is called long-term potentiation (LTP). Conversely, stimulation protocols initiating postsynaptic membrane removal of AMPARs result in a weakening of the synapse and are known as long-term depression (LTD) (3). Several decades of research demonstrate that both LTP and LTD, widely considered the molecular correlates of learning and memory, can be explicitly modulated by the phosphorylation/dephosphorylation state of AMPAR subunits (3).

Members of the Rho GTPase family of proteins are molecular switches, transitioning between inactive/active states to control numerous physiological functions (4, 5). Canonical members include Rho, Rac1, and Cdc42, which regulate many cellular mechanisms, but particularly those involving actin cytoskeletal reorganization, such as cell polarity, migration, and membrane trafficking (4, 6). To selectively integrate extracellular and intracellular signals, Rho GTPases bind to specific targets, known as effector molecules, which in turn propagate downstream signaling cascades. The first Rho GTPase effector identified was p21-activated kinase-1 (PAK1), a member of a superfamily of kinases (7). The PAK protein family comprises six members, falling into two categories: group I (consisting of PAKs 1, 2, and 3) and group II (includes PAKs 4, 5, and 6), which are distinguished based on sequence homology and are functionally regulated by distinct mechanisms. Although PAK proteins differ in their specific developmental/subcellular distribution, they are primarily implicated in regulating actin cytoskeletal dynamics (8). However, the possibility that PAK signaling controls AMPAR localization and/or trafficking has not been investigated.

In this study, we identified a novel site in the GluA1 subunit of AMPAR (serine 863) that is rapidly phosphorylated following brief disruption of neuronal activity and which critically regulates surface expression of AMPARs in neurons. Moreover, activation of p21-activated kinase-3 (PAK3) through Cdc42 greatly enhances phosphorylation of S863. In contrast, targeted loss of PAK3 expression disrupted activity-dependent phosphorylation of S863 in neurons. To delineate upstream modulators of this phosphorylation we focused on Eph receptor (EphR) mediated-signal transduction, which regulates both Cdc42 and PAK activity and is known to modulate synaptic recruitment of AMPAR (9–13). Using heterologous and neuronal cell culture assays we demonstrate that activation of EphB2 receptor enhances GluA1 phosphorylation at S863 in vivo. Additionally, we identified the guanine–nucleotide exchange factor Zizimin1 as a novel binding partner of EphB2. Our study reveals that Zizimin1-mediated in vivo activation of Cdc42 is sufficient to stimulate GluA1 phosphorylation at S863. Our findings identify a novel signaling cascade involving EphB2, Zizimin1, Cdc42, and PAK3 that controls GluA1-S863 phosphorylation and thereby regulates neuronal trafficking of AMPARs.

Significance

Precise choreography of AMPA-type glutamate receptor (AMPAR) movements within neurons is critical for brain function; aberrant AMPAR trafficking is associated with impaired synaptic plasticity and cognitive deficits. We identified a previously unidentified phosphorylation site in AMPAR subunit GluA1 (serine 863) that regulates neuronal trafficking of GluA1. We define a specific signal transduction pathway that controls GluA1-S863 phosphorylation, mediated by EphB2, Zizimin1, Cdc42, and p21-activated kinase-3 (PAK3). These signaling proteins are associated with modulation of neuronal morphology and AMPAR recruitment to dendritic spines. EphB2 and PAK3 are implicated in cognitive disorders, including Alzheimer's and X-linked intellectual disability. Collectively, the GluA1-S863 phosphorylation signal cascade delineates a novel pathway regulating AMPAR trafficking that may be important in the modulation of learning and memory.

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Results

AMPAR Subunit GluA1 Is Phosphorylated at Serine 863 Through Activation of Rho GTPases. Several AMPAR-phosphorylation sites are critical for modulating receptor recycling across internal endosomal compartments and to the neuronal cell surface/synapse (14). We recently identified one such JNK-mediated phosphorylation site conserved within the C-terminal regions of the GluA2L (T912) and GluA4 (T855) subunits that regulates reinsertion of AMPARs to the neuronal surface (15). Sequence alignment of AMPAR subunits revealed a serine in GluA1 (S863) that specifically diverges from the threonine (T912/T855) in GluA2L/GluA4 (Fig. 1*A*). Given the notable deviation of GluA1 from this conserved site in GluA2L/GluA4, we asked whether GluA1-S863 plays any role in regulating AMPAR trafficking. We designed a phosphorylated peptide immunogen to generate rabbit polyclonal antibodies against the GluA1-S863 putative phosphorylation site as previously described (15). GluA1 immunoprecipitates from cortical neurons showed strong immunoreactivity with α-S863(P), and lambda phosphatase treatment confirmed phosphospecificity of this signal (Fig. 1*B*). To further characterize the α-S863(P) antibody we transiently transfected HEK cells with different forms of GFP-tagged GluA1 harboring point mutations at S863. Following 24 h overexpression each construct was immunoprecipitated at levels comparable to WT GluA1 [GFP-GluA1 (WT)], as detected by Western blot analysis using total α-GFP (Fig. 1*C*). Although immunoreactivity to phosphomimetic proteins is uncommon for phosphospecific antibodies, we determined that α-S863(P) antibody was capable of detecting a phosphomimetic form of GFP-GluA1 where S863 was mutated to encode an aspartate (S863D). However, α-S863(P)–mediated detection of phosphorylation was abolished in a phosphodeficient mutant of GFP-GluA1 at S863 (S863A, serine to alanine mutation) and only weakly detectable in immunoprecipitates isolated from GFP-GluA1 (WT) expressing cell lysates (Fig. 1*C*).

Phosphorylation events are dynamic and result from highly regulated processes in neurons. Having established precedence for activity-dependent modulation of GluA2L (T912) and GluA4 (T855) phosphorylation (15), we examined whether neuronal activity regulates the analogous GluA1-S863 region. Western blot analyses of endogenous GluA1 immunoprecipitated from cultured 18 d in vitro (DIV 18) cortical neurons revealed that under basal (control) conditions, GluA1-S863 phosphorylation was only weakly detected by α-S863(P) antibody (Fig. 1 *D* and *E*). However, brief inhibition of spontaneous neuronal activity upon bath application of tetrodotoxin (TTX, 2 μM for 20 min) resulted in robust phosphorylation of endogenous GluA1 at S863 (Fig. 1 *D* and *E*). Activation of NMDA-dependent glutamatergic receptors was not required for GluA1-S863–dependent phosphorylation because incubation with the NMDA-R antagonist, 2-amino-5-phosphonovalerate (APV), before TTX treatment had no effect on the significant level of phosphorylation detected (Fig. 1 *D* and *E*).

Which kinase(s) might phosphorylate GluA1-S863? GluA2L (T912) and GluA4 (T855) are phosphorylated by JNK (c-Jun N-terminal kinases), but the sequence surrounding GluA1-S863 does not conform to a proline-directed kinase consensus sequence (Fig. 1*A*), suggesting that S863 phosphorylation is unlikely to be targeted by JNK, nor by other MAPKs (mitogen-activated protein kinases), CDKs (cyclin-dependent kinases), GSK3 (glycogen synthase kinase 3), or CLK (cdc2-like kinase) family kinases (16). To narrow this search we sought to identify prospective signal transduction pathways contributing to GluA1-S863 phosphorylation. We used a candidate approach to this effort, beginning with the Rho-family of small GTPases. In neurons, Rho GTPase mediated-transduction affects actin-dependent modulation of dendritic spine morphogenesis, AMPAR recycling, and synaptic plasticity (5, 17–19). To explore the potential for Rho GTPases to stimulate phosphorylation of GluA1-S863, we immunoprecipitated total GluA1 that had been exogenously coexpressed with constitutively active (CA) Rac1, RhoA, or Cdc42 for 24 h in HEK cells. Coexpression of GluA1 with Rac1 CA and Cdc42 CA, but not RhoA CA, significantly increased GluA1 S863 phosphorylation relative to a vector control (Fig. 1 *F* and *G*). These findings

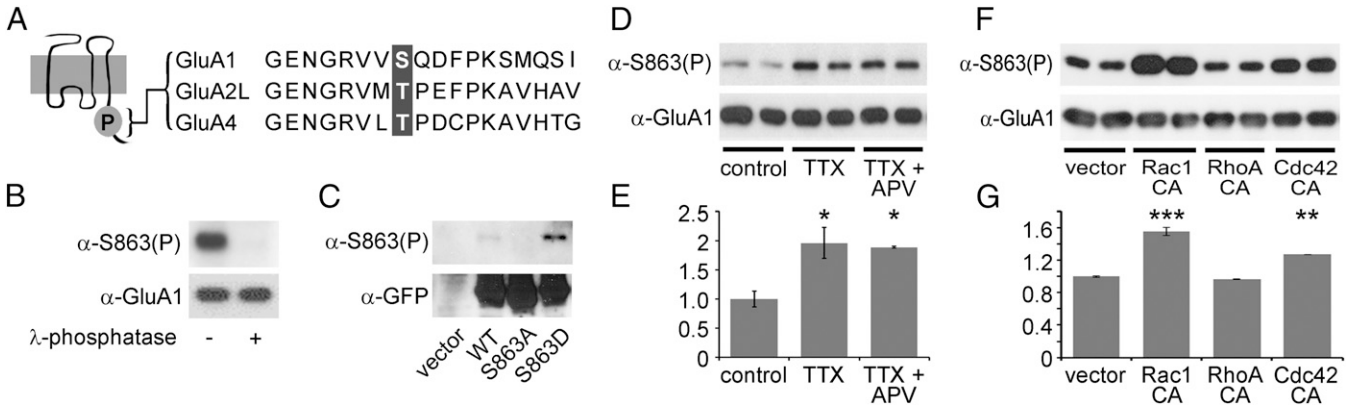

Fig. 1. AMPAR subunit GluA1 is phosphorylated at Serine 863 and regulated by Rho GTPases. (*A*) C-terminal sequences from AMPAR subunits aligned to indicate sequence divergence of serine 863 in GluA1 (highlighted in gray) from the conserved JNK-phosphorylated threonine in GluA2L and GluA4 (threonine 912 and 855, respectively). (*B*) GluA1 immunoprecipitates from cortical neurons were incubated in phosphatase assay buffer with (+) or without (–) lambda phosphatase before Western blotting with α-S863(P) (*Top*) or GluA1 antibody (*Bottom*). (*C*) Western blot analysis using α-S863(P) antibody specifically identifies phosphomimetic (S863D) and not phosphodeficient (S863A) GluA1, as well as weakly detecting basal phosphorylation of WT GluA1 tagged with GFP and immunoprecipitated from HEK cells using GFP antibodies (*Top*). Total protein levels were detected using GFP antibody (as indicated at *Bottom*). (*D*) Rapid modulation of neuronal activity affects GluA1-S863 phosphorylation state. After 18 d in vitro, cortical neurons were untreated (control), briefly stimulated 20 min with 1 μM TTX, or pretreated 1 h with 100 μM APV before 20 min with 1 μM TTX in the continued presence of APV. Lysates were prepared for immunoprecipitation of endogenous GluA1, followed by Western blot analyses using α-S863(P) or total GluA1 antibodies (indicated at *Left*). (*E*) Quantification of S863(P) levels relative to control as detected by Western blotting of *D*. Error bars indicate ± SEM. *$p < 0.04$, ANOVA. (*F*) Activated Rho GTPases regulate GluA1-S863 phosphorylation. Constitutively active forms of Rac1 (Rac CA) and Cdc42 (Cdc42 CA), but not RhoA (RhoA CA), increased WT GluA1-S863 phosphorylation when coexpressed in HEK cells. Lysates were prepared for immunoprecipitation of GluA1, followed by Western blot analyses using α-S863(P) or total GluA1 antibodies (indicated at *Left*). (*G*) Quantification of S863(P) levels relative to vector control as detected by Western blotting of *F*. Error bars indicate ± SEM. **$p < 0.002$ and ***$p < 0.0001$, ANOVA.

demonstrate that specific Rho GTPase-mediated signal transduction is involved in phosphorylation of GluA1 at S863.

Activated Cdc42 Stimulates PAK3 Kinase to Phosphorylate GluA1 at Serine 863. We next sought to identify the kinase(s) that function downstream of Rac1 and Cdc42 to mediate GluA1-S863 phosphorylation. Although the human genome includes in excess of 500 protein kinases (20), we limited our search to those which encode a signature Cdc42/Rac1 interactive-binding motif (CRIB) (21) and are also capable of targeting serine/threonine amino acids for phosphorylation (16). These criteria are uniquely fulfilled by the PAK (p21 activated kinases), as well as MRCK (Myotonic dystrophy kinase-related Cdc42-binding kinase) enzyme families. Therefore, we overexpressed either Myc-PAK3 or hemagglutinin (HA) tagged MRCKα along with GluA1 in HEK cells to test whether either kinase group stimulates GluA1-S863 phosphorylation downstream of constitutively active Cdc42 or Rac1. Negligible phosphorylation was detected following immunoprecipitation of GluA1 expressed alone in HEK cells (Fig. 2*A*). However, coexpression of either Rac1 CA or Cdc42 CA and GluA1 along with Myc-PAK3 greatly enhanced S863 phosphorylation relative to GTPase expression with GluA1 alone (Fig. 2*A*). Conversely, expression of HA-MRCKα with active GTPase did not enhance GluA1-S863 phosphorylation compared with expression of active GTPases in the absence of a kinase (Fig. 2*A*). These data demonstrate that signal transduction mediated by activated Rac1 and Cdc42 stimulates PAK3, and not MRCKα, to phosphorylate GluA1-S863. Furthermore, pharmacological inhibition of PAK in cultured cortical neurons completely abolished TTX-mediated increase in S863 phosphorylation (Fig. 2*B*). These data support the finding that PAKs, rather than MRCK, are the primary kinase family mediating GluA1-S863 phosphorylation.

To determine whether GluA1-S863 phosphorylation is selectively mediated by one PAK family category versus the other, we coexpressed GluA1 with representative group I (Myc-PAK1 and 3) or group II (Myc-PAK6) PAK members in HEK cells. Western blot analyses with α-S863(P) antibody following immunoprecipitation of GluA1 revealed no increase in phosphorylation upon coexpression with either group I or group II PAK enzymes alone. However, when coupled with either Cdc42 CA or Rac1 CA, expression of Myc-PAK3 stimulated robust phosphorylation of GluA1-S863 beyond that seen with Cdc42 CA or Rac1 CA alone, whereas Myc-PAK1 coupled with Rac1 CA only slightly increased S863 phosphorylation (Fig. 2*C*). Myc-PAK6 failed to trigger phosphorylation downstream of either Rho GTPase (Fig. 2*C*), consistent with evidence that group II PAK function is regulated independent of direct interaction with Cdc42 or Rac1 (22). Moreover, these data suggest that downstream of Cdc42 and Rac1 activation, S863 phosphorylation is principally mediated by PAK3.

To determine whether PAK3 directly phosphorylates GluA1, we performed in vitro kinase assays using purified GST-GluA1 C-tail fusion protein (which includes S863) or GST alone as a control. Equal expression and loading of purified protein levels was confirmed by Western blotting with antibodies against GST (Fig. 2*D*, *Middle*). As expected, autophosphorylation of purified active PAK3 was detected by ^{32}P incorporation (Fig. 2*D*, *Bottom*). Moreover, autoradiography detected a lower molecular weight band specifically upon inclusion of purified GST-GluA1 C-tail protein but not GST protein control (Fig. 2*D*, *Bottom*). Western blot analyses with α-S863(P) antibody confirmed that this in vitro phosphorylated substrate of PAK3 is GluA1-S863 (Fig. 2*D*, *Top*).

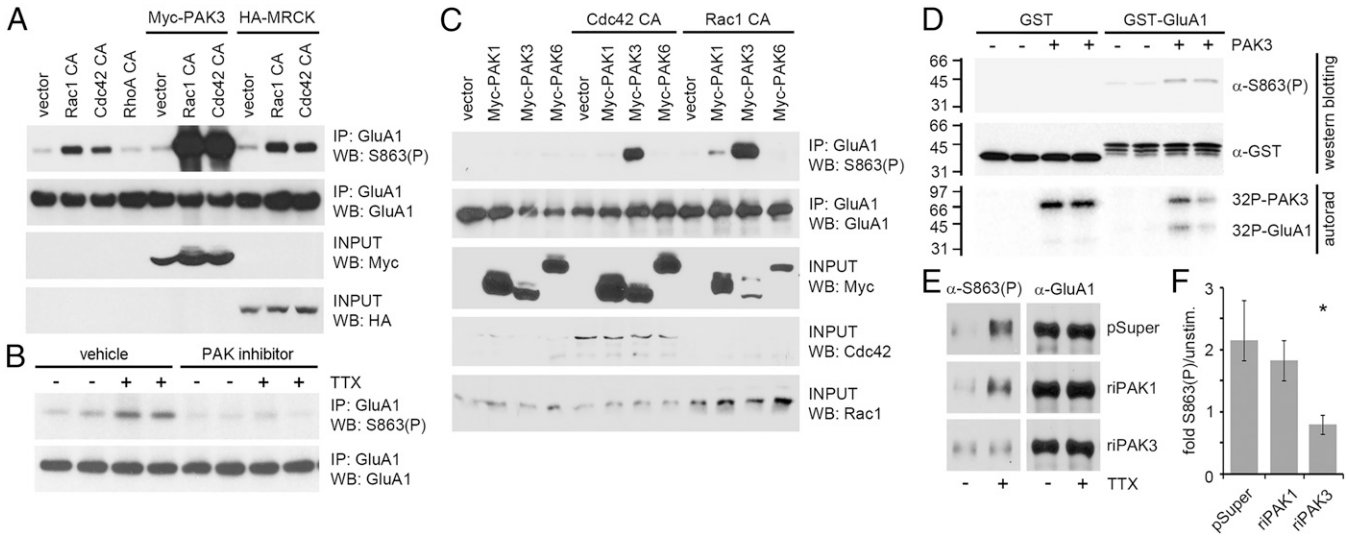

Fig. 2. PAK3 kinase phosphorylates GluA1-S863 in heterologous cells, in vitro, and in neurons. (*A*) GluA1 was immunoprecipitated (IP) from HEK cells following coexpression with an empty vector, constitutively active (CA) Rac1, Cdc42, or RhoA either alone or in conjunction with Myc-PAK3 or HA-MRCK (as indicated at *Top*). Lysates were prepared and processed for Western blot (WB) analyses using the indicated antibodies (*Right*). (*B*) Pharmacological inhibition of PAK disrupts GluA1-S863 phosphorylation. Cortical neurons cultured 18 d in vitro were pretreated for 1 h with vehicle (DMSO) or 10 μM PAK inhibitor before 20 min application of 1 μM TTX. Lysates were prepared for immunoprecipitation of endogenous GluA1, followed by Western blot analyses (as indicated at *Right*). (*C*) GluA1 and either PAK Type I (Myc-PAK1 and Myc-PAK3) or Type II (Myc-PAK6) family members were coexpressed HEK cells in conjunction with Cdc42 CA or Rac CA. Lysates prepared for immunoprecipitation of GluA1(IP) and total extract aliquots (INPUT) were analyzed by Western blot with α-S863(P), total GluA1, Myc, and endogenous Cdc42 or Rac1 antibodies (indicated at *Right*). (*D*) Purified PAK3 phosphorylates GluA1 in vitro. Immunoblots using α-S863(P) (*Top*) or GST antibody (*Middle*) and autoradiography (*Bottom*) of purified GST and GST-GluA1 C-terminal used in kinase assays in the presence (+) or absence (–) of active PAK3. (*E* and *F*) Loss of endogenous PAK3 from cortical neurons abrogates TTX-mediated enhancement of GluA1-S863 phosphorylation. (*E*) Rat cortical neurons transfected by electroporation at DIV 0 with vector control (pSuper) or shRNA directed against either rat PAK1 (riPAK1) or rat PAK3 (riPAK3) remained untreated (control) or were stimulated with 1 μM TTX for 20 min following 18 d in vitro. Lysates were prepared for immunoprecipitation of endogenous GluA1, followed by Western blot analyses using α-S863(P) (*Left*) or total GluA1 (*Right*) antibodies (as indicated at *Right*). (*F*) Quantification of TTX stimulated versus unstimulated fold increases in S863(P) levels normalized to total immunoprecipitated GluA1. Error bars indicate ± SEM. *$p < 0.03$, ANOVA. $n = 11$.

Having determined that group I PAK expression is sufficient to phosphorylate S863 in vitro, we next investigated whether group I PAKs are required for GluA1 S863 phosphorylation in neurons. Specifically, we knocked down expression of either PAK1 or PAK3 using plasmid-based short-hairpin RNA (shRNA) interference in cortical neurons and briefly inhibited spontaneous neuronal activity to stimulate phosphorylation of GluA1-S863. Endogenous AMPAR phosphorylation was monitored by Western blot following immunoprecipitation of total GluA1. Relative to vector control (pSuper), knockdown of PAK1 (riPAK1) had no effect on TTX-mediated GluA1 S863 phosphorylation (Fig. 2 *E* and *F*). In contrast, knockdown of PAK3 (riPAK3) caused a significant block of TTX-induced enhancement of GluA1-S863 phosphorylation (Fig. 2 *E* and *F*). Collectively, our results demonstrate that S863 of GluA1 is a novel activity-dependent phosphorylation site selectively targeted by PAK3 kinase acting downstream of activated Cdc42 and Rac1.

Zizimin1 Activates Cdc42 to Regulate S863 Phosphorylation. Rho-family GTPases are activated by guanine nucleotide exchange factors (GEFs), which catalyze their exchange of GDP for GTP (23). Fundamentally, these enzymes are cellular gatekeepers of Rho GTPase-mediated signal transduction. We recently identified Zizimin1/Dock9, a member of the DOCK (Dedicator of Cytokinesis) superfamily of GEFs, as a novel interactor of AMPARs in the brain (24). This finding was of particular interest for this study because Zizimin1 targets and activates Cdc42 to regulate dendritic growth in cortical and hippocampal neurons (25). We hypothesized that Zizimin1 interaction with AMPAR combined with its regulation of Cdc42 could delineate a novel signaling cascade stimulating PAK3-mediated phosphorylation of GluA1-S863 in neurons. To test this, we generated constructs encoding full-length HA-tagged Zizimin1 or its catalytic domain fused to GFP (GFP-CZH2) (Fig. 3*A*). Transiently transfected GluA1 was immunoprecipitated from HEK cells coexpressing GFP-CZH2, and immunopurified proteins were processed by SDS/PAGE. Western blot analyses with GFP and GluA1 antibodies revealed that the C-terminal region of Zizimin1 is sufficient to mediate interaction with GluA1 in cells (Fig. 3*B*). To investigate its downstream signal transduction activity we expressed Zizimin1, WT Cdc42, or Rac1 alone or in combination with GluA1 and probed for phosphorylation of S863. Although coexpression of HA-Zizimin1 with GluA1 modestly increased S863 phosphorylation, this was not enhanced upon concomitant expression of Rac1 (Fig. 3*C*). In contrast, Cdc42 and GluA1 coexpressed with either full-length (HA-Zizimin1) or the isolated catalytic domain (GFP-CZH2) of Zizimin1 resulted in robust S863 phosphorylation (Fig. 3*C*). Together these findings are consistent with the reported preferential enzymatic activity of Zizimin1 toward Cdc42 over Rac1 (26). Moreover, our data demonstrate that Zizimin1-mediated activation of Cdc42 in vivo stimulates a signal transduction pathway that increases phosphorylation of GluA1 at S863.

To investigate the functional significance of S863 phosphorylation we transfected GFP-tagged WT, phosphodeficient (S863A), or phosphomimetic (S863D) forms of the GluA1 subunit into DIV 18 hippocampal neurons for 48 h and assessed their effect on surface AMPAR expression (Fig. 4*A*). Quantification of the integrated intensity of surface-expressed GFP-tagged GluA1 (sGFP) dendritic clusters revealed that both mutations of S863 robustly increased surface trafficking compared with WT GluA1 (Fig. 4*B*). These data demonstrate that the integrity of S863 is a crucial component controlling surface GluA1 expression in hippocampal neurons.

EphR Signaling Stimulates S863 Phosphorylation and Enhances EphB2-GluA1 Interaction in Neurons. Collectively, our data delineate a novel Zizimin1-Cdc42-PAK3 cascade capable of modulating S863-dependent surface trafficking of GluA1. What upstream factors might regulate this pathway? Several factors directed our focus toward EphR tyrosine kinases and their cognate ephrin ligands in addressing this question. Notably, ephrinB-mediated activation of EphB2 receptor stimulates excitatory synaptogenesis through recruitment of AMPARs to synaptic sites (9–11). In addition, EphB2 forward signaling stimulates Cdc42 and PAK activation to regulate filipodia motility and dendritic spine maintenance (10, 12, 13). Therefore, we examined whether EphR signaling in cortical neurons affects phosphorylation of GluA1-S863. Total GluA1 was immunoprecipitated from cell lysates isolated from untreated cultured cortical neurons (DIV 18) or from neurons treated with clustered EphB2-Fc (EphB2), ephrinB2-Fc (EphrinB2), or control Fc alone (IgG), TTX, or KCl (Fig. 5*A*, *Bottom*). Western blotting with α-S863(P) revealed that EphrinB2 as well as TTX treatment increases GluA1-S863 phosphorylation, whereas each of the other treatments failed to do so (Fig. 5*A*, *Top*). We further reasoned that EphR signaling might also regulate the formation of GluA1-mediated protein complexes in neurons. GluA1 coimmunoprecipitated EphB2 from cortical neurons, in which EphR signaling was specifically activated by treatment of clustered EphrinB2 (Fig. 5*B*). These data indicate that stimulus-dependent EphrinB2-induced

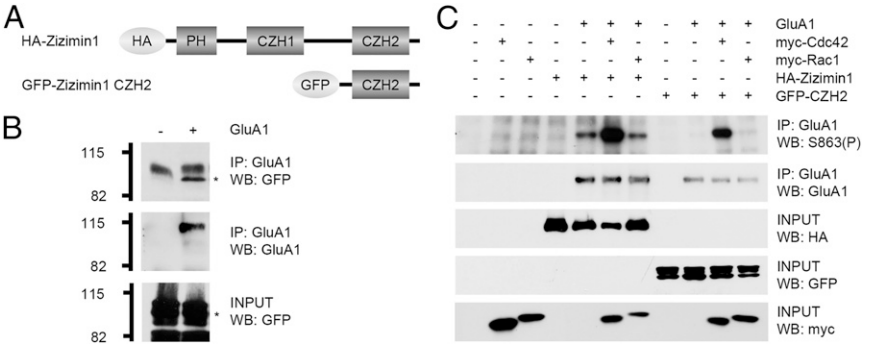

Fig. 3. Zizimin is a guanine nucleotide exchange factor that increases S863 phosphorylation on GluA1. (*A*) Schematic diagram demarcating the pleckstrin homology (PH) and CDM-Zizimin homology 1 and 2 (CZH1 and CZH2) domains encoded by full-length HA-tagged Zizimin1, in contrast to a truncated GFP-tagged Zizimin1 (GFP-CZH2) construct used for overexpression in heterologous cells. (*B*) HEK 293T cells were cotransfected with GFP-CZH2 Zizimin1 and either empty vector (–) or GluA1 (+). Total cell extract (INPUT) and GluA1 immunoprecipitate (IP) were Western-blotted with antibodies to either GFP or total GluA1 (indicated at *Right*). (*C*) Activation of Rac1, Cdc42, by Zizimin1 stimulates GluA1 S863 phosphorylation in cells. Myc-tagged GTPases, full-length (HA-Zizimin1), or truncated Zizimin1 (GFP-CZH2) were transiently expressed alone or in combination with GluA1 in HEK 293T cells (as indicated at *Top*). Total cell extract (INPUT) and GluA1 immunoprecipitate (IP) were Western-blotted (WB) with the indicated antibodies (*Right*).

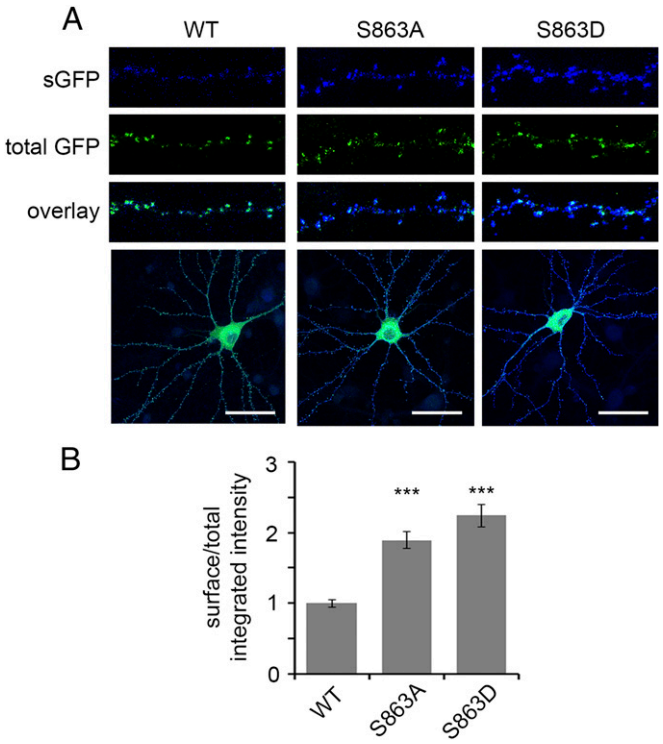

Fig. 4. Serine 863 mutation modulates surface expression of AMPARs. (*A*) Representative images of 20-d-old hippocampal neurons transfected for 48 h with GFP-tagged GluA1 WT or with those harboring S863 mutation to alanine (S863A) or aspartic acid (S863D) (indicated at *Top*). Surface (sGFP) and total expression of receptors were detected by immunostaining with GFP antibodies (indicated at *Left*). (Scale bars, 40 μm). Enlarged dendritic regions are 40-μm segments. Modification of S863 increases surface AMPA expression. (*B*) Bar graph of mean integrated intensity of dendritic surface GFP-tagged S863A and S863D clusters normalized to WT GFP-GluA1 (WT) transfected hippocampal neurons. Error bars indicate ± SEM. ***$p < 0.001$ relative to WT-transfected neurons, ANOVA. $n \geq 12$ neurons for each.

phosphorylation of S863 enhances the interaction between GluA1 and EphB2 in cortical neurons.

EphR-Ephrin signal transduction stimulates interaction of EphRs with GEFs and other adaptor molecules to subsequently activate members of the Rho GTPase family (10, 11, 13). Our data indicate that activation of EphR-Ephrin and modulation of S863 each function in a convergent signal transduction pathway. To test this hypothesis we sought to identify further links between EphRs signaling and the Zizimin1-Cdc42-PAK3–signaling cascade. Heterologous EphB2 expression stimulates clustering and activation of EphB2 receptors (27). Using this approach to activate EphR signaling in HEK cells, we coexpressed Flag-EphB2 with HA-Zizimin1 or with an empty vector control for 48 h followed by cell lysate preparation for coimmunoprecipitation assays. Immunoprecipitation assays demonstrate that HA-Zizimin1 specifically copurifies EphB2 from cells coexpressing Flag-EphB2, and not an empty Flag-vector control (Fig. 5*C*, *Center*). The inverse immunoprecipitation of Flag-EphB2 confirmed that a specific complex with HA-Zizimin1 is readily formed in cells (Fig. 5*C*, *Right*). The interaction between EphB2 and Zizimin1 supports a molecular mechanism whereby EphR-Ephrin signaling through Zizimin1, Cdc42, and PAK3 ultimately regulates S863 phosphorylation.

EphR-Ephrin activation enhances synaptic expression of AMPAR (9–11). Because mutation of S863 also regulates surface clustering of GluA1 receptor, we addressed whether modification of this residue effects EphR-Ephrin dependent recruitment of AMPAR. Cultured hippocampal neurons (DIV 18) transfected for 48 h with GFP-GluA1 (WT) were treated with clustered EphrinB2-Fc (EphrinB2) or Fc control alone (IgG) and processed for immunohistochemical analyses of surface relative to total GluA1 expression (see representative images, Fig. 5*D*). Consistent with published findings, EphrinB2-Fc significantly increased surface WT GluA1 cluster intensity relative to control treatment (Fig. 5*D*, bar graph). In contrast, regardless of EphrinB2 or IgG treatment, neurons transfected with either S863 mutation (S863A or S863D) had significantly increased surface GluA1 cluster intensity compared with WT-IgG–treated cells (Fig. 5*D*). Thus, consistent with our findings from Fig. 4 *A* and *B*, mutation of S863 enhances surface GluA1 and occludes the effect of EphrinB2 (Fig. 5*D*). Finding that EphB2 signal activation and S863 mutation effects on surface AMPAR expression are not additive suggests that a shared signal transduction pathway exists between EphR-Ephrin and S863-mediated modulation of AMPARs trafficking.

Discussion

Several studies demonstrate that aberrant membrane trafficking of AMPARs impairs memory and cognition and is correlated with neurological disorders, including Alzheimer's disease, epilepsy, and Parkinson's disease (3, 28). AMPARs are tetrameric channels formed by assembly of the subunits GluA1–4. In adult rat hippocampal neurons, AMPARs mainly consist of combinations of GluA1/2 or GluA2/3 heteromers or GluA1 homomers (29, 30). Each of these subunits are phosphorylated at multiple sites by several kinases to differentially effect channel conductance and/or interaction with other synaptic proteins (1). This dynamic modulation of subunit phosphorylation is a salient component of AMPAR trafficking and regulation of activity-dependent plasticity (31, 32). For instance, long-term potentiation (LTP), a well-characterized form of synaptic plasticity, is expressed as an enrichment of synaptic AMPARs and is accompanied by modulation of specific GluA1 phosphorylation sites (33–36). Mutation of these phosphorylation sites in mice leads to disrupted LTP and LTD as well as spatial learning and alters the biophysical properties of AMPARs during excitatory neurotransmission (33, 36). Collectively, these findings provide functional links between posttranslational modification and the trafficking of AMPARs to synaptic plasticity, learning, and memory.

In this study we identified a carboxyl-terminal serine (S863) in the GluA1 receptor subunit that is phosphorylated in an activity-dependent manner and which regulates surface targeting of AMPARs in neurons. Previously, we found that the analogous residues within GluA2L (T912) and GluA4 (T855) are also phosphorylation sites that are modulated by neuronal activity. Further, these GluA2L/4 phosphoresidues regulate neuronal surface trafficking of AMPAR complexes (15). We determined that both the nonphosphorylatable and phosphomimetic forms of GluA1-S863 (i.e., S863A and S863D, respectively) increase surface AMPAR expression in neurons. Although ostensibly surprising, this result is not without precedent, as mutational analyses of GluA2L-T912 and of GluR4-S842 to alanine or aspartate each produce mirrored effects on receptor trafficking (15, 37). This sort of parallel regulatory effect has been proposed to arise if internalized receptors bind to a protein that preferentially interacts with the dephosphorylated form of AMPAR and retains it from the surface. For example, the AMPAR interacting protein GRIP1 has been shown to interact with GluA2 exclusively when dephosphorylated at S880 and fails to bind either nonphosphorylatable or phosphomimetic mutants (38–40). In the case of this investigation, a retention protein unable to interact with GluA1-S863A or GluA1-S863D could cause these mutants to selectively accumulate at the membrane surface. Although our data are suggestive of a retention protein preferentially interacting with dephosphorylated GluA1-S863, the identification of such a protein remains an intriguing course for future study.

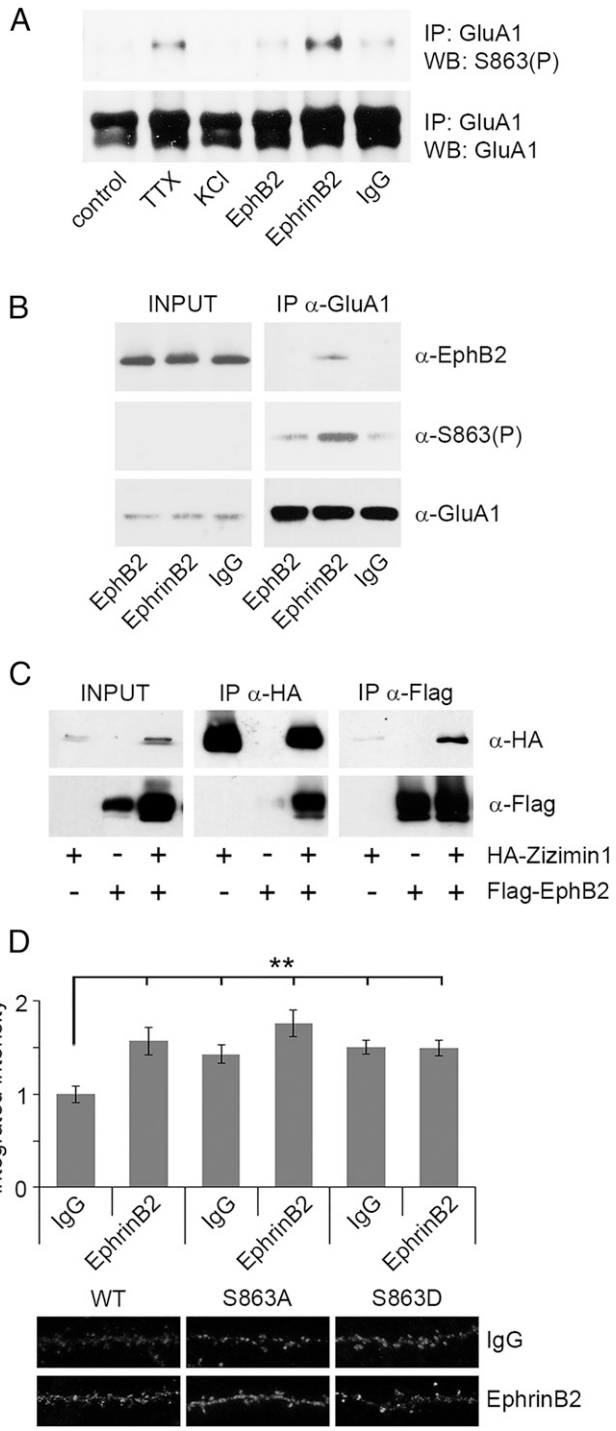

Fig. 5. Activation of EphB2 in neurons leads to phosphorylation of GluA1 S863. (*A*) DIV 21 cortical neurons were unstimulated (control) or treated with 1 μM TTX (20 min), 90 mM KCl (3 min), or incubated with either hIgG (control) alone or clustered using 4 μg of Fc-EphB2 or Fc-ephrinB2 per milliliter for the 3 h before lysis and immunoprecipitation of GluA1. GluA1 immunoprecipitates (IP) were Western-blotted with α-S863(P) or total GluA1 antibodies (indicated at *Right*). (*B*) Ephrin-B2 mediated activation of EphB2 enhances interaction with GluA1. Cortical neurons (DIV 21) were stimulated as described above and processed for immunoprecipitation of GluA1. Total cell extract (INPUT) and GluA1 immunoprecipitate (IP) were Western-blotted (WB) with the indicated antibodies (*Right*). (*C*) Interaction between Zizimin1 and EphB2 occurs in vivo. Solubilized cell extract prepared from HEK 293T cells transiently transfected with HA-Zizimin1 and Flag-EphB2 for 24 h (indicated at *Bottom*) were used for immunoprecipitation using antibodies against HA and Flag. Extracted proteins (INPUT) and precipitated proteins (IP) were detected by Western blot (antibodies indicated at *Left*). (*D*) Activated EphB2-mediated surface recruitment of AMPAR is not further enhanced by mutation of S863. Hippocampal neurons (DIV 20) transfected for 48 h with GFP-tagged GluA1 WT or with S863 mutants (S863A or S863D) were stimulated with hIgG (control) alone or with 4 μg of Fc-ephrinB2 per milliliter for the 3 h before immunohistochemical labeling of surface GFP. (*D*, *Top*) Mean integrated intensity bar graph of dendritic surface AMPAR clusters normalized to control (hIgG-treated) WT GFP-GluA1 transfected neurons. Error bars indicate ± SEM. $^{**}p < 0.005$ relative to WT-transfected neurons, ANOVA. $n \geq 12$ neurons for each. (*D*, *Bottom*) Representative images of surface AMPAR expression used for graphical analyses following control (hIgG) or Fc-ephrinB2 mediated activation of endogenous EphB2 in hippocampal neurons.

An important distinction between phosphorylation of GluA1 (S863) and that of GluA2L/4 (T912/T855) is the kinase family mediating each of these phosphoevents. We determined that unlike GluA2L and GluA4, which are targets of the JNK family, the signal transduction mechanism modulating GluA1 S863 distinctly relies upon group I PAK kinase family. Both Rho GTPases and PAK enzymes are highly expressed in the central nervous system. Importantly, these protein families dramatically modulate the structure and function of dendritic spines, membranous protrusions emanating from dendrites that are sites of neuronal contact for excitatory synaptic communication. Because dendritic spines are highly enriched in actin, they are acutely sensitive to factors that alter cytoskeletal dynamics. For instance, actin-remodeling through Cdc42 and Rac1 promotes dendritic development, spine morphogenesis, and maintenance, whereas stimulation of Rho results in retraction and loss of spines (6, 41, 42). Disruption of PAK expression or kinase activity also disturbs the formation and maintenance of dendritic spines and synapses (43). Structural integrity of synapses is a critical aspect of neuronal function; aberrant synapses are a hallmark of dysfunctional synaptic plasticity and are associated with several disorders of cognition, learning, and memory (44, 45). Dynamic regulation of Rac1 and Cdc42 expression is required to induce structural changes that accompany modulation of synaptic plasticity (46). Further, deregulation of Rho GTPase-signaling components, particularly Cdc42, Rac1, and PAK3, is associated with autism and Alzheimer's disease and these components have been identified as causal genes for X-linked intellectual disability (47, 48).

Several upstream regulators of group I PAK kinases are well-characterized for their ability to affect neuronal structure and function (49). For instance, EphR-Ephrin signal transduction through PAKs plays a critical role in synaptogenesis and spine morphogenesis; activated EphRs bind to Kalirin, a neuronal exchange factor, which in turn stimulates Rac1-PAK–mediated signal transduction to trigger changes in dendritic spine morphology (10). EphR activation also impinges on the trafficking of AMPARs and thereby critically modulates synaptic plasticity (9, 12). In this study we identified a novel activity-dependent EphR-signaling pathway that also regulates AMPAR trafficking but is distinct from the aforementioned Kalirin-Rac1 pathway. We determined that EphB2 and the guanine nucleotide exchange factor Zizimin1 are both GluA1 interacting proteins that can independently bind to each other. These layered interactions between EphB2-Zizimin and GluA1 position these proteins in proximity of synapses, providing they are optimally poised to modulate activity-dependent signal transduction. Indeed, we provide evidence for the involvement of these two signaling molecules, along with Cdc42, in PAK3-dependent phosphorylation of GluA1-S863 to regulate cell surface targeting of AMPARs (Fig. 6). Although each of the group I PAK kinases have been implicated in neurodegenerative diseases and play an important role in synaptic plasticity, PAK3 is particularly known for its association with X-linked intellectual disability (48). Based on our findings, it is conceivable that PAK3-specific phosphorylation of S863 contributes to selective targeting and/or

maintenance of GluA1-containing receptor combinations at the membrane. Because subunit regulation by site-specific phosphorylation has effects on synaptic transmission, it is possible that crosstalk among signaling pathways might fine-tune AMPAR function/composition within the synapse. Thus, rather than a single signaling pathway, it is likely that several factors that control the dynamic trafficking of AMPAR converge to modulate structural and functional plasticity. Both mutation of PAK3 and phosphomodulation of GluA1 receptor subunits have been found to have dramatic effects on synaptic plasticity and in cognitive function. Thus, the identification of S863 as a novel PAK3 modulatory site provides a focus for future characterization of activity-dependent regulation of AMPARs and cognition.

Materials and Methods

Antibodies. Commercial antibodies used in this study include those against HA.11 (Covance), c-myc (Santa Cruz), GFP (Abcam), Rac and Cdc42 (Millipore), and EphB2 and Flag M2 (Sigma–Aldrich). Specific monoclonal antibodies against N-terminal GluA1 (4.9D) and polyclonal antibodies against GFP (JH4030), total GluA1 (JH4294), and phosphorylation-specific S863 (JH3823) were generated in-house after antisera for each was purchased from Covance. EphrinB2-Fc, EphB2-Fc, and control Fc were obtained from R&B Systems.

Plasmids. Full-length rat GluA1 was subcloned into GFP-pRK5 mammalian expression vector, and point mutations were introduced using Quickchange (Stratagene). The C-terminal tail of GluA1 was subcloned into the mammalian expression vector pCIS, downstream of an N-terminal GST tag. GST-GluA1 fusion protein was prepared as previously described for the homologous GST-GluA2L and -GluA4 (15). Full-length HA-MRCKα and Flag-EphB2 were kindly provided by Louis Lim (University College London, London) and Matthew Dalva (Thomas Jefferson University, Philadelphia), respectively. Constitutively active Rho GTPases (RhoA, Rac1, Cdc42) were constructed as described (50). HA-tagged Zizimin1, generously gifted by Nahum Meller (Cleveland Clinic, Cleveland), was used as a PCR template to subclone a prenylated C-terminal segment (amino acids 1605–2069) into pEGFP-C1 (Clontech). Full-length EST clones of PAK1, 3, and 6 obtained from Open Biosystems were used as PCR templates for subcloning into modified myc-pRK5 vector. Oligonucleotides were annealed and cloned into HindIII and BglII sites of pSuper to make PAK shRNA. riPAK1 sense 5′-GATCCCCCCAAGCCTTCTATGAAATAAATTCAAGAGATTTATTTCATAGAAGGCTTGGTTTTTTC-3′ and antisense 5′-TCGAGAAAAAACCAAGCCTTCTATGAAATAAATCTCTTGAATTTATTTCATAGAAGGCTTGGGGG-3′. riPAK3 sense 5′-GATCTTAGCAGCACATCAGTCGAATACTCGAGTATTCGACTGATGTGCTGCTA TTTTTC-3′ and antisense 5′-TCGAGAAAAATAGCAGCACATCAGTCGAATACTCGAGTATTCGACTGATGTGCTGCTAA-3′.

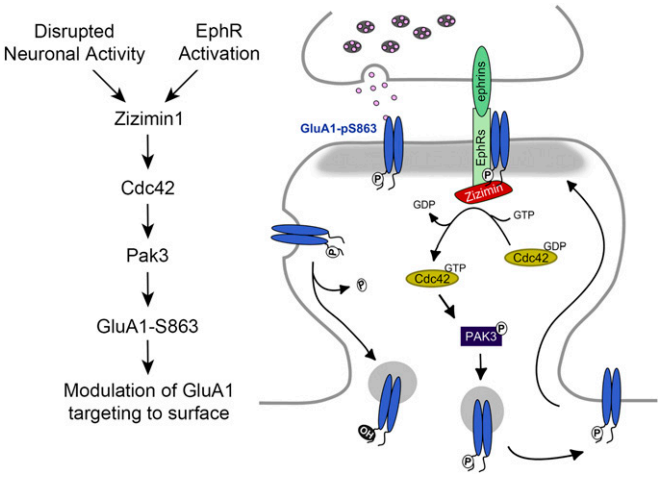

Fig. 6. Proposed signal cascade mediating phosphorylation of GluA1-S863 and model of regulated AMPAR trafficking. Stimulation of EphB2 enhances its binding to Zizimin1 and GluA1. Subsequent Zizimin1-mediated activation of Cdc42 directs PAK3 to phosphorylate GluA1-S863 (*Left*). GluA1-S863 specific phosphorylation contributes to selective targeting and/or maintenance of GluA1-containing receptor combinations at the membrane (*Right*).

Cell Culture, Electroporation, and Transient Transfection. Hippocampal neurons were dissected from embryonic day 19 (E19) Sprague–Dawley rat embryos, plated onto coated glass coverslips (30 μg/mL poly-D-lysine and 2.5 μg/mL laminin), and cultured in neurobasal medium with B27, 0.5 mM glutamine, and 12.5 μM glutamate. Neurons were transfected after 17–19 d in vitro using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, and processed 24–48 h later. Rat cortical neurons isolated from E18 pups were plated onto poly-L-lysine–coated dishes in NM5 media [Neurobasal growth medium (Invitrogen), supplemented with 2% (vol/vol) B27 (Invitrogen), 2 mM Glutamax (Gibco), 50 U/mL PenStrep (Gibco), and 5% (vol/vol) Fetal Horse Serum (HyClone)]. At DIV 3–4, neurons were treated with 5 uM uridine and 5 uM (+)-5-fluor-2′-deoxyuridine in NM1 (Neurobasal growth medium supplemented with 2% (vol/vol) B27, 2 mM Glutamax, 50 U/mL PenStrep, and 1% Horse Serum) for 3 d. Every 3–4 d in vitro thereafter, half of the culture media was changed with glia-conditioned NM1 until DIV 18–20. Electroporation of dissociated cortical culture was performed at DIV 0 using Rat Neuron Nucleofector Kit according to manufacturer protocol (Lonza Group Ltd.). HEK 293T cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 2 mM Glutamax, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Small-molecule PAK inhibitor (FRAX 120, Afraxis), resuspended in DMSO, was applied to cortical neurons at 1 μM for 1 h before TTX treatment and cell harvest for specified experiments. Cultured cells were harvested 24 h posttransfection (HEK cells) or at DIV 18–20 (cortical neurons) and processed similar to brain fractionation experiments. Briefly, cells were extracted in NL buffer (1× PBS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 5 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100 supplemented with 1 μg/mL leupeptin, 0.1 μg/mL aprotinin, 1 μg/mL phenylmethanesulfonyl fluoride, and 1 μg/mL pepstatin) and rocked at 4 °C for 30 min before 16,000 × *g* centrifugation for 15 min. Supernatants were then incubated with antibodies coupled to protein A- or G-Sepharose overnight at 4 °C, followed by three washes with ice-cold NL buffer and elution in 2× SDS sample buffer. The immunoprecipitated proteins were resolved by SDS/PAGE and visualized by Western blot analysis.

Lambda Phosphatase Assay. GluA1 immunoprecipitates from cortical neurons were washed into lambda phosphatase assay buffer (50 mM Tris·HCl pH 7.8, 5 mM DTT, 2 mM $MnCl_2$, 100 μg/mL BSA) with or without 1,600 U of lambda phosphatase. After 60 min at 30 °C, reactions were terminated by addition of SDS sample buffer and processed for immunoblotting.

Immunostaining, Microscopy, and Quantification. Hippocampal neurons fixed in 4% (vol/vol) paraformaldehyde and 4% (vol/vol) sucrose for 8 min were incubated with primary antibodies overnight at 4 °C in 1× genuine diesel buffer (GDB) buffer (30 mm phosphate buffer, pH 7.4, containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl), followed by secondary antibodies for 2–4 h. For surface staining, neurons transfected with GFP-tagged AMPARs were fixed for 5 min and incubated with rabbit GFP antibody (JH4030) overnight in 1× GDB buffer lacking Triton X-100, followed by immunostaining of total GFP-AMPARs upon incubation of chicken GFP antibody (Abcam) in regular 1× GDB buffer for 2–4 h. Subsequent secondary antibody incubations were done in regular 1× GDB buffer as described above.

An LSM510 confocal microscope system (Zeiss) was used to acquire fixed neuron *z*-series image stacks that encompassed entire dendrite segments compressed into a single plane and analyzed using MetaMorph software (Universal Imaging). For surface-integrated intensity quantification, immunostained channels were parsed into separate images. Five dendritic segments of 30 μm collected from at least 20 neurons per condition were outlined, and a threshold level for each channel was set manually to exclude diffuse background staining. Identical settings were applied to each image acquired within an experiment. Statistical significance between samples was calculated using ANOVA.

Recombinant Eph-Ephrin Activation in Neurons. Recombinant extracellular domains of EphB2 receptor and EphrinB2 ligand fused to the Fc fragment of human immunoglobulins and control human immunoglobulins (4 μg/mL) were clustered using anti-Fc antibodies (0.2 μg/mL) in conditioned cortical or hippocampal media for 1 h at room temperature. Twenty-four hours after Lipofectamine 2000 (Invitrogen) mediated transfection, hippocampal neurons (DIV 17), or nontransfected cortical neurons (DIV 18) had either clustered Fc-recombinant or control Fc proteins applied to the media for a 4-h treatment. Hippocampal neurons were subsequently fixed and prepared for surface immunostaining, whereas cortical neuron soluble cell extracts were processed for immunoprecipitation of endogenous GluA1 as described above.

In Vitro Kinase Assay. GST and GST-GluA1 C-terminal region were expressed in HEK 293T cells and affinity-purified as described (15). Reactions (40 μL) were set

up containing a final concentration of 2 μM GST or GST-GluA1 in kinase assay buffer [25 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 0.1% (vol/vol) β-mercaptoethanol, 0.03% (wt/vol) Brij-35] plus either 5 ng active PAK3 (EMD Millipore) or blank buffer. Tubes were placed at 30 °C and reactions initiated by adding 10 μL 50 mM MgCl2, 0.5 mM [γ]32P-ATP (specific activity, 200,000–400,000 cpm/nmol). Reactions were terminated by addition of SDS sample buffer, boiled and electrophoresed on SDS-polyacrylamide gels, and transferred to PVDF membrane (GE Amersham). Total fusion proteins and phosphorylated GluA1-S863 expression were visualized by Western blot analyses. The ^{32}P radioactivity incorporated was detected by autoradiography.

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