GluN3A expression restricts spine maturation via inhibition of GIT1/Rac1 signaling

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NMDA-type glutamate receptors (NMDARs) guide the activity-dependent remodeling of excitatory synapses and associated dendritic spines during critical periods of postnatal brain development. Whereas mature NMDARs composed of GluN1 and GluN2 subunits mediate synapse plasticity and promote spine growth and stabilization, juvenile NMDARs containing GluN3A subunits are thought to inhibit these processes via yet unknown mechanisms. Here, we report that GluN3A binds G protein-coupled receptor kinase-interacting protein (GIT1), a postsynaptic scaffold that assembles actin regulatory complexes, including the Rac1 guanine nucleotide exchange factor βPIX, to promote Rac1 activation in spines. Binding to GluN3A limits the synaptic localization of GIT1 and its ability to complex βPIX, leading to decreased Rac1 activation and reduced spine density and size in primary cultured neurons. Conversely, knocking out GluN3A favors the formation of GIT1/βPIX complexes and increases the activation of Rac1 and its main effector p21-activated kinase. We further show that binding of GluN3A to GIT1 is regulated by synaptic activity, a response that might restrict the negative regulatory effects of GluN3A on actin signaling to inactive synapses. Our results identify inhibition of Rac1/p21-activated kinase actin signaling pathways as an activity-dependent mechanism mediating the inhibitory effects of GluN3A on spine morphogenesis.

synaptic refinement | structural plasticity | actin cytoskeleton

During the development of neural circuits, a phase of intense synaptogenesis is followed by a period of activity-dependent remodeling (or "synaptic refinement") in which more than half of the initially formed synapses are eliminated, whereas other connections will mature and be kept (1, 2). The subunit composition of NMDA-type glutamate receptors (NMDARs) expressed by individual synapses during this critical period is a key factor influencing functional and structural synaptic plasticity and, in turn, synapse fate (3). Mature NMDARs composed of GluN1 and GluN2 subunits drive the maturation of active synapses by detecting coincident pre- and postsynaptic activity and coupling this activity to signaling pathways that trigger the enlargement and stabilization of synapses and associated dendritic spines (4–6). This structural plasticity is critical for coupling the wiring of neural circuits to experience and supporting the long-term maintenance of spines and memories. During the refinement stage, NMDARs additionally contain GluN3A subunits that serve as a brake on synapse maturation and stabilization, which might provide a counterbalance to limit synapse numbers. Supporting this idea, loss of GluN3A increases spine density and size (7) and accelerates the expression of markers of synaptic maturation (8), whereas overexpression reduces synapse and spine density and yields a higher proportion of smaller, immature spines (9). However, the downstream mechanisms by which GluN3A inhibits synapse and spine maturation remain unknown.

Spines are actin-rich, and their structural remodeling relies on rearrangements of the actin cytoskeleton (10–13). Cytoskeletal rearrangements are regulated by the Rho family of small GTPases, and two members of this family, Rac1 and RhoA, are major regulators of spine remodeling (14, 15). Rho-GTPases act as molecular switches that cycle between an inactive GDP-bound conformation and an active GTP-bound conformation (16). Their activation state is controlled by guanine exchange factors (GEFs), which promote the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), which catalyze GTP hydrolysis. Several Rac1-specific GEFs, including Kalirin7, Tiam1, and βPIX, are targeted to synapses via interactions with scaffolding proteins, which allows local regulation of actin remodeling in spines and its coupling to synaptic activity (12, 17–22). Although many studies have shown that NMDAR activation induces cytoskeletal and spine remodeling by activating Rac1-GEFs (23–25), much less is known about pathways that restrict excitatory synapse maturation and/or promote elimination.

Here, we identify a physical association between the intracellular C-terminal domain of GluN3A subunits and G protein-coupled receptor kinase-interacting protein (GIT1), a postsynaptic scaffold that assembles a multiprotein signaling complex with Rac1 and the Rac1-GEF βPIX to regulate actin dynamics in spines (19, 26). GIT1 selectively bound juvenile NMDARs containing GluN3A but not mature NMDAR subtypes, and binding was regulated by activity because it could be enhanced or reduced, respectively, by brief episodes of synaptic inactivity or synaptic stimulation. A functional analysis demonstrates that binding to GluN3A interferes with the synaptic localization of GIT1 and its ability to recruit βPIX, leading to decreased Rac1 activation. We finally show that GluN3A-induced reductions in spine density and size critically require GIT1 binding. We propose that the coupling of GIT1/GluN3A binding to synapse use might provide an effective mechanism with which to restrict the maturation and growth of inactive synapses in a selective manner.

Significance

More than 95% of excitatory synaptic contacts form on spines, highly motile dendritic protrusions that emerge, grow, or disappear in response to specific patterns of synaptic activity. This physical rearrangement is most prominent during critical periods of early postnatal life, when young brains are reshaped by experience to encode certain kinds of information. We reveal a mechanism whereby juvenile NMDA-type glutamate receptors containing GluN3A subunits regulate spine rearrangements by controlling the function of G protein-coupled receptor kinase-interacting protein (GIT1). GIT1 is a postsynaptic scaffold that couples stimuli promoting spine maturation to sustained cytoskeletal rearrangements. Binding to GluN3A limits the synaptic targeting of GIT1 and its ability to recruit actin regulators in an activity-dependent manner, providing a mechanism to limit the maturation of nonused synapses.

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Results

GluN3A Binds the Postsynaptic Scaffold Protein GIT1. To identify GluN3A-interacting proteins that mediate its negative regulatory effects on spine maturation, we conducted a yeast two-hybrid screen using a rat hippocampus cDNA library and the intracellular C-terminal tail of GluN3A as bait (amino acids 952–1,115). Two of the clones isolated corresponded to the signaling adaptor GIT1 (27) (Fig. 1*A*). Further interaction assays in yeast showed that GIT1 binds the C-terminus (Ct) of GluN3A but not GluN1, GluN2A or GluN2B (Fig. 1*A*). The minimal GIT1-binding region was mapped to the distal GluN3A Ct (amino acids 1,082–1,115) (Fig. 1*B*). This was confirmed in pull-down assays using full-length and truncated GluN3A Ct constructs fused to GST. GST-GluN3A Ct, but not GST alone, precipitated GIT1, and GST-GluN3A Ct (amino acids 1,082–1,115) was sufficient to precipitate endogenous GIT1 from mouse forebrain lysates (Fig. 1*C*). Smaller portions of the GluN3A Ct failed to pull down GIT1 (Fig. 1*C*). Coimmunoprecipitation experiments in HEK 293T cells coexpresing full-length GluN3A tagged to green fluorescent protein (GFP) or GluN3A lacking the 1,082–1,115 amino acid strech (GFP-GluN3AΔGIT1), and GIT1 further verified the interaction and the requirement for the distal 33 amino acids of GluN3A (Fig. 1*E*).

GIT1 is a multimodular protein that contains an N-terminal ADP ribosylation factor-GAP domain involved in receptor endocytosis, a Spa2 homology domain that binds βPIX, a central synaptic localization domain (SLD), and a paxillin-binding domain (PBD) (26, 28, 29) (Fig. 1*D*). Our initial yeast two-hybrid data showed that a GIT1 region comprising the SLD and PBD domains was sufficient for GluN3A binding (amino acids 445–770; Fig. 1*A*). To map the interaction site further, we performed coimmunoprecipitation experiments from HEK 293T cells cotransfected with GluN1, GluN3A, and Flag-GIT1 or Flag-GIT1 lacking the SLD (Flag-GIT1ΔSLD; Fig. 1*D*). Deletion of the SLD abolished GluN3A/GIT1 binding (Fig. 1*E*). Together, these results show that GluN3A subunits selectively bind GIT1 and that the interaction involves the SLD of GIT1 and the last 33 C-terminal amino acids of GluN3A.

To evaluate the physiological significance of GluN3A/GIT1 interactions, we examined the spatiotemporal expression patterns of both proteins in rat brain. In situ hybridization showed that GIT1 is broadly expressed in young rats [postnatal day 8 (P8)]; high levels of both GluN3A and GIT1 mRNAs were found in cortical regions, the CA1 hippocampal region, the amygdala, and thalamic nuclei (Fig. S1*A*). Immunoblot analyses of cortical and hippocampal extracts showed that GIT1 protein is expressed continuously from P0 to adult age, whereas GluN3A was prominently expressed during periods of synapse/spine reorganization (P8–P15) and declined after P25 (30) (Fig. S1 *B* and *C*). These experiments demonstrate overlapping distribution of both proteins during critical periods of synaptic development and suggest a potential role for GluN3A in modulating actin rearrangement via GIT1 interaction.

To test whether GluN3A and GIT1 form a complex in vivo, we performed coimmunoprecipitation experiments from P8 mouse forebrain lysates. An anti-GluN3A antibody coprecipitated GIT1 but not the AMPA receptor subunit GluA1 or the presynaptic protein synaptophysin (Fig. 1*F*). In addition, both GIT1 and GluN3A were present in protein complexes pulled down by an antibody against the GluN1 subunit, an obligatory component of NMDAR complexes. No GIT1 coprecipitated with GluN1 in mice lacking GluN3A (*GluN3A*$^{-/-}$), indicating that GIT1 interactions with NMDARs are mediated by GluN3A subunits and confirming selectivity for juvenile NMDARs containing GluN3A (Fig. 1*G*).

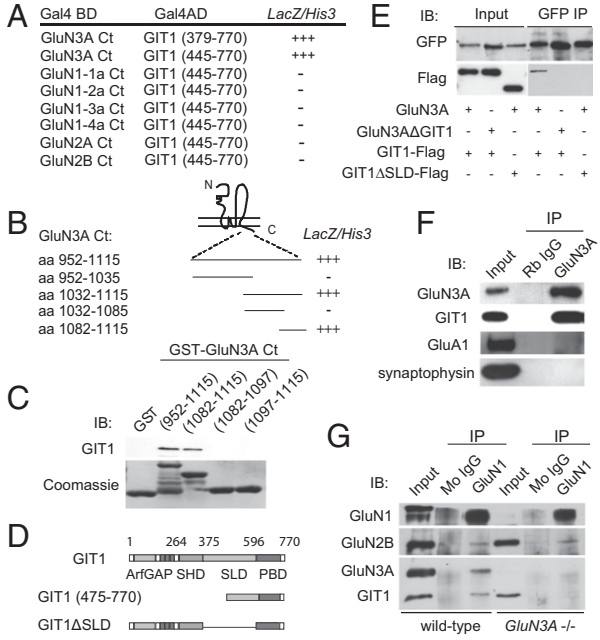

Fig. 1. GluN3A binds the synaptic scaffold GIT1. (*A*) Two independent clones encoding GIT1 were isolated by yeast two-hybrid screening using the intracellular carboxyl-terminal domain of GluN3A (GluN3A Ct, amino acids 952–1,115) as bait. Interactions were tested by induction of the reporter genes *LacZ* (β-Gal) and *His3*. (*B*) Amino acids 1,082–1,115 in the distal GluN3A tail were sufficient for GIT1 binding. Amino acid numbers refer to positions C-terminal to the last transmembrane domain. (*C*) Whole extracts of P8 mouse forebrain were incubated with control GST beads or beads bound to GST fused to the entire GluN3A Ct or smaller fragments of the Ct as indicated. Precipitated proteins were detected by immunoblot (IB) using an antibody to GIT1, and Coomassie staining was used to visualize GST-fusion proteins in the same gel. (*D*) Diagram of GIT1 and truncations used. ArfGAP, ADP ribosylation factor-GAP domain; PBD, paxillin-binding domain; SHD, Spa2 homology domain; SLD, synaptic localization factor. (*E*) Extracts of HEK 293 cells transfected with GluN1 plus the indicated constructs were incubated with anti-GFP antibody, and immunoprecipitated proteins were analyzed by IB with the indicated antibodies. GluN3A full-length, or lacking amino acids 1,082–1,115 responsible for GIT1 binding (GluN3AΔGIT1), were tagged with GFP; entire GIT1 or GIT1 lacking the synaptic localization domain (GIT1ΔSLD) was Flag-tagged. Input is 5% of the lysate used for the immunoprecipitate (IP). (*F*) Solubilized mouse forebrain membrane extracts were incubated with GluN3A antibody or control IgG. Rb, rabbit. (*G*) Lysates from WT and *GluN3A*$^{-/-}$ P8 mouse forebrain were incubated with GluN1 antibody or control IgG, and immunoprecipitated proteins were analyzed by IB. Mo, mouse. In *F* and *G*, input is 10% of the lysate used for immunoprecipitation.

GluN3A Inhibits GIT1/βPIX/Rac1 Signaling. We next asked whether binding to GluN3A influences the assembly and function of GIT1 actin regulatory complexes. To test this, we infected rat cortical neurons cultured 9 d in vitro (DIV9) with a neurotropic Sindbis virus expressing GFP-tagged GluN3A or GluN3AΔGIT1 and analyzed GIT1/βPIX complex formation 24 h later by coimmunoprecipitation. Overexpression of GluN3A significantly reduced the amount of βPIX binding to GIT1, whereas overexpression of GFP-GluN3AΔGIT1 had no effect (Fig. 2*A*). Inhibition of GIT1/βPIX complex formation by GFP-GluN3A was associated with decreased activation of Rac1 (as measured by the levels of GTP-bound Rac1), whereas Rac1 activation was not affected in neurons infected with GFP-GluN3AΔGIT1 (Fig. 2*B*). No changes in total Rac1 levels were observed in either condition (Fig. 2*B*). GFP-GluN3A and GFP-GluN3AΔGIT1 displayed similar subcellular distributions, and surface biotinylation assays showed that both were expressed at the surface at similar levels (Fig. S2). Coimmunoprecipitation assays in cultured neurons confirmed that virally delivered GFP-GluN3AΔGIT1 did not bind endogenous GIT1, as seen in recombinant systems (Fig. S2*C*). These results demonstrate that GluN3A subunits work as negative regulators of Rac1 activity by binding GIT1 and inhibiting its ability to assemble actin regulatory complexes (Fig. 2*C*).

Supporting this notion, the association between GIT1 and βPIX was stronger, and Rac1 activation was enhanced, in lysates from *GluN3A*$^{-/-}$ mouse forebrain at P8 (Fig. 2 *D* and *E*). The levels of

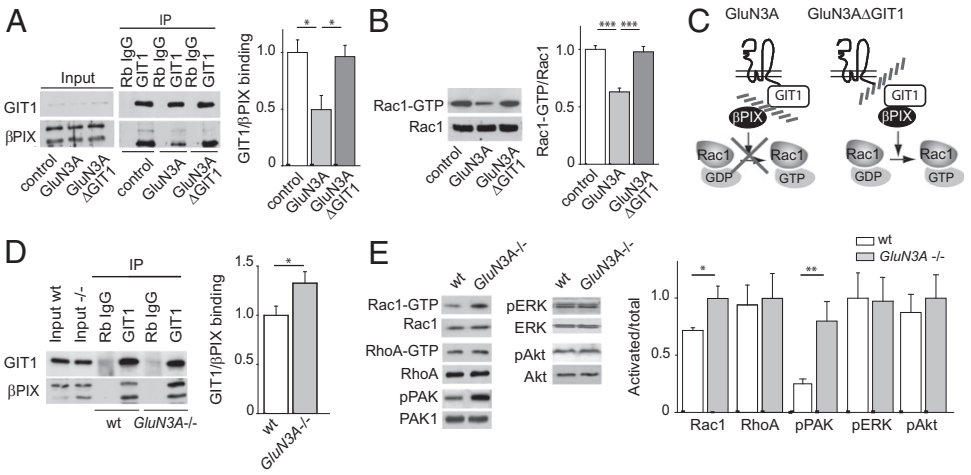

Fig. 2. Inhibition of GIT1/βPIX assembly and Rac1 signaling by GluN3A. (*A*) Extracts from cortical neurons infected with Sindbis virus expressing GFP (control), GFP-GluN3A, or GFP-GluN3AΔGIT1 were immunoprecipitated with GIT1 antibody or control IgG and probed for GIT1 and βPIX. Representative blots and quantification are shown. Bound βPIX levels were normalized to GIT1 levels in the IP ($n = 5$ independent experiments; $^*P < 0.05$, ANOVA followed by Tukey's test). Here, and in all subsequent figures, error bars indicate the mean ± SEM. (*B*) Inhibition of Rac1 activation in neurons expressing GFP-GluN3A ($n = 5$ independent experiments; $^{***}P < 0.001$, ANOVA followed by Tukey's test). (*C*) Diagram depicts the effects of GluN3A on GIT1 function. GIT1 forms a complex with the Rac1-GEF βPIX that promotes the exchange of Rac1-bound GDP for GTP; the assembly of this complex is disrupted by GluN3A (*Left*) but not by GluN3A lacking the GIT1-binding domain (*Right*). (*D*) Forebrain extracts from P8 WT (wt) and *GluN3A*$^{-/-}$ mice were immunoprecipitated with GIT1 antibodies and blotted for GIT1 and βPIX. Representative blots and quantification are shown ($n = 9$ independent experiments; $^*P < 0.05$, Student's *t* test). (*E*) Increased Rac1-GTP and pPAK levels in extracts from P8 *GluN3A*$^{-/-}$ mouse forebrain without changes in other NMDAR-dependent signaling pathways ($n = 6$–10 independent experiments; $^*P < 0.05$ and $^{**}P < 0.01$, Student's *t* test).

phosphorylated p21-activated kinase (pPAK), a major downstream effector of Rac1, were also increased in *GluN3A*$^{-/-}$ forebrain without changes in total protein levels (Fig. 2*E* and Fig. S3*A*). Consistent with GluN3A negatively regulating Rac1/PAK activation, pPAK levels were significantly increased upon developmental GluN3A down-regulation (Fig. S3*B*). Other NMDAR-dependent signaling pathways were not affected, as shown by a lack of changes in RhoA activation and phosphorylation levels of Akt or extracellular-regulated kinases 1 and 2 (ERK1/2) (Fig. 2*E*).

GluN3A Inhibits the Synaptic Targeting of GIT1/βPIX Complexes. GIT1 is enriched at postsynaptic densities, and its synaptic localization has been reported to be critical for targeting actin regulators, including βPIX and Rac1, to synapses and promoting the local activation of Rac1/PAK signaling (19). Because (*i*) GIT1 is targeted to synapses via the SLD, which we have identified as the GluN3A-interacting site, and (*ii*) GluN3A-containing subtypes are less anchored to postsynaptic densities than classical NMDARs and predominate in peri- and extrasynaptic plasma membrane locations (31), we investigated whether inhibition by GluN3A of the assembly of GIT1 actin regulatory complexes is due to altered subcellular localization. Biochemical fractionation of P8 mouse forebrain from WT and *GluN3A*$^{-/-}$ mice was performed as described (32, 33), and the enrichment of GIT1 and βPIX in postsynaptic fractions was quantified by Western blot analysis. Both GIT1 and βPIX were enriched in Triton X-100 insoluble postsynaptic fractions (TIFs), but their synaptic enrichment was enhanced in *GluN3A*$^{-/-}$ mice (Fig. 3 *A* and *B*), suggesting that GluN3A normally prevents their targeting or stabilization at synaptic compartments. pPAK levels were correspondingly increased in TIFs from P8 *GluN3A*$^{-/-}$ mice (Fig. S3*C*). The presence and absence of post- and presynaptic markers [postsynaptic density protein 95 (PSD-95) and synaptophysin] was used to assess the purity of our postsynaptic fractions. No changes in GluN2B were detected (Fig. 3*B*) in agreement with previous work (8).

Experiments in cultured neurons from *GluN3A*$^{-/-}$ mice demonstrated that the enhanced synaptic targeting of GIT1 and βPIX could be fully reversed by reexpressing GluN3A. Here, hippocampal neurons were infected with Sindbis viruses expressing GFP, GFP-GluN3A, or GFP-GluN3AΔGIT1 at DIV16 and fixed at DIV17, and synaptic localization was assessed by immunofluorescence quantification of the colocalization with the postsynaptic marker Homer. Whereas in control *GluN3A*$^{-/-}$ neurons, GIT1 and βPIX accumulated in clusters that colocalized with Homer along the neuronal processes, reexpression of GluN3A inhibited clustering and decreased the synaptic concentration of both proteins (Fig. 3*C* and Fig. S4*A*). Differences could be quantified as (*i*) a shift in the frequency distribution of GIT1 and βPIX clusters toward smaller sizes in GluN3A-transfected neurons ($P = 0.003$ and $P = 0.00007$, respectively, vs. control GFP-transfected neurons; Fig. 3*D* and Fig. S4*B*), (*ii*) decreased ratios of clustered/nonclustered GIT1 and βPIX fluorescence intensities (Fig. 3*D* and Fig. S4*B*), and (*iii*) decreased colocalization of GIT1 and βPIX with Homer (Fig. 3*E* and Fig. S4*C*). In contrast, GluN3AΔGIT1 expression did not affect the colocalization of GIT1 or βPIX with Homer, or any of the other clustering parameters (Fig. 3 and Fig. S4), indicating that direct binding to GluN3A prevents the synaptic clustering of GIT1 and, consequently, its ability to recruit βPIX.

Activity Dependence of GluN3A/GIT1 Assembly. Neuronal activity reduces the membrane expression of GluN3A-containing NMDARs and has been shown to induce the clustering of GIT1 at synapses and to promote GIT1/βPIX assembly (25, 31, 34). Thus, we next asked whether activity modulates the binding of GIT1 to GluN3A and, in turn, the assembly and clustering of GIT1/βPIX complexes. To do so, we first exposed cultured neurons to brief episodes of synaptic blockade (using the sodium channel blocker tetrodotoxin (TTX)] or synaptic stimulation (using the GABA antagonist bicuculline) and evaluated whether such bidirectional activity manipulation effectively modulates GIT1/βPIX clustering. Bicuculline treatment increased GIT1 and βPIX clustering compared with control neurons, whereas a more diffuse distribution was observed after blocking activity with TTX (Fig. 4 *A* and *B*). Coimmunoprecipitation assays demonstrated that activity blockade enhances GIT1 binding to GluN3A and concomitantly reduces GIT1/βPIX binding, whereas synaptic stimulation reduced GluN3A/GIT1 binding and augmented GIT1/βPIX interactions (Fig. 4 *C* and *D*). Brief glutamate stimulation (50 μM for 5 min), which was previously shown to induce clustering of GIT1 at synapses (34), similarly decreased GluN3A/GIT1 binding (Fig. 4 *E* and *F*). The effect was NMDAR-dependent because it was blocked by the NMDAR antagonist DL-(-)-2-amino-5-phosphovaleric acid (APV) but not by cyano-nitroquinoxaline-dione (CNQX), an antagonist of AMPA-type glutamate receptors (Fig. 4 *E* and *F*). Based on these data, we hypothesized that enhanced GIT1/GluN3A interactions might serve a role to limit the maturation and growth of inactive synapses by reducing Rac1 signaling. Synaptic activity, by releasing GIT1 from GluN3A, would allow the resultant GIT1 to interact with βPIX and activate Rac1 pathways modulating spine structure (model in Fig. 4*G*).

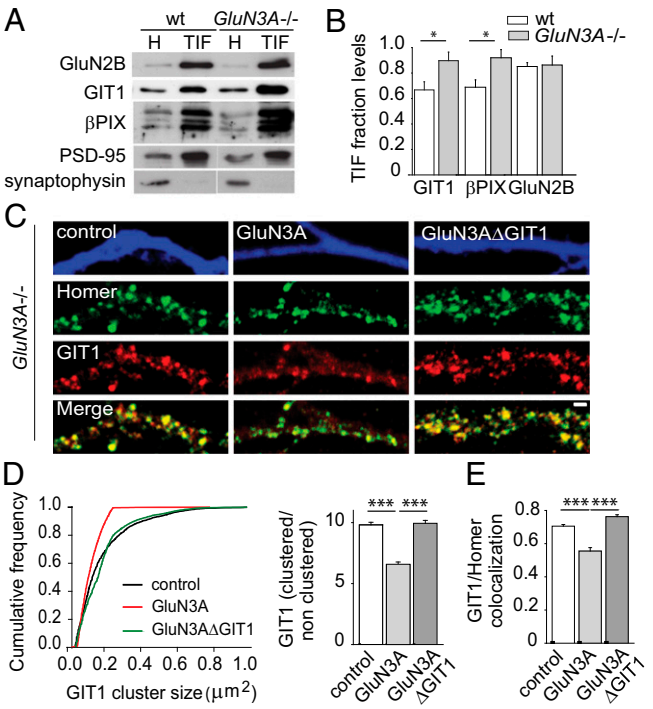

Fig. 3. GluN3A alters the targeting of GIT1 to synapses. (*A* and *B*) Biochemical fractionation analysis of WT and *GluN3A*$^{-/-}$ mouse forebrain at P8. Shown are representative IBs of whole homogenates (H) and TIFs probed with the indicated antibodies (*A*) and quantification (*B*) (n = 7–9 independent experiments; *P < 0.05, Student's t test). Plotted are protein levels in TIFs normalized to levels in homogenates. (*C*) Representative single confocal images of Homer and GIT1 immunostaining in *GluN3A*$^{-/-}$ cultured hippocampal neurons infected with Sindbis virus expressing GFP (control), GFP-GluN3A, or GFP-GluN3AΔGIT1. (Scale bar, 1 μm.) (*D*) Quantification of the effects of GFP, GFP-GluN3A, or GFP-GluN3AΔGIT1 on GIT1 cluster size (n = 11–13 neurons from four independent cultures; P < 0.001, Kolmogorov–Smirnov test) and ratio of GIT1 clustered/nonclustered fluorescence intensities (n = 11–13 neurons from four independent cultures; ***P < 0.001, ANOVA followed by Tukey's test). (*E*) Colocalization with the postsynaptic marker Homer measured using Pearson's coefficient (n = 11–13 neurons from four independent cultures; ***P < 0.001 ANOVA followed by Tukey's test).

GIT1 Binding Is Required for GluN3A Effects on Spine Density and Morphology. To test this model, we examined whether the ability of GluN3A to bind GIT1 underlies its negative regulatory effects on dendritic spine morphogenesis. Hippocampal neurons in culture were transfected with GFP, GFP-GluN3A, or GFP-GluN3AΔGIT1, together with mCherry, to visualize dendritic and spine morphology (Fig. 5*A*). Confirming data in transgenic mice (9), overexpression of GFP-GluN3A, which is associated with decreased GIT1/βPIX assembly and diminished Rac1 activity, caused a large reduction in the total number of spines (Fig. 5*B*) and across all morphological spine categories (Fig. 5*B*). Spine size was also altered, as quantified by decreased spine head diameters upon GluN3A expression (Fig. 5*C*). The effect required GIT1 binding because expression of GluN3A lacking the GIT1-binding domain did not affect spine density and morphology. We conclude from these data that GluN3A restricts spine maturation and growth and interferes with spine maintenance via the disassembly of GIT1 actin regulatory complexes.

Discussion

Here, we identify a link between juvenile NMDARs containing GluN3A subunits and inhibition of Rac1/PAK actin signaling pathways critical for spine remodelling. Our results reveal a bidirectional regulation of actin signaling by GluN3A and support a model whereby the presence of GluN3A restricts synapse maturation by binding the actin scaffolding protein GIT1, interfering with its targeting or stabilization at synapses and inhibiting its ability to associate with βPIX, which diminishes Rac1 activation. Consistent with this model, (*i*) the synaptic clustering of both GIT1 and βPIX, GIT1/βPIX binding, and Rac1/PAK activation were enhanced in *GluN3A*$^{-/-}$ mice and inhibited by GluN3A overexpression and (*ii*) binding to GIT1 was required for the ability of GluN3A to inhibit Rac1 activation and restrict spine growth and maintenance. Activation of GIT1 actin-regulating complexes has previously been shown to promote spine morphogenesis, whereas inhibition of GIT1 signaling decreased spine density and maturation (19, 25), and our work indicates that changes in GluN3A levels can initiate or limit these events (Fig. 4*G*). Because of its activity dependence, regulation of GluN3A/GIT1 binding might unleash GIT1/βPIX/Rac1/PAK signaling at only active synapses during critical periods of synaptic development, and thus couple persistent changes in spine structure and the wiring of adult neural circuits to experience.

A number of features point toward GluN3A expression as an activity-dependent switch that controls the timing and extent of synapse maturation and associated spine remodelling during this period. First, GluN3A expression peaks during a narrow window of postnatal development when massive synaptic rearrangement takes place (P8–P25) and is down-regulated in adult brains. By electron microscopy (EM), juvenile GluN3A subunits were found at small, immature spines but were excluded from large synapses (PSD length >250 nm) (9). Second, loss- and gain-of function studies support a role for GluN3A subunits in limiting synapse and spine maturation and growth (7, 9). Third, neuronal activity drives the removal of GluN3A-containing NMDARs from synapses and their exchange for mature, Ca^{2+}-permeable GluN1/GluN2 subtypes (31, 35). Inactivation of the GIT1/βPIX/Rac1 pathway by GluN3A might fulfill unique functions at developing synapses by providing a mechanism to restrict the maturation of nonused synaptic connections selectively, perhaps eventually ending in synapse elimination. Via its SLD, GIT1 senses stimuli that promote spine maturation and links them to sustained cytoskeletal rearrangements. The SLD interacts with the adaptor protein Grb4 and with CaMKII; these interactions transduce ephrin reverse signaling and BDNF stimulation, respectively, into the recruitment of GIT1 and βPIX to synapses (36, 37). At inactive synapses, SLD interactions with GluN3A might mask this domain and compete for synaptic targeting mechanisms. Alternatively, impairments in the synaptic clustering of GIT1 and βPIX might reflect GluN3A/GIT1 interactions occurring in peri- and extrasynaptic locations rather than synaptic locations, which could be expected, given the limited anchoring of GluN3A to postsynaptic densities. Developmental and activity-dependent removal of GluN3A would be predicted to relieve this inhibition and enable GIT1/βPIX assembly and Rac1/PAK activation only at activated synapses (Fig. 4*G*). GluN3A down-regulation might also enable other βPIX targeting mechanism, such as Shank (18), or roles of other Rac1-GEFs, such as Kalirin-7 (24), that promote structural plasticity in mature neurons.

Rac1/PAK signaling is thought to be essential for the formation, growth, and maintenance of dendritic spines (38–42). In hippocampal neurons, augmenting Rac1 activation increases spine densities, whereas inactivation of Rac1 or PAK by dominant negative approaches or by genetic suppression of PAKs reduces spine number and yields a decreased proportion of mature, mushroom-shaped spines (14, 21, 43–45). These reciprocal phenotypes mimic the increased spine density reported in *GluN3A*$^{-/-}$ mice, where Rac1/PAK activity is enhanced, and the immature spine morphology and decreased spine numbers found in transgenic mice or cultured neurons overexpressing GluN3A, which are linked to deficits in Rac1 activation. Disrupting the synaptic localization of GIT1 or inhibiting GIT1/βPIX assembly has similarly been shown to trigger the elimination of mature spines (26). Nevertheless, brute force or prolonged hyperactivation of Rac1 with constitutively active mutants causes a proliferation

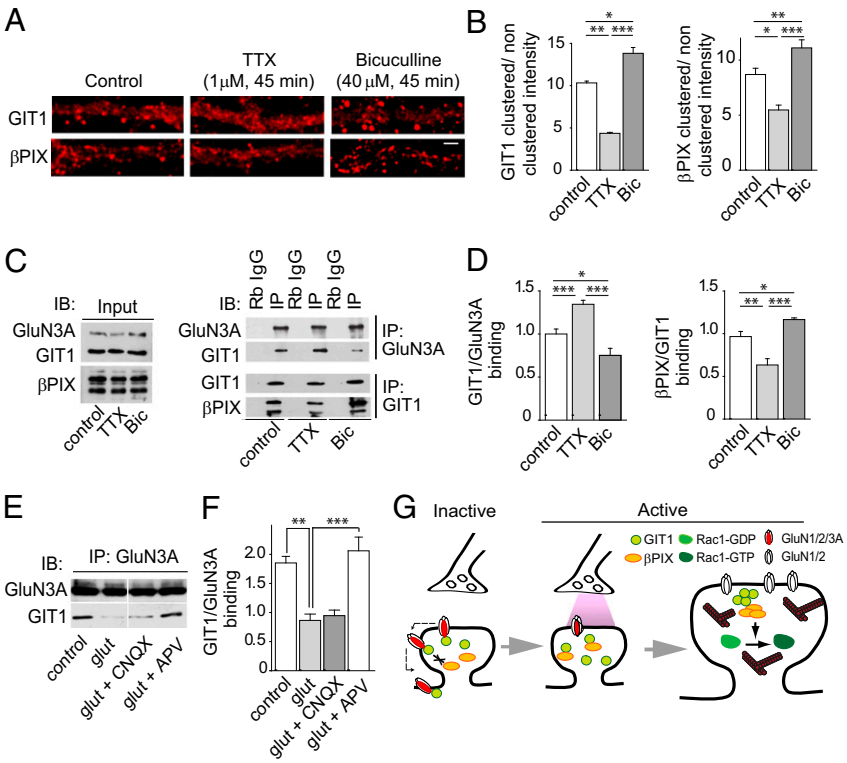

Fig. 4. Synaptic activity regulates GluN3A binding to GIT1. (*A*) Representative images of cultured hippocampal neurons, either untreated (control) or exposed to TTX or bicuculline for 45 min and stained with the indicated antibodies. (Scale bar, 1 μm.) (*B*) Quantification of the ratios of GIT1 and βPIX clustered/nonclustered fluorescence intensities (n = 10–13 neurons from three independent cultures; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, ANOVA followed by Tukey's test). Bic, bicuculline. (*C*) Cultured neurons treated with TTX or bicuculline were lysed, and lysates were immunoprecipitated with GluN3A or GIT1 antibodies or control IgG. Inputs (10% of lysate) and IPs were blotted with the indicated antibodies. (*D*) Quantification of GIT1/GluN3A and GIT1/βPIX binding. Bound GIT1 levels were normalized to GluN3A levels in the IP, and bound βPIX was normalized to GIT1 levels in the IP (n = 5 independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, ANOVA followed by Tukey's test). (*E*) Representative IBs of cortical neurons pretreated with TTX and stimulated with 50 μM glutamate for 5 min in the presence or absence of 10 μM CNQX or 50 μM APV. Lysates were immunoprecipitated with GluN3A and immunoblotted with the indicated antibodies. (*F*) Quantification of GIT1/GluN3A binding (n = 6 independent experiments; $^{**}P < 0.01$ and $^{***}P < 0.001$, ANOVA followed by Tukey's test). (*G*) Model depicts the potential role of activity-dependent regulation of GluN3A/GIT1 binding on spine maturation. During postnatal development, GluN3A limits the maturation of inactive synapses by preventing the localization of GIT1 to synapses via mechanisms that might involve a limited anchoring of GluN3A itself to postsynaptic compartments (arrows) or by inhibiting GIT1 interactions with βPIX. Synaptic activity releases GIT1 from GluN3A, allowing its synaptic localization and the recruitment of actin regulators. Local activation of Rac1 promotes actin cytoskeleton remodeling and, in turn, spine growth and stabilization.

of minispines (43), whereas spines are larger in *GluN3A*$^{-/-}$ mice (7). This could be caused by differences in activation levels or because disrupting GluN3A alters a modulatory pathway that is engaged by neurons to achieve spatiotemporal control of Rac1 signaling, rather than drastically altering Rac1 activation, which initially causes rapid spine growth but triggers spine shrinkage in the long term (21). Studies have suggested that Rac1/PAK activation promotes the stabilization of newly formed actin filaments upon synaptic stimulation, and is thus required for the persistence of spine structural changes (40, 42). Supporting this idea, defective Rac1/PAK activation has been linked to altered memory consolidation without deficits in acquisition (39), which closely resembles the selective deficits in memory consolidation displayed by GluN3A-overexpressing mice (9).

Disturbances in the balance between synapse maturation and elimination have been implicated in a number of disorders of cognition, including mental retardation, autism, and schizophrenia, that exhibit reduced densities of spines and synapses or a predominance of immature spine morphologies. Interestingly, reduced spine density and elevated levels of GluN3A have been reported in the brain of schizophrenic patients (46). Our study reveals a mechanism that could explain the inhibitory effects of GluN3A subunits on spine morphogenesis under physiological and pathological conditions and provides insight into Rho-GTPase control in neurons.

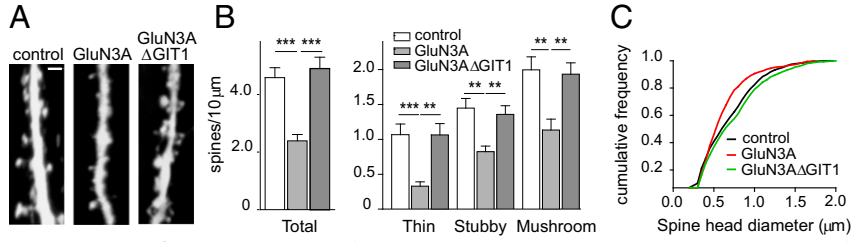

Fig. 5. GIT1 binding is required for the negative regulatory effects of GluN3A on spine morphogenesis. (*A*) Representative confocal images of cultured hippocampal neurons cotransfected with GFP (control), GFP-GluN3A, or GFP-GluN3AΔGIT1, as well as mCherry at DIV10, and fixed at DIV17. (Scale bar, 2 μm.) (*B*) Quantitative analysis of total spine densities or spine densities across different morphological categories (secondary and tertiary dendrites of n = 16–18 neurons from three different cultures; $^{**}P < 0.01$ and $^{***}P < 0.001$, ANOVA followed by Tukey's test). (*C*) Morphometric analysis of spine head diameter (n = 750–1,600 spines; $P < 0.005$, Kolmogorov–Smirnov test).

Materials and Methods

Detailed experimental methods are described in *SI Materials and Methods*.

Yeast Two-Hybrid Screening. Yeast two-hybrid screening was performed as described previously (31) using a rat hippocampal cDNA library fused to the GAL4 DNA-activating domain of pACT2 (Clontech). A cDNA encoding the GluN3A carboxyl terminus fused with the GAL4 DNA-binding domain of pAS2-1 (Clontech) was used as bait.

Cell Culture and Transfection. Cultured cortical and hippocampal neurons were prepared from E19 Sprague–Dawley rat embryos as described (31). Neurons were transfected using calcium phosphate or infected with Sindbis virus at the indicated times. Details are provided in *SI Materials and Methods*.

Biochemistry. Methods for immunoprecipitation, GST pull-down, biochemical fractionation, and surface biotinylation of crude membrane or total extracts from mouse forebrain or cultured cortical neurons are described in detail in *SI Materials and Methods*.

Immunofluorescence. Cultured neurons were fixed in 2% (wt/vol) paraformaldehyde and stained with primary antibodies in blocking/permeabilizing solution containing 5% (vol/vol) horse serum and 0.05% saponin for 1 h. Cells were washed in PBS, incubated with Cy3 or Cy5-conjugated secondary antibodies, washed, and mounted in Mowiol supplemented with DABCO 2.5% (wt/vol) (Sigma).

Image Analysis and Quantification. Image acquisition was performed by means of an inverted confocal fluorescence microscope (Zeiss LSM 510 META confocal system) using an oil objective with a magnification of 63×. Fluorescence excitation was achieved with a 488/543/633-nm laser light, and emission was detected through a 560- to 615-nm bandpass and 650-nm long-pass filters when imaged simultaneously. 3D volumes of Z-stacks (spacing of 0.3 μm between single confocal slices) were analyzed using ImageJ (National Institutes of Health). Details are provided in *SI Materials and Methods*.

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