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The Nogo Receptor Family Restricts Synapse Number in the Developing Hippocampus

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SUMMARY

Neuronal development is characterized by a period of exuberant synaptic growth that is well studied. However, the mechanisms that restrict this process are less clear. Here we demonstrate that glycosylphosphatidylinositol-anchored cell-surface receptors of the Nogo Receptor family (NgR1, NgR2, and NgR3) restrict excitatory synapse formation. Loss of any one of the NgRs results in an increase in synapse number in vitro, whereas loss of all three is necessary for abnormally elevated synaptogenesis in vivo. We show that NgR1 inhibits the formation of new synapses in the postsynaptic neuron by signaling through the coreceptor TROY and RhoA. The NgR family is downregulated by neuronal activity, a response that may limit NgR function and facilitate activity-dependent synapse development. These findings suggest that NgR1, a receptor previously shown to restrict axon growth in the adult, also functions in the dendrite as a barrier that limits excitatory synapse number during brain development.

INTRODUCTION

The establishment of the appropriate number of synaptic connections during development is critical for proper brain function. Failures in this process may underlie neurological disorders including mental retardation, autism, and schizophrenia (Bassell and Warren, 2008; Eastwood, 2004; Südhof, 2008). Recent work has identified several of the cell-cell recognition molecules that promote synapse formation (Dalva et al., 2007), but much less is known about the mechanisms that restrict synapse number to ensure the exquisite specificity in organization of neural circuits that occurs throughout the brain.

Excitatory synaptic development begins as contacts are made between passing axons and dendritic filopodia (Ziv and Smith, 1996), actin-rich protrusions along dendritic shafts. Dendritic filopodia rapidly discriminate between potential partners and appear to stabilize contacts with the appropriate presynaptic axons (Lohmann and Bonhoeffer, 2008). The rapid nature of this process suggests that signaling by cell-surface receptors is likely to be involved in determining when and where synapses form. These cell-surface receptor/ligand complexes include neurexins/neuroligins, EphB/EphrinBs, N-cadherins, and NGL3/LAR, which are thought to contribute to the stabilization of nascent synaptic contacts through recruitment of scaffolding molecules and neurotransmitter receptors (Dalva et al., 2007). It is not known whether analogous mechanisms exist to restrict synapse formation so that synapses form at the right time and place and in the correct number.

We hypothesized that there might be cell-surface receptors that function to restrict one or more steps in the process of synaptic maturation, thereby balancing the process of synapse formation so that synapses form in the correct number. These steps could include determining when and where synaptogenesis begins by preventing the inappropriate recruitment of synaptic components to asynaptic sites, limiting the activity-dependent growth of synapses, or mediating the pruning of weak synaptic contacts during synapse elimination.

We report here the discovery that one subfamily of leucine-rich repeat receptors, the Nogo receptor family, functions to restrict the number of excitatory synapses that form during brain development. Much is known about the function of Nogo Receptor 1 (NgR1) in the adult central nervous system (CNS) (reviewed in Yiu and He, 2006); in contrast, far less is known about Nogo receptor 2 (NgR2) and Nogo receptor 3 (NgR3). NgR1 binds to several ligands, including Nogo-A, MAG, and OMgp, as well as FGF-1 and FGF-2 (Lee et al., 2008). Several of these ligands were isolated from CNS myelin, where they are thought to induce growth-cone collapse and axon retraction following CNS injury, a function suggested to be mediated by NgR1 and several coreceptors, including P75, Lingo, and TROY (reviewed in Yiu and He, 2006). NgR1 signaling in axons has been shown to activate the small GTPase RhoA as well as Rho kinase (ROCK), important cytoskeletal regulatory proteins thought to mediate axon outgrowth inhibition (Niederöst et al., 2002). While there is an emerging appreciation that NgR1 plays a role in restricting dendritic growth and plasticity in several brain regions, the mechanism of this process has not been understood (McGee et al., 2005; Lee et al., 2008, Zagrebelsky et al., 2010, Delekate

et al., 2011), nor has the functional role of the various NgR family members during brain development been established.

Here we show that members of the NgR family function in the dendrite to restrict synapse number in vivo. This effect appears to be due to synapse addition, not synapse elimination, and is mediated by RhoA, which reduces overall synapse number in part by constraining dendritic growth, thereby limiting the number of synaptic contacts made during development. Our expression studies show that the NgR family is downregulated by neuronal activity, suggesting a possible mechanism by which the NgR barrier for synapse development is relieved. These findings define a family of cell surface receptors that restrict the number of synaptic connections that form in the mammalian brain and thus ensure the proper development of neural circuits.

RESULTS

The NgR Family Is Dendritically Localized and Inhibits Excitatory Synapse Formation In Vitro

NgR1 was first identified based on its ability to bind Nogo-66, an inhibitor of axon outgrowth (Fournier et al., 2001). However, NgR1 expression is not limited to the axon. Upon examining NgR1 expression in dissociated hippocampal neuron cultures using an NgR1-specific antibody (Figures 1A and 1C, and Figure S1A available online), we found that NgR1 is expressed from 7 to 18 days in vitro (DIV), a time when the majority of synapses are forming in these cultures (Figure 1B). We used immunocytochemistry to investigate the subcellular distribution of NgR1 and found that it is broadly expressed on dendrites as well as axons (Figure 1D), consistent with biochemical fractionation studies demonstrating that NgR1 is present in both pre- and postsynaptic density fractions (Lee et al., 2008). Experiments using antibodies to specific synaptic proteins revealed that, while NgR1 is in close apposition to synaptic proteins such as PSD95, GluR2, SV2, and GAD67, NgR1 seldom overlaps with these proteins (Figure 1E and quantified in Figure S1B). Whereas PSD95 and GluR2 are expressed in dendritic spines, NgR1 is expressed primarily in the dendritic shaft (outlined in white in Figure 1E_{i-v}), where it colocalizes with filamentous actin (Figure 1E_v). These observations suggest that NgR1 is largely excluded from excitatory synapses and instead is concentrated in nonsynaptic sites along the dendritic shaft. Importantly, staining under nonpermeabilizing conditions demonstrates that ~40% of NgR1 is on the cell surface of dendrites (Figures S1C–S1D). Given these findings, we considered the possibility that NgR1 might define regions of dendrites where synaptic development is suppressed.

To assess the function of NgR1 during synapse development, we examined the effect of reducing the expression of NgR1 in cultured hippocampal neurons. Two distinct RNAi-based approaches were used to knockdown NgR1 expression, either direct transfection with short interfering RNA duplexes (siNgR1) or a plasmid encoding a short hairpin RNA to NgR1 (shNgR1) that targets a distinct region of NgR1 mRNA. These RNAis were tested in heterologous cells and primary neuronal cultures, where they selectively reduced NgR1 protein levels while leaving NgR2 and NgR3 expression unaffected (Figures S2A-S2C). To investigate the effect of reducing NgR1 expression on synapse number, hippocampal neurons were cultured, transfected at 9 days in vitro with a plasmid encoding green fluorescent protein (GFP) together with an RNAi to NgR1 or a control RNAi, and fixed 5 days later for staining with antibodies that recognize the presynaptic protein synapsin1 (Syn1) and the postsynaptic protein PSD95. To quantify the number of synapses formed on the transfected neuron, we counted the number of apposed Syn1/PSD95 puncta along dendrites of GFP-expressing neurons (see Experimental Procedures). Using this approach we found that knockdown of NgR1 resulted in a significant increase in excitatory synaptic number (Figures 2A–2C; all data are listed in Table S1). Similar results were obtained using alternative sets of synaptic markers (GluR2/Syt1 or NR2B/Syt1) (Figures 2E, 2F, and S2D). Furthermore, we also observed an increase in the average size and intensity of synaptic puncta after NgR1 knockdown (Figures S2E and S2F).

We verified the specificity of the NgR1 RNAi phenotype by testing the ability of an RNAi-resistant form of NgR1 (ResNgR1) to rescue the increase in synapse density observed upon knockdown of NgR1. ResNgR1 was validated in heterologous cells (Figure S1B) and then cotransfected in culture neurons along with shNgR. We found that ResNgR1was sufficient to reverse the increase in synaptic number observed with knockdown of NgR1 (Figure 2D), suggesting that the increase in synapse number in NgR1 RNAi-treated neurons is due to the specific knockdown of NgR1 by RNAi.

NgR1 belongs to a family that includes two highly homologous proteins, NgR2 and NgR3. All three NgRs are expressed at high levels in the dorsal telencephalon during synaptic development (Figure S2G). To investigate whether NgR2 and NgR3 also function as negative regulators of synapse development, we examined the effect of reducing expression of either NgR2 or NgR3 in cultured hippocampal neurons. Short hairpin RNAs to NgR2 (shNgR2) or NgR3 (shNgR3) were validated in heterologous cells (Figure S2H) and then expressed in neurons, where they resulted in a significant increase in excitatory synapse density (Figure S2I). To extend this finding, we acquired knockout mice for *NgR1* (Zheng et al., 2005), *NgR2*, and *NgR3* (Lexicon Genetics), validated that these animals are null for the respective NgR genes (Figures S5A and S5B), and assessed the contribution of NgR1, NgR2, and NgR3 to synaptic development in cultured hippocampal neurons. These experiments revealed that loss of any single NgR family member (*NgR1*$^{-/-}$, *NgR2*$^{-/-}$, or *NgR3*$^{-/-}$) results in an increase in the number of excitatory synapses relative to littermate controls (Figure 2G). Thus, all three NgR family members have similar functions in restricting synaptic development in vitro, regardless of whether they are removed acutely in individual neurons with RNAi, or constitutively removed throughout neuronal cultures using genetic loss-of-function approaches.

Since eliminating expression of members of the NgR family results in an increase in synapse number, we asked whether overexpression of NgR1 results in a decrease in synapse number. Cultured hippocampal neurons were transfected with varying concentrations of a wild-type NgR1 expression construct (WTNgR1) and synaptic puncta were quantified. When expressed at a low concentration such as that used to rescue the NgR1 shRNA phenotype (100 ng), WTNgR1 had no effect on

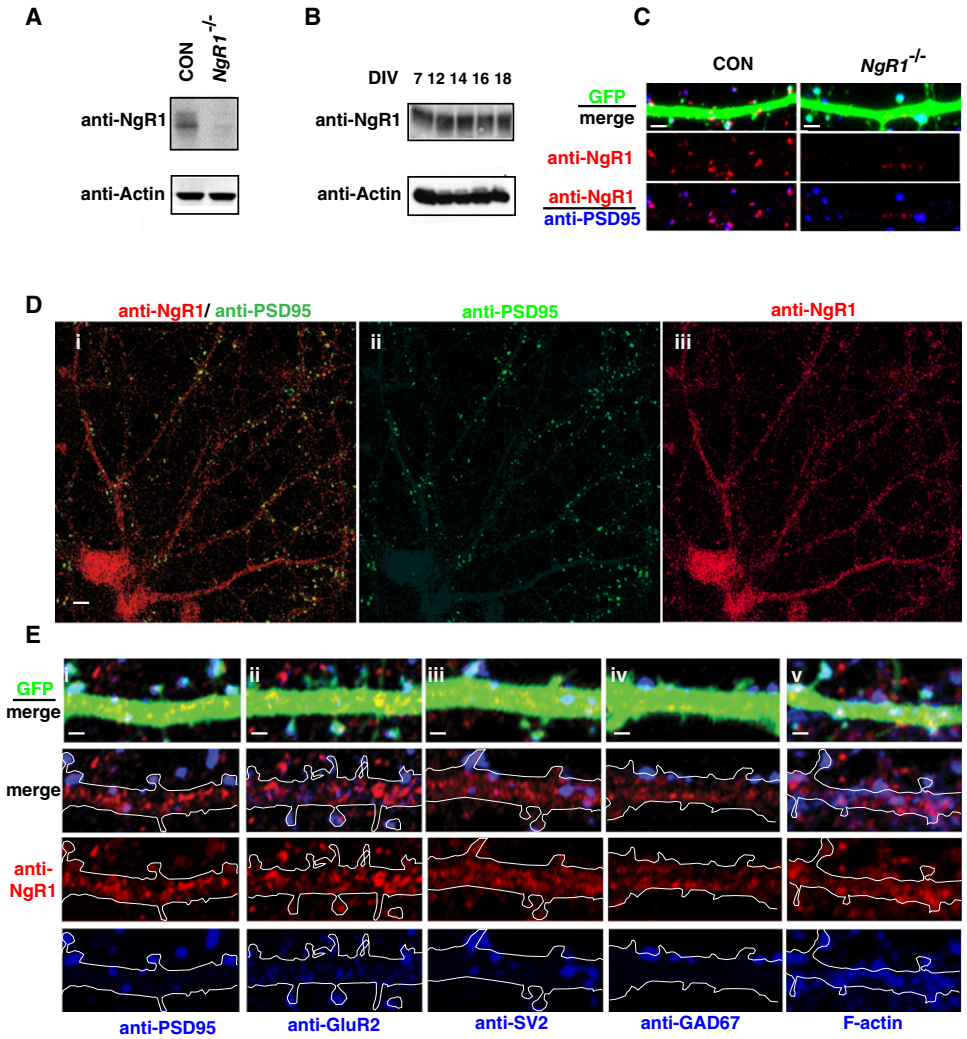

Figure 1. NgR1 Is Expressed on Dendrites of Hippocampal Neurons during Synaptic Development

(A) Specificity of NgR1 antibody in immunoblotting. Lysates from hippocampal neurons (18 DIV) were analyzed by immunoblot using an antibody directed against NgR1. NgR1 protein is expressed in cultures derived from heterozygous *NgR1*$^{+/-}$ embryos (CON) and is selectively lost in neurons derived from *NgR1*$^{-/-}$ embryos. Samples were blotted for actin as a loading control.

(B) Time course of NgR1 expression. Immunoblot analysis of lysates from wild-type hippocampal neurons using an NgR1 antibody demonstrates that NgR1 is highly expressed from 7 to 18 DIV, a time of abundant synapse formation.

(C) Specificity of NgR1 antibody in immunostaining. Hippocampal neurons derived from either heterozygous embryos (CON; *NgR1*$^{+/-}$) or *NgR1* mutant (*NgR1*$^{-/-}$) embryos were transfected with GFP and analyzed at 18 DIV by immunostaining for NgR1 (red) and PSD95 (green). A representative dendrite is shown, revealing punctate NgR1 expression that is specifically lost in neurons from *NgR1*$^{-/-}$ mice. Scale bar is 2.5 μm. Further validation is shown in Figure S1A.

(D) NgR1 is broadly expressed in dendrites. Wild-type hippocampal neurons were analyzed by immunostaining (18 DIV) for NgR1 (red) and PSD95 (green). NgR1 (red puncta in i and iii) is expressed broadly along developing dendrites and axons but is largely excluded from overlapping with the excitatory synaptic marker, PSD95 (green puncta in i and ii). Note that apparent NgR1 staining in the soma is likely nonspecific, since this signal is also present in neurons from *NgR1*$^{-/-}$ embryos. Scale bar is 10 μm. NgR1 cell-surface staining is shown in Figure S1C.

(E) NgR1 is dendritic, but is excluded from synapses. Hippocampal neurons were transfected with GFP (9 DIV) and then subsequently analyzed (18 DIV) by costaining for NgR1 (red) and the indicated markers (blue). Representative dendrites illustrate that NgR1 rarely colocalizes with synaptic markers, including PSD95 (i), GluR2 (ii), SV2 (iii), or GAD67 (iv), though it frequently overlaps with broadly expressed proteins such as filamentous actin (Phalloidin) (v). Scale bar is 1 μm. Quantification of NgR1 overlap with synaptic proteins is presented in Figure S1B.

synapse number; however, a 2-fold higher concentration of WTNgR1 (200 ng) significantly reduced synapse density (Figures 2H and 2I). Similarly, overexpression of WTNgR2 (Figures 2H and 2I) or WTNgR3 (Figure S7A) significantly reduced synapse number. Thus, results from a number of different experiments support that members of the NgR family restrict the number of excitatory synapses that form on hippocampal neurons in culture.

We next asked whether NgR1 inhibits the development of synapses in the context of an intact hippocampal circuit.

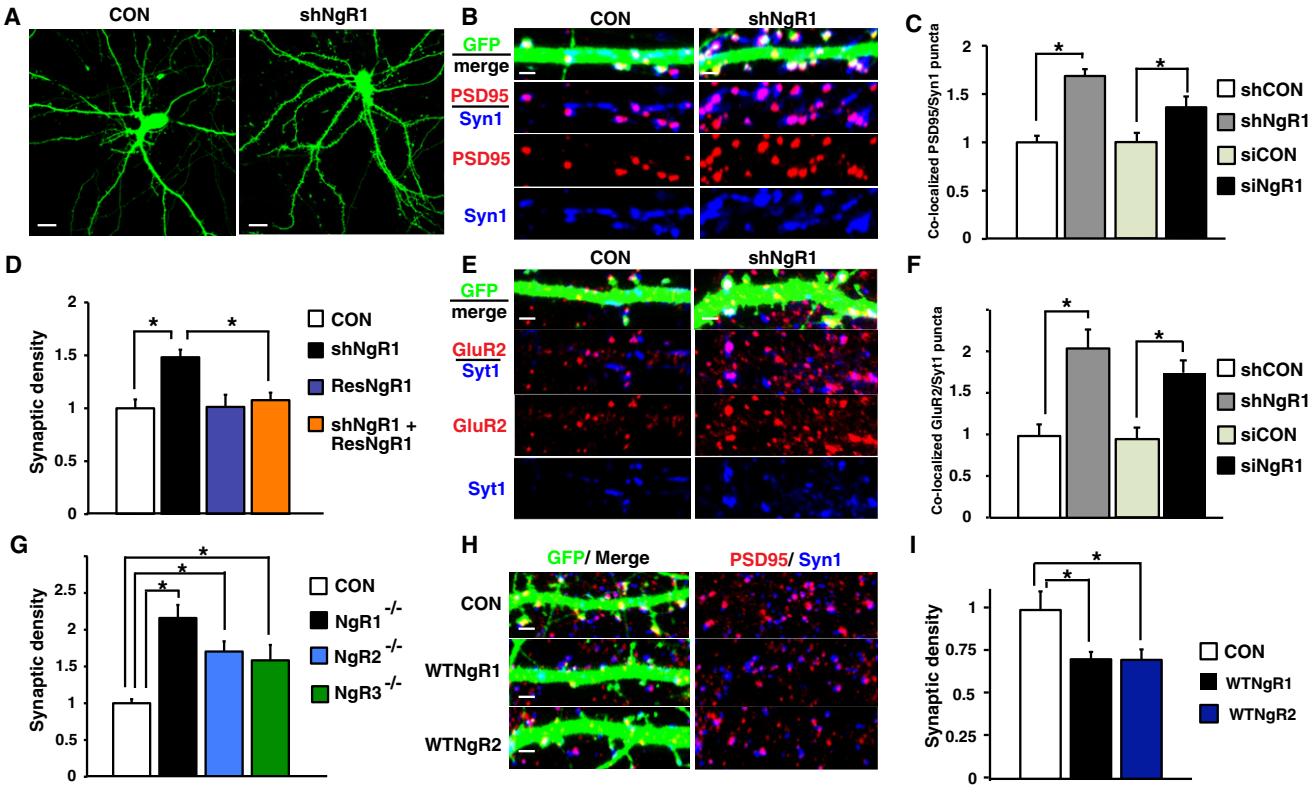

Figure 2. The NgR Family Restricts Synapse Formation in Cultured Neurons

(A–C) Knockdown of NgR1 increases the density of PSD95/Syn1 puncta in cultured hippocampal neurons. Cultured hippocampal neurons were cotransfected with GFP and either shCON (white bar), shNgR1 (gray bar), siCON (green bar), or siNgR1 (black bar) and subsequently immunostained with antibodies against the excitatory postsynaptic marker PSD95 (red) and the presynaptic marker Syn1 (blue). (A) Representative examples of control or shNgR1-transfected neurons. Scale bars represent 15 μm. (B) Representative dendrites from control or shNgR1-transfected neurons. Scale bars represent 2 μm. (C) Quantification of the density of colocalized PSD95 and Syn1 puncta along dendrites of transfected neurons. Data are normalized to respective controls. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are mean ± SEM from three to five experiments; total numbers of neurons analyzed (n) range from 28 to 54 cells per condition. Note that shNgR1 and siNgR1 were tested in heterologous cells and neurons, where they were found to selectively and efficiently knock down NgR1 protein (see Figure S2).

(D) RNAi-resistant NgR1 rescues the effect of shNgR1 on the density of PSD95/Syn1 puncta. Cultured hippocampal neurons were transfected with GFP and either shCON (white bar), shNgR1 (black bar), shCON and ResNgR1 (blue bar), or shNgR1 and ResNgR1 (orange bar). Neurons were immunostained with antibodies against PSD95 and Syn1, and the normalized density of apposed PSD95/Syn1 puncta along the dendrites of transfected neurons was quantified. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 27 to 31cells per condition.

(E and F) A distinct set of synaptic markers (GluR2/Syt1) also reveals increased synapse density upon knockdown of NgR1. Cultured hippocampal neurons were transfected with GFP and either shCON (white bar), shNgR1 (gray bar), siCON (green bar), or siNgR1 (black bar), and subsequently immunostained with antibodies against the postsynaptic marker GluR2 (red) and the presynaptic marker Syt1 (blue). (E) Representative dendrites. Scale bars represent 2 μm. (F) Quantification of the density of colocalized GluR2 and Syt1 puncta along dendrites of transfected neurons. Data are normalized to respective controls. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 28 to 34 cells per condition. Note that knockdown of NgR1 was also found to increase synapse intensity and size in vitro (see Figure S2).

(G) Hippocampal neurons from mice lacking any member of the NgR family (*NgR1*, *NgR2*, or *NgR3*) show elevated synapse density. Cultured hippocampal neurons from littermates of the indicated genotype were transfected with GFP and immunostained antibodies against PSD95 and Syn1 to determine the normalized synapse density. Embryos that were heterozygous for *NgR1*, *NgR2*, and *NgR3* were used as a control (CON; white bar). Neuronal cultures from $NgR1^{-/-}$ (black bar), $NgR2^{-/-}$ (blue bar), and $NgR3^{-/-}$ (green bar) mutant embryos were heterozygous for the other respective NgR family members. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 45 to 60 cells per condition. Note that acute knockdown of NgR1, NgR2, or NgR3 also resulted in an increase in synapse density (see Figure S2I). Western blotting and quantitative RT-PCR were used to confirm that NgR mutant mice were protein or mRNA nulls (see Figure S5).

(H and I) Overexpression of WTNgR1 or WTNgR2 reduces synapse density. Cultured hippocampal neurons were transfected with GFP alone (CON; white bar) or GFP and either WTNgR1 (black bar) or WTNgR2 (blue bar). Neurons were subsequently immunostained with antibodies against PSD95 (red) and Syn1 (blue). (H) Representative dendrites. Scale bars represent 2 μm. (I) Quantification of the normalized density of colocalized PSD95 and Syn1 puncta along dendrites of transfected neurons. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 21 to 49 cells per condition.

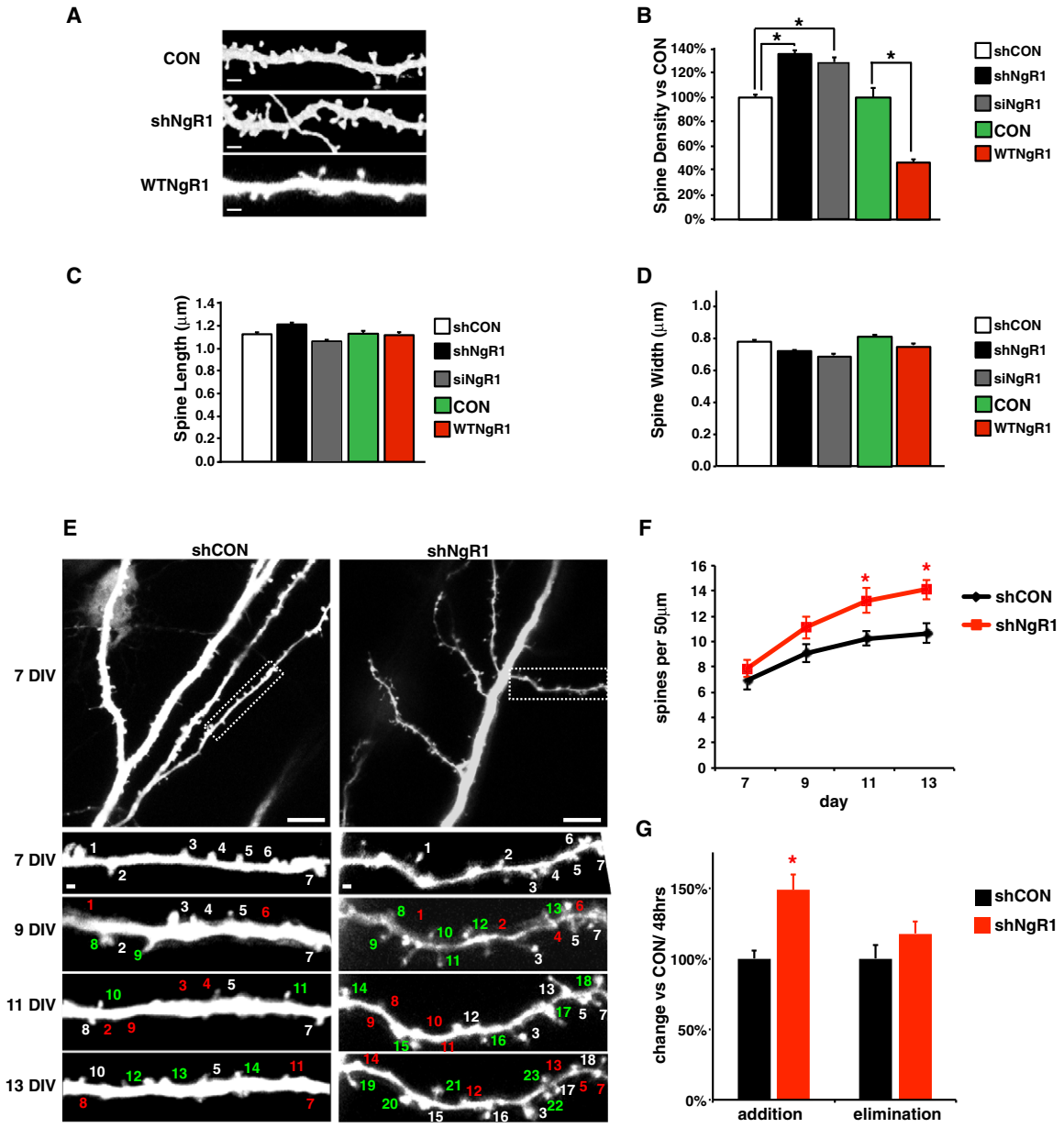

Figure 3. NgR1 Restricts Spine Formation in a Hippocampal Circuit

(A–D) Effects of NgR expression on dendritic spine morphology. Organotypic hippocampal slices were biolistically transfected with GFP alone (CON; green bar) or GFP along with either a control shRNA construct (shCON, white bars), shNgR1 (black bars), siNgR1 (gray bars), or WTNgR1 (red bars), as indicated, and fixed. (A) Representative images of proximal secondary apical dendrites from GFP-labeled CA1 pyramidal neurons from CON, shNgR1, and WTNgR1 are shown. Scale bar is 1 μm. Secondary apical dendrites were analyzed following biolistic introduction of GFP together with the indicated constructs, and the average dendritic spine density (B), spine length (C) and spine width (D) were determined. Spine density is normalized to respective controls; spine length and width are averages. * indicates $p < 0.01$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from three experiments; total numbers of neurons analyzed (n) range from 20 to 30 cells per condition.

(E) Extended live imaging of spine density changes over time. Organotypic hippocampal slices were biolistically transfected with GFP along with either a control shRNA construct (shCON) or an shRNA construct targeting NgR1 (shNgR1). Representative images of proximal secondary apical dendrites from GFP-labeled CA1 pyramidal neurons that were repeatedly imaged are shown. Top panels show the entire proximal dendritic region of representative neurons at 7 DIV, while a higher magnification image of the boxed regions (white dotted rectangles) is shown below following spine development over 7 days in vitro (7–13 DIV). White numbers mark persistent spines (older than 48 hr), green numbers mark newly formed spines (<48 hr old) and red numbers mark eliminated spines (lost within the last 48 hr). Scale bars in top images indicate 10 μm, and those in the bottom images mark 1 μm.

(F) Quantification of spine density over time. The average spine density of dendritic regions repeatedly imaged following the introduction of shCON (black line) or shNgR1 (red line) is quantified at four time points (7, 9, 11, and 13 DIV). * indicates $p < 0.01$ with a Student's t test. Data are means, with error bars representing ±SEM from three experiments; total number of neurons analyzed (n) was 26 per condition.

Hippocampal slices were cultured from wild-type P6 rats and biolistically transfected with GFP alone or GFP along with control RNAi, shNgR1, or WTNgR1 to assess the effect of NgR1 expression on spine formation in a neuronal circuit. Knockdown of NgR1 through the introduction of either shNgR1 or siNgR1 into hippocampal slices for 5 days resulted in a significant increase in the number of dendritic spines relative to control (Figures 3A and 3B), with no effect on spine width or length (Figures 3C and 3D). In contrast, overexpression of WTNgR1 in organotypic hippocampal slices resulted in a substantial reduction in spine number (Figures 3A and 3B). These observations suggest that in an intact neuronal circuit, NgR1 restricts the number of dendritic spines, the sites where the majority of excitatory synapses form.

Our experiments thus far raise the possibility that NgRs either prevent the initiation of new synapses or mediate synapse elimination. To distinguish between these possibilities, we quantified spine addition and elimination over time by repeatedly imaging cultured hippocampal slices that were biolistically transfected with GFP and a control shRNA or shNgR1. We observed that NgR1 knockdown results in a significant increase in spine density following repeated imaging of cultured hippocampal slices (Figures 3E and 3F). A quantification of spines added or eliminated following NgR1 knockdown revealed a significant increase in spine addition but no change in spine elimination (Figures 3G, S3A, and S3B), lending support to the idea that NgR1 functions to suppress the establishment of new synapses rather than by mediating synapse elimination.

NgR1 Functions Postsynaptically

Several NgR1 ligands and coreceptors are expressed on axons and dendrites; thus, the potential exists for NgR1 to signal bidirectionally. To address whether NgR1 functions pre- or postsynaptically, we quantified changes in synapse density observed upon knockdown or overexpression of NgR1 and then deconvolved these same data sets to determine whether there was a change in the number of pre- and/or postsynaptic specializations. This analysis revealed that the effects of NgR1 on synapse density were due to changes in the number of postsynaptic (PSD95 or GluR2) puncta rather than the presynaptic (Syn1 or Syt1) puncta (Figures 4A–4C; data not shown). Similarly, deconvolution of synapse density measurements following RNAi targeting of NgR2 and NgR3 also revealed a specific increase in PSD95 puncta number, size, and intensity (Figures S2G and S2H). Importantly, simulated modeling studies confirmed that the changes in synapse density following NgR1 knockdown could not be accounted for by random overlap due to increased numbers of postsynaptic puncta (Figures S4B and S4C).

To determine whether changing the level of NgR1 throughout neuronal cultures affects the levels of specific synaptic proteins, we infected neurons with lentiviruses to drive the expression of NgR1 throughout neuronal cultures and found that WTNgR1 overexpression results in a significant reduction in PSD95 protein levels as assessed by quantitative western blotting (Figures 4D and 4E). Moreover, the opposite effect was observed upon NgR1 knockdown, which resulted in a significant increase in both PSD95 and GluR2 levels (Figures 4D, 4E, and S4A). In contrast, the level of Syn1 was unaffected by NgR1 overexpression or knockdown (Figures 4D, 4E, and S4A). Thus, analysis of both single cells and neuronal cultures suggests that NgR1 inhibits the development of excitatory synapses through its action in the postsynaptic cell, where it causes reduced expression of specific postsynaptic proteins. These findings suggest that NgR1 has a cell-autonomous role in the dendrite that is distinct from its previously described function in the axon.

NgR1 Functions Together with a Coreceptor, TROY, to Restrict Excitatory Synapse Number

NgR1 functions by activating intracellular signaling cascades via transmembrane coreceptors such as P75, TROY, and Lingo-1 (Yiu and He, 2006). To investigate whether coreceptor signaling is required for the inhibition of synapse formation by NgR1, we tested the effect of an NgR1 mutant that lacks a co-receptor-binding region (DNNgR1 [Wang et al., 2002a]). Unlike the effect of WTNgR1, overexpression of DNNgR1 did not result in decreased synapse density but rather caused a small but significant increase in synapse density relative to control, presumably due to its ability to sequester ligands away from endogenous NgR1 (Figures 4F and 4G). This finding suggests that NgR1 requires a coreceptor to inhibit synapse development.

Genome-wide RNA sequencing revealed that of the known NgR1 coreceptors, only Lingo-1 and TROY are expressed at appreciable levels in 7 DIV neuronal neurons (data not shown). Since Lingo-1 is largely expressed on axons (Lee et al., 2008), we focused on TROY as a potential NgR1 coreceptor that might function in dendrites to inhibit synapse development.

Immunostaining with protein-specific antisera revealed that TROY is expressed along the dendrites of cultured neurons and overlaps significantly with all NgR family members (Figure S4E). In addition, TROY knockdown (Figures S4E, S4I, S4J, and S8B) caused a significant increase in synapse density in cultured hippocampal neurons (Figure 4H). Together, these findings are consistent with TROY being the coreceptor that mediates the inhibitory effects of NgR1 on synapse development.

To determine whether TROY is required for NgR1-dependent suppression of synapse development, WTNgR1 was overexpressed with or without TROY knockdown (shTROY) and synapse density was quantified. TROY knockdown reversed the reduction in synapse number observed with NgR1 overexpression (Figure 4I). An increase in synapse density was observed, similar to that seen upon TROY knockdown alone. Similar epistasis studies with WTNgR2 and WTNgR3 overexpression revealed that TROY is required for the suppression of synapse development by NgR2 and NgR3 (Figure S4K). Moreover, binding experiments using recombinant TROY protein incubated with heterologous cells expressing different NgR family members

(G) Quantification of spine addition and elimination. The average number of spines added or eliminated over all four time points was quantified on a per micron basis following biolistic introduction of shCON (black) or shNgR1 (red). Data are presented as percent change relative to control. * indicates $p < 0.01$ with a Student's t test. Data are means, with error bars representing ±SEM from three experiments. Quantification of spine addition and elimination for each time point is illustrated in Figure S3.

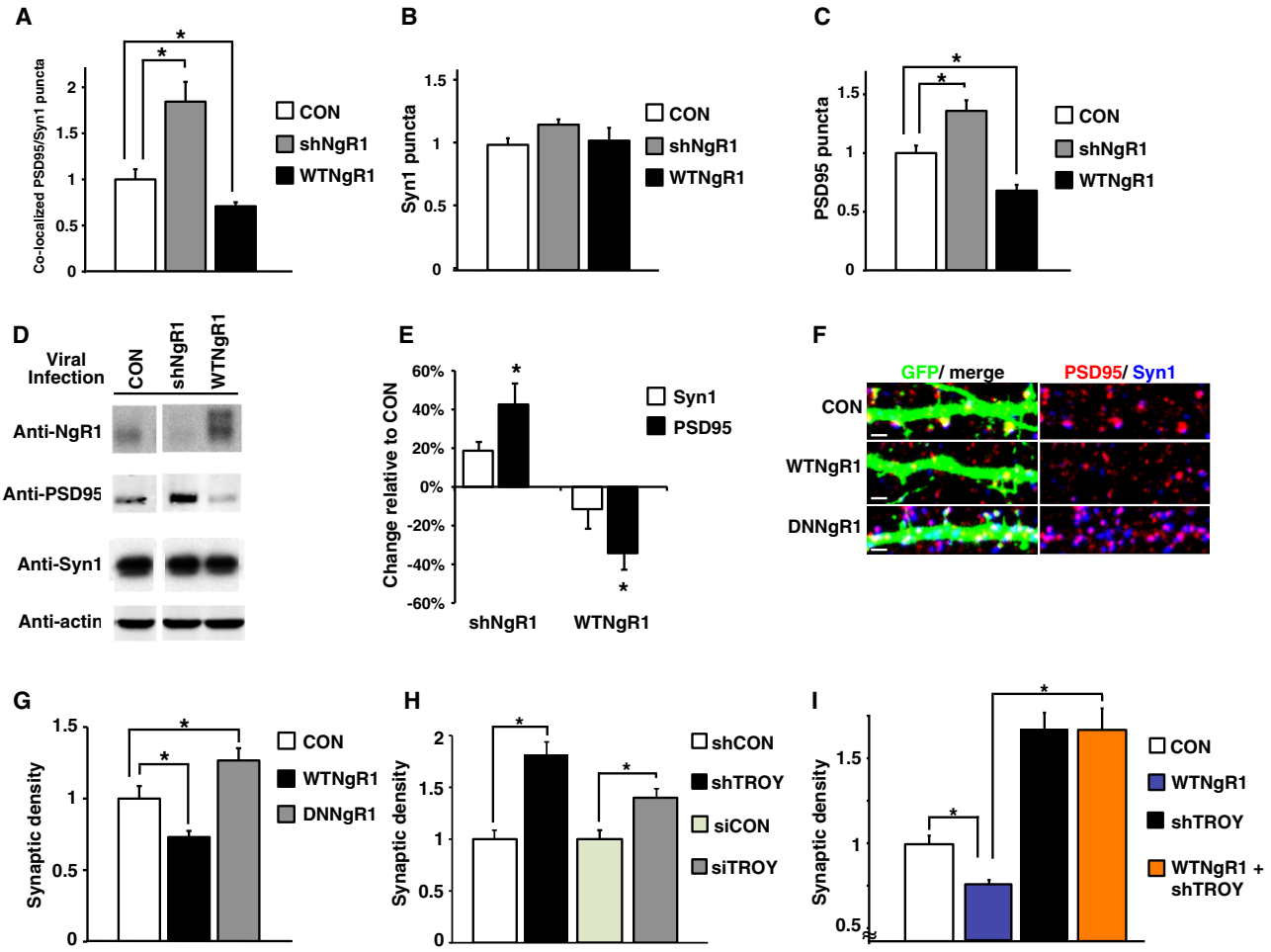

Figure 4. NgR1 Functions Postsynaptically through the Coreceptor TROY to Restrict Synapse Formation

(A–C) Effect of NgR1 on the density of PSD95/Syn1 puncta is due to changes in the postsynaptic protein PSD95. Cultured hippocampal neurons were transfected with GFP alone (CON; white bar) or GFP and either shNgR1 (gray bar) or WTNgR1 (black bar), and subsequently immunolabeled with antibodies against PSD95 and Syn1. (A) Synapse density was quantified by assessing the number of colocalized PSD95/Syn1 puncta along the dendrites of transfected neurons. These data were subsequently deconvolved to determine the density of Syn1 (B) or PSD95 (C) puncta along the dendrites. Similar results were observed upon deconvolution of the Syt1/GluR2 puncta (data not shown). * indicates $p < 0.01$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from three experiments; total numbers of neurons analyzed (n) range from 32 to 49 cells per condition.

(D and E) Modulation of NgR1 expression throughout neuronal cultures affects total levels of PSD95. Cultured hippocampal neurons (5 DIV) were infected with a control lentivirus (CON), an shNgR1 lentivirus, or a WTNgR1 lentivirus. Neurons were lysed at 14 DIV and analyzed by immunoblotting for NgR1, PSD95, Syn1 or actin (used as a loading control). (D) Representative immunoblots. (E) Quantification of protein levels. Integrated intensities were measured by infrared fluorescence detection of immunoblots. Syn1 (white bar) and PSD95 (black bar) protein levels were normalized to actin and expressed as change relative to CON. * indicates $p < 0.01$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from three to six experiments. Note that knockdown of NgR1 also resulted in an increase in the total level of a second postsynaptic protein, GluR2 (see Figure S4).

(F and G) Overexpression of a mutant form of NgR1 (DNNgR1) causes a significant increase in synapse density. Cultured hippocampal neurons were transfected with GFP alone (CON; white bar), or cotransfected with GFP and either WTNgR1 (black bar) or DNNgR1 (gray bar). Samples were immunolabeled for PSD95 and Syn1. (F) Representative dendrites. Scale bar is 1 μm. (G) Quantification of normalized synapse density. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from three experiments; total numbers of neurons analyzed (n) range from 32 to 49 cells per condition.

(H) Knockdown of the NgR1 coreceptor TROY causes a significant increase in synapse density. Cultured hippocampal neurons were transfected with GFP and control RNAis shCON (white bar) or siCON (green bar), or GFP and RNAis targeting TROY, shTROY (black bar) or siTROY (gray bar), as indicated. Five days later, cells were fixed, immunolabeled for PSD95 and Syn1, and quantified to determine the normalized synapse density. * indicates $p < 0.01$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from three experiments; total numbers of neurons analyzed (n) range from 24 to 51 cells per condition.

(I) Inhibition of synapse formation by NgR1 requires TROY. Cultured hippocampal neurons were transfected with GFP alone (CON; white bar), or cotransfected with GFP and WTNgR1 (blue bar), shTROY (black bar), or both (orange bar). Neurons were immunolabeled with antibodies against PSD95 and Syn1 to determine the normalized synapse density. * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from three experiments; total numbers of neurons analyzed (n) range from 25 to 67 cells per condition. Note that TROY is highly expressed in cultured hippocampal neurons (data not shown) and that knockdown of TROY significantly reduces its mRNA levels (see Figure S4).

show that TROY is capable of binding NgR1 and NgR2, but not NgR3 (Figure S4F), suggesting that NgR1 and NgR2 may signal through TROY directly. It remains unclear whether the affinity of the NgR3-TROY interaction falls below the detection limit of this assay or whether NgR3 acts through an alternative coreceptor. Taken together, these findings identify TROY as a potential coreceptor for the NgR family that mediates their ability to restrict excitatory synapse number.

The NgR Family Cooperates in Restricting Synaptic Development In Vivo

To address whether the NgR family contributes to synaptic development in vivo, we crossed NgR mutant mice with the GFPM line (Feng et al., 2000), in which a small subset of neurons are genetically labeled with the Thy1-GFP allele, thus enabling visualization of dendritic spines from hippocampal pyramidal neurons. Knockout of any one NgR family member alone was not sufficient to affect the density of dendritic spines in vivo (Figure 5B). Given our previous finding that all three NgR family members play a similar role in limiting synapse development in vitro (Figures 2G and S2I), we hypothesized that these family members might functionally compensate for one another in vivo. To address this possibility, we generated triple knockout mice (*NgRTKO*$^{-/-}$). *NgRTKO*$^{-/-}$ mice were born with the appropriate Mendelian frequencies and appear largely normal, with no obvious defects in formation of the hippocampus. Analysis of GFP-expressing neurons at P18 revealed a significant increase in the number of dendritic spines on CA1 pyramidal neurons in *NgRTKO*$^{-/-}$ mice relative to their triple heterozygous littermate controls (Figures 5A and 5B). These findings are consistent with the idea that the NgR family members function together in vivo to limit the number of excitatory synapses.

To extend this analysis using an independent approach, we performed transmission electron microscopy to visualize the ultrastructural features of excitatory synapses. In micrographs from *NgRTKO*$^{-/-}$ mice, we observed asymmetric synapses of typical morphology, suggesting that the overall structure and vesicle content of excitatory synapses are normal in the absence of NgRs. However, quantification of the number of excitatory synapses in the apical dendritic regions of CA1 revealed that *NgRTKO*$^{-/-}$ mice had a significant increase in the density of excitatory synapses relative to heterozygous littermate controls (Figures 5C and 5D). Furthermore, this effect was not limited to CA1 neurons, since analysis of CA3 neurons also revealed a clear increase in the number of PSDs in *NgRTKO*$^{-/-}$ animals (Figure 5E). Thus, analysis by confocal and electron microscopy suggests that the NgR family functions to limit the number of excitatory synapses in vivo.

To address whether the observed increase in synapse number reflects an increase in functional synapses, we performed whole-cell patch-clamp electrophysiology on CA1 pyramidal neurons from acute hippocampal slices obtained from *NgRTKO*$^{-/-}$ mice and control littermates to quantify the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs). This analysis revealed a significant increase in the frequency of mEPSCs in *NgRTKO*$^{-/-}$ mice relative to littermate controls (Figure 5F and S5C), suggesting that the NgR family restricts the development of functional excitatory synapses. Interestingly, there was a small but significant decrease in the amplitude of mEPSCs (Figure 5G and S5D), consistent with the immature spine types observed in *NgR1* knockouts (Lee et al., 2008; Zagrebelsky et al., 2010). Thus, reducing the expression of the NgR family results in an increase in functional synapses that are slightly reduced in strength.

The NgR Family Inhibits Dendritic Growth and Complexity

The question remained as to how NgRs work at a mechanistic level to restrict excitatory synapse number. One possibility was that NgRs limit the formation of new synapses in part by inhibiting dendritic growth, thereby reducing the possibility of contact between axons and dendrites. Therefore, we asked whether loss of NgR family members affects dendritic branching. Specifically, we analyzed dendritic branch complexity in GFP-expressing neurons by Sholl analysis, which quantifies the number of dendritic branches intersecting concentric circles of increasing radii centered on the cell body (Sholl, 1953). We found that neurons from mice lacking *NgR1* showed a significant increase in dendritic complexity relative to littermate controls, whereas overexpression of WTNgR1 resulted in a decrease in complexity of the dendritic arbor (Figures 6A and 6B; all Sholl data are listed in Table S2). Similarly, there was a significant increase in dendritic complexity and total dendritic length in hippocampal slices upon knockdown of NgR1 (Figure S6). Moreover, this effect was also observed in vivo, where analysis of GFP-expressing CA1 pyramidal neurons from *NgRTKO*$^{-/-}$ animals revealed an increase in both the complexity of basal dendrites (Figures 6C–6E) and total dendritic length (Figure 6F). Taken together, these findings provide evidence that NgR family members inhibit the growth and decrease the complexity of the dendritic arbor and suggest that, in addition to decreasing synapse density, a second way that NgR family members may restrict synapse number is by inhibiting dendritic growth, reducing the overall area for potential synaptic inputs.

NgR1 Restricts Dendritic Growth and Synapse Number through Activation of RhoA

We asked if NgR/TROY limits dendrite and spine/synapse development by inhibiting the polymerization of the actin cytoskeleton, a process that is essential for dendritic and spine growth. Previous studies have shown that RhoA is a critical regulator of actin assembly (Maekawa et al., 1999). To investigate the involvement of RhoA in the inhibition of dendritic growth and synapse development by NgR1, we tested whether NgR1 activates RhoA in hippocampal neurons during synaptic development. Hippocampal neurons were infected with lentivirus expressing WTNgR1, and RhoA activity was assessed using a Rhotekin-binding domain (RBD) assay, which utilizes the Rho-binding domain of Rhoteckin as an affinity reagent to precipitate active Rho (Rho-GTP) from cells. We found that the level of active RhoA was reduced by reduction of NgR1 and elevated upon NgR1 overexpression (Figures 7A and 7B). Thus, NgR1 signaling activates RhoA in hippocampal neurons during synapse formation.

To test whether the inhibitory effect of NgR1 on synapse development is mediated by RhoA, we blocked the activity of

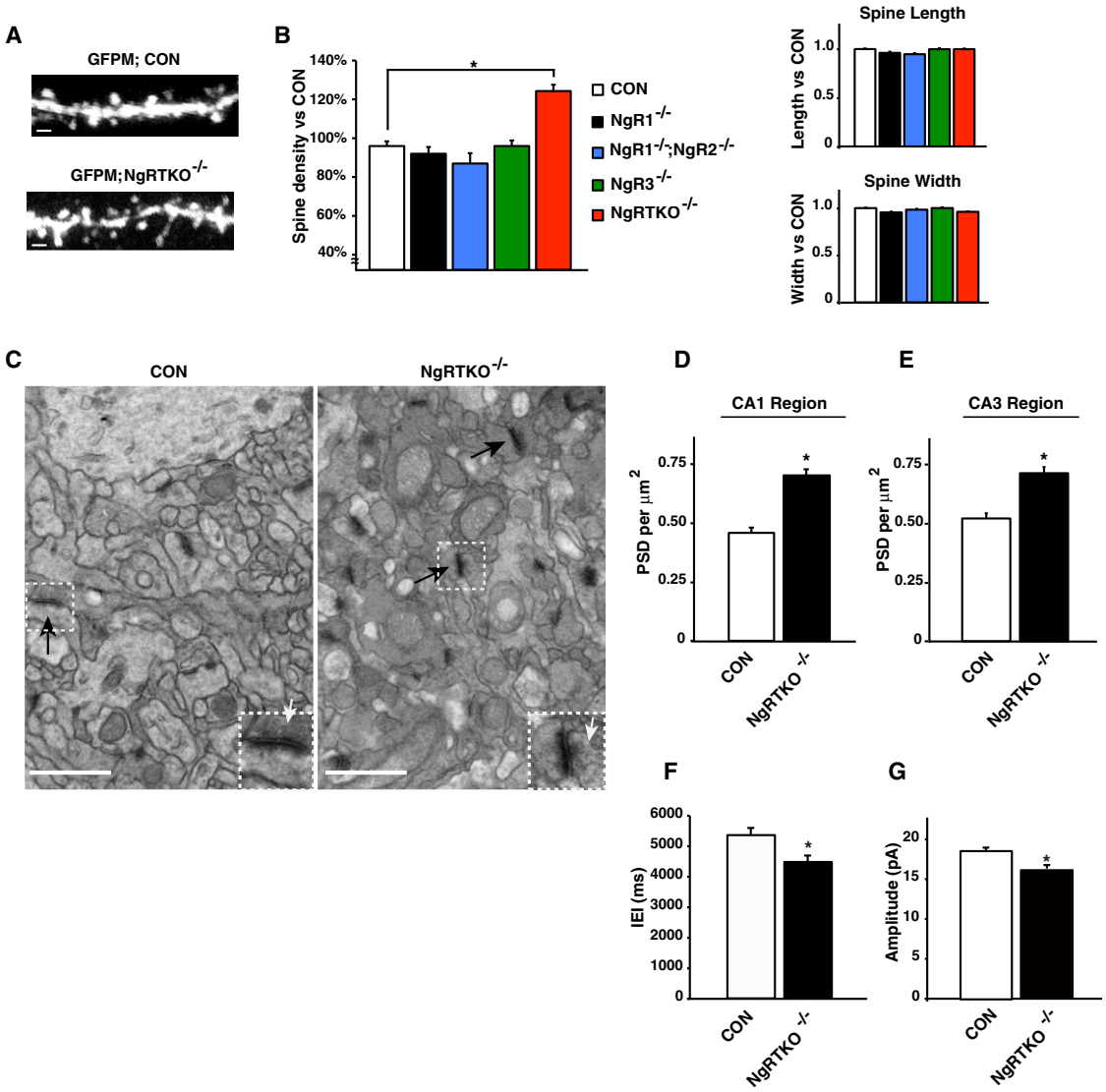

Figure 5. The NgR Family Restricts Synapse Formation in Vivo

(A and B) Mice lacking all three *NgR* family members have elevated spine density. Neurons were labeled genetically by breeding the GFPM allele into triple NgR knockout or control mice. Triple NgR knockout mice (*NgRTKO*$^{-/-}$ [red bar]) and triple heterozygous littermates (CON; white bar) were perfused at P18, sectioned by vibratome and immunolabeled with an anti-GFP antibody. (A) Representative images from proximal secondary apical dendrites. Scale bar is 1 µm. (B) Quantification of spine features. Dendritic spine density, spine length, and spine width from secondary apical dendrites of neurons expressing GFP were determined. Spine density values are expressed as percentage of the control value. Spine length and spine width values for the various genotypes are normalized to the mean value of the triple heterozygotes. Data are mean ±SEM from three independent experiments; total numbers of neurons analyzed (n) range from 30 to 60 cells per condition. * indicates $p < 0.01$, one-way ANOVA with pairwise comparison by Tukey's post hoc test.

(C–E) Ultrastructural analysis reveals that triple *NgR* knockouts have an abnormally high density of synaptic sites. (C) Representative transmission electron micrographs. Hippocampi of either triple heterozygous (CON) or triple knockout (*NgRTKO*$^{-/-}$) P18 littermates were prepared for electron microscopy and apical CA1 regions were analyzed. Black arrows mark examples of asymmetric excitatory postsynaptic densities (PSDs). White dashed boxes mark example PSDs magnified in lower righthand corner, with white arrows highlighting presynaptic vesicles. Scale bar represents 1 µm. (D and E) Quantification of asymmetric PSDs. Asymmetric PSDs from either the CA1 (D) or CA3 (E) regions of the hippocampus were quantified, analyzing 5 × 5 µm regions. Data are mean ± SEM from two littermate pairs, analyzing 30 regions per condition. * indicates $p < 0.01$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test.

(F and G) Acute slice recordings from triple *NgR* knockouts reveal an increase in the frequency but decrease in the amplitude of mEPSCs. Mini-excitatory postsynaptic currents (mEPSCs) were recorded from acute hippocampal slice preparations obtained from P15 triple heterozygous (CON) or triple knockout (*NgRTKO*$^{-/-}$) animals. Mean interevent intervals (IEI) or amplitudes are represented ± SEM from 10–14 animals. * indicates $p < 0.05$ using a KS test from analysis of cumulative probability plots of mEPSCs IEIs and amplitudes (Figure S5).

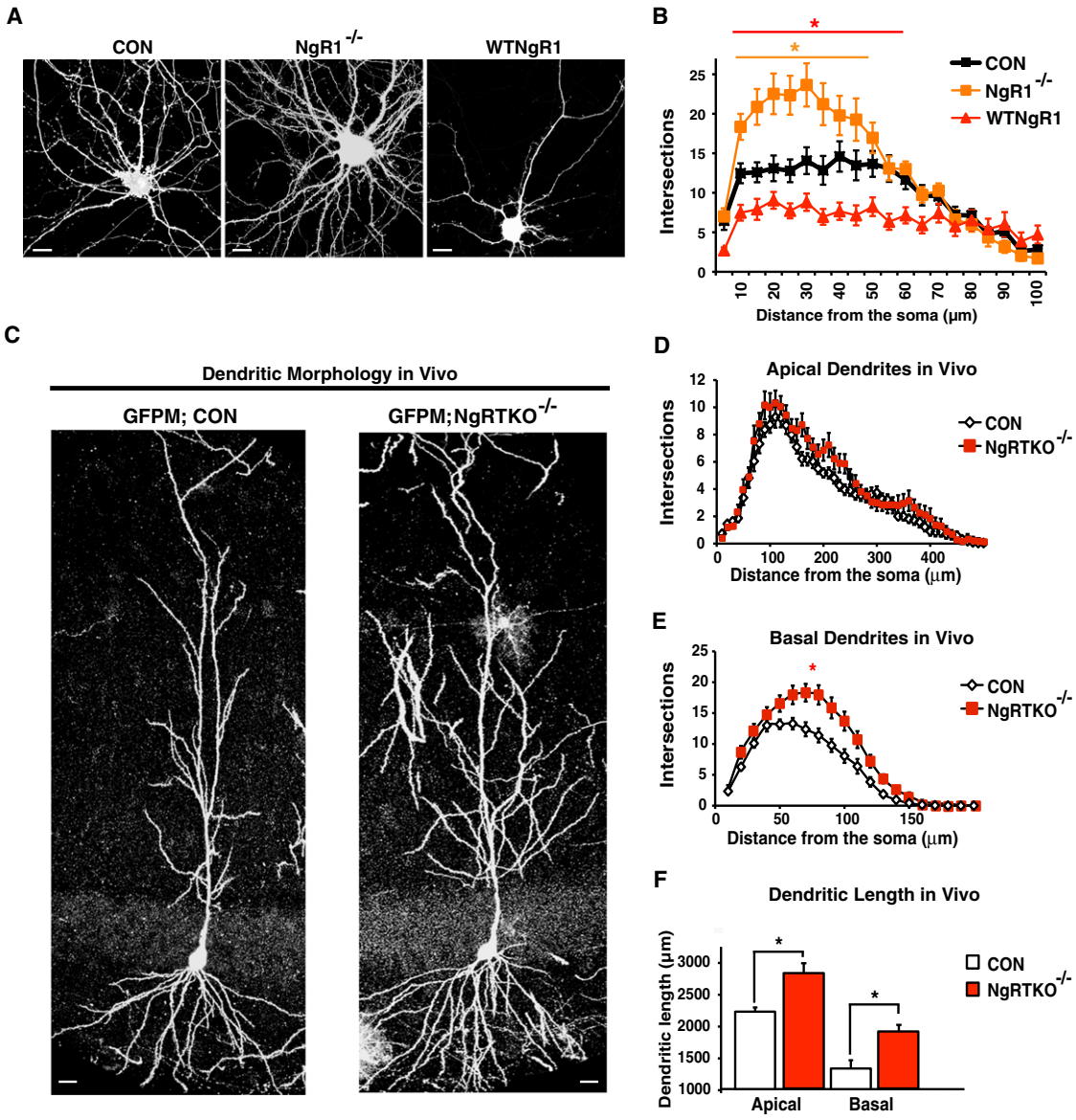

Figure 6. The NgR Family Restricts Dendritic Growth In Vitro and In Vivo

(A and B) NgR1 inhibits the dendritic complexity of hippocampal neurons in vitro. GFP was expressed by transfection of cultured hippocampal neurons from wild-type mice (CON; black bar), embryos lacking *NgR1* (*NgR1*$^{-/-}$; orange bar), or neurons in which WTNgR1 was cotransfected along with GFP (red bar). Subsequently, neurons were fixed and analyzed by confocal microscopy. (A) Representative neurons. Scale bar is 15 μm. (B) Quantification of dendritic complexity by Sholl analysis. Note that knockdown of NgR1 also increased dendritic complexity and length in organotypic slice culture (see Figure S6). Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) are 23 cells per genotype. * indicates $p < 0.05$, repeated-measures two-way ANOVA with pairwise comparison by Bonferroni post hoc.

(C–F) The length and complexity of dendrites is significantly increased in *NgR* triple knockout mice in vivo. Neurons were genetically labeled by breeding the GFPM allele (Feng et al., 2000) into the background of mice lacking NgR family members. Triple NgR knockout mice (*NgRTKO*$^{-/-}$; red bar) and triple heterozygous littermates (CON; white bar) were perfused at P18, sectioned by vibratome, and labeled with an anti-GFP antibody. (C) Representative neurons from the CA1 region of the hippocampus. Scale bar is 10 μm. (D–F) Quantification of dendritic complexity. Sholl analysis of apical (D) and basal (E) dendrites was performed, and total dendritic length (F) was measured. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 20 to 22 cells per genotype. * indicates $p < 0.05$, repeated-measures two-way ANOVA with pairwise comparison by Bonferroni post hoc.

RhoA or one of its downstream effectors, ROCK, using selective inhibitors. Treatment of hippocampal cultures with either the Rho inhibitor (C3 Transferase) or the ROCK inhibitor (Y27632) led to a significant increase in synapse number (Figure 7C), suggesting that RhoA signaling acts downstream of NgR1 to restrict synapse number. Further, Rho or ROCK inhibition entirely rescued WTNgR1 suppression of synapse development (Figure 7C). These findings also extended to NgR2, NgR3, and TROY, all of

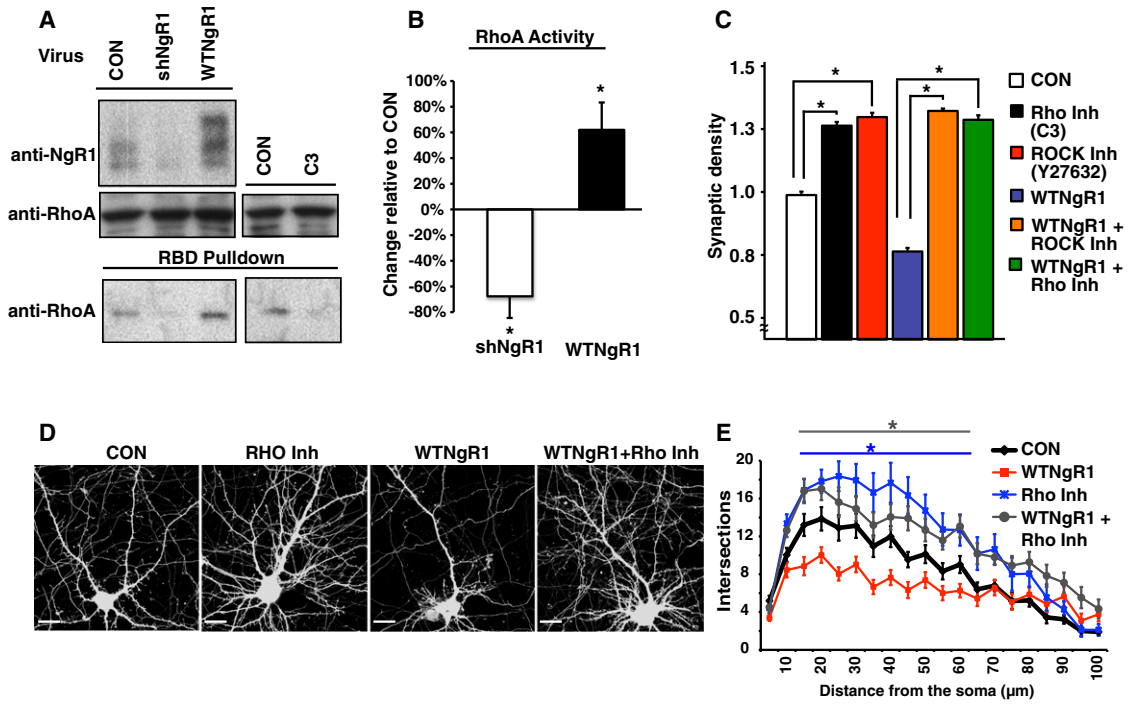

Figure 7. NgR1 Restricts Synaptic and Dendritic Growth through RhoA

(A) NgR1 regulates RhoA activity in hippocampal neurons. Cultured hippocampal neurons were infected with lentivirus expressing a control shRNA (CON), shNgR1, or WTNgR1. Alternatively, hippocampal cultures were treated with the Rho inhibitor C3 (200 ng/ml) or vehicle (CON) for 12 hr. Active Rho was then isolated by performing rhoteckin-binding domain (RBD) pull-downs and visualized by western blotting with antibodies to RhoA (bottom). Total protein levels were also assessed by immunoblot, using antibodies against NgR1 (top) and RhoA (middle).

(B) Quantification of active RhoA. Integrated intensities were measured by densitometry measurements of immunoblots in ImageJ (see Methods). Protein levels were normalized to actin and expressed as change relative to CON. * indicates $p < 0.01$–0.05, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are means, with error bars representing ±SEM from five independent experiments.

(C) Inhibition of synapse formation by NgR1 is reversed by blocking ROCK or Rho activity. Cultured hippocampal neurons were transfected with GFP alone (CON; white bar) or cotransfected with GFP and WTNgR1 (purple bar). Subsequently, transfected neurons were either mock-treated (white and purple bars) or treated with the Rho inhibitor C3 (200 ng/ml, black and green bars) or the ROCK inhibitor Y27632 (1 μM; red and orange bars) for 12 hr prior to immunolabeling with antibodies against PSD95 and Syn1 to determine normalized synapse density. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 50 to 70 cells per condition. * indicates $p < 0.05$, repeated-measures two-way ANOVA with pairwise comparison by Bonferroni post hoc.

(D and E) Inhibition of dendritic complexity by NgR1 is reversed by blocking Rho activity. Cultured hippocampal neurons were transfected with GFP alone (black bar) or cotransfected with GFP and WTNgR1 (red bar). Subsequently, neurons were mock-treated or treated with the Rho inhibitor C3 (200 ng/ml, blue and gray bars) for 12 hr, fixed and subjected to Sholl analysis. (D) Representative neurons. Scale bar is 15 μm. (E) Quantification of dendritic complexity. Data are mean ± SEM from three experiments; 30 neurons were analyzed per condition. * indicates $p < 0.05$, repeated-measures two-way ANOVA with pairwise comparison by Bonferroni post hoc. Similar results were observed using the ROCK inhibitor (see Figure S7).

which require Rho and ROCK to suppress synaptic development (Figure S7A). Similarly, inhibition of RhoA or ROCK blocked, albeit not completely, the effect of WTNgR1 overexpression on dendritic growth (Figures 7D, 7E, and S7B). Together, these findings suggest that the NgR family regulates synapse number in part by activating RhoA, potentially restricting actin polymerization that underlies the growth of dendrites and spines.

Neuronal Activity Downregulates the NgR family and TROY

The finding that the NgR family restricts dendritic and spine development raised the possibility that NgR family members function together with TROY as a barrier that limits neural connectivity during development. However, these receptors are highly expressed at a time when neurons are beginning to form synapses, raising the question: what limits the inhibitory effect of NgR family members to allow for synaptogenesis? We hypothesized that stimuli such as neuronal activity that promote dendritic growth and synaptogenesis (Sin et al., 2002; Peng et al., 2009) might trigger the downregulation of the NgR family and/or TROY, thus relieving the barrier to excitatory synapse formation. To test this hypothesis, we analyzed the expression of NgR1, NgR2, NgR3, and TROY mRNA in response to changes in neuronal activity. Increasing neuronal activity resulted in a significant decrease in the mRNA level in all three NgR family members and TROY (Figures 8C–8F). To confirm these observations at the level of NgR protein expression, GFP-expressing hippocampal neurons were stained with anti-NgR1 antibodies and the total number of NgR1 puncta (cell surface and intracellular) on dendrites was quantified. When neurons were depolarized, either by elevation of levels of potassium chloride, addition of N-methyl-D-aspartic acid (NMDA), or inhibition of GABA

receptors with the antagonist bicuculline, the number of NgR1 puncta along dendrites was significantly reduced relative to untreated neurons (Figures 8A and 8B). A similar decrease in TROY and NgR1 protein levels was observed in vivo in response to kainite-induced seizure (Figures 8H and 8I) or enriched environment (Figures S8C and S8D). Conversely, blocking neuronal activity by treatment of neurons with a combination of the NMDA receptor antagonist amino-5-phosphonovaleric acid (APV) and the sodium channel blocker tetrodotoxin (TTX) had the opposite effect, causing a significant increase in the number of dendritic NgR1 puncta (Figures 8A and 8B). Importantly, cell-surface staining confirmed that modulation of neuronal activity altered NgR1 levels present at the cell surface (Figure S8A). While significant levels of the NgR family members persist throughout the period of synaptic development, TROY expression was found to decrease upon the onset of pronounced synaptogenesis (Figure 8G). Thus, neuronal activity and/or reduced expression of the coreceptor TROY may relieve the NgR-dependent barrier to synaptic growth, facilitating synaptogenesis during development and plasticity in the adult.

DISCUSSION

The formation of synaptic connections during development is a highly regulated process that is mediated in part by cell-surface proteins that promote initial contact between developing axons and dendrites. We hypothesized that neural connectivity might also be limited by cell-surface proteins that function to restrict excitatory synapse development so that synapses form at the right time and place and in the correct number. Here we show that the NgR family of proteins serves this important function. Our study suggests that NgRs function along the arbor of dendrites as a barrier that limits synapse formation. Loss of any one member of the NgR family is sufficient to reveal their inhibitory influence in vitro, whereas loss of all three NgRs is required for abnormally elevated excitatory synaptogenesis in vivo. These findings broaden our understanding of NgR1's function, since they identify a dendritic role for receptors whose function was hitherto ascribed mainly to the axon.

At a mechanistic level, NgRs appear to work through the coordinated inhibition of synaptic and dendritic growth. These findings are consistent with those of recent studies of more mature neuronal circuits, demonstrating that both Nogo and the Nogo receptor constrain dendritic growth (Zagrebelsky et al., 2010). The effects of NgR loss on synaptogenesis and dendrogenesis are coupled. Unlike Neuropilin-2, which has a more selective role in regulating the spatial distribution of synapses on a specific region of the dendrite, the primary apical shaft (Tran et al., 2009), the NgR family functions broadly on the dendrite to restrict dendritic growth and limit the number of excitatory synapses that form.

It will be important to identity the ligand or ligands that regulate the activity of the NgR family members in this developmental context. Several ligands have been shown to regulate NgR1 signaling. Recent work provides evidence that Nogo may promote synaptic maturation in more established neuronal circuits (Zagrebelsky et al., 2010; Pradhan et al., 2010). Consistent with these findings, we observe a significant increase in synapse density following Nogo-Fc (Nogo-66) addition to cultured hippocampal neurons (Z. Wills and M. Greenberg, unpublished observations), raising the possibility that Nogo may inhibit rather than activate NgR in this context. These findings suggest that NgR1 signaling may fulfill multiple roles in synaptogenesis depending on its mechanism of activation and developmental period. Given that Nogo is highly enriched in the PSD (Peng et al., 2004; Raiker et al., 2010), a better understanding of how ligand binding to NgR1 affects its downstream signaling may help to reveal how NgR1 regulates synapse number. It is noteworthy that of the known NgR1 ligands, only MAG can activate NgR2 (Venkatesh et al., 2005), and none have affinity for NgR3. These findings raise the possibility that NgR family members may bind different ligands, allowing each receptor to be tuned to distinct extracellular cues that function in parallel to inhibit synapse formation. Alternatively, these receptors may share a common ligand that remains to be be identified.

NgR1 was originally discovered as a receptor that mediates the inhibition of axon regrowth after injury in the adult (Fournier et al., 2001). More recent studies have also revealed developmental functions of NgR1 in the closure of the critical period in adolescent mice (McGee et al., 2005) and in regulating activity-dependent synaptic strengthening in the hippocampus (Lee et al., 2008). However, robust expression of NgR family members begins in newborn mice (Lee et al., 2008), and its function at this stage of growth was unknown. Our study clarifies this issue by uncovering a role for the NgR family in the early postnatal brain, where it functions in the dendrite to restrict synapse number. What might be the purpose of synaptic restriction by NgR family members? Our live-imaging studies suggest that the NgR family inhibits the formation of new synapses, possibly preventing premature synaptogenesis so that synapses are established at the correct time and place. In addition, the NgR family may provide inhibition to counterbalance prosynaptic factors. Therefore, synapse formation might involve the concurrent activation of signaling pathways that promote synaptogenesis and a relief of inhibition of synapse formation by the NgR family. Consistent with these possibilities, we provide evidence that NgR1 mediates its effects through the activation of RhoA, a GTPase that restricts actin polymerization and thereby limits dendritic growth and spine development (Elia et al., 2006; Sin et al., 2002).

Signaling through RhoA to regulate actin assembly may be a common feature of NgR signaling. Previous work has shown that NgR1 regulates actin dynamics in the axon through TROY, RhoA, and ROCK (Yiu and He, 2006). In the present study, we provide evidence that a similar signaling pathway mediates the effects of NgR1 in the dendrite. While we have found that TROY can bind both NgR1 and NgR2 in heterologous cells (Figures S4E and S4F), future work will be required to demonstrate the presence of a protein complex comprised of these signaling components in developing dendrites. Further, the signals promoting synaptic and dendritic growth may not be identical. Preliminary work suggests that while TROY inhibits synapse development, it does not inhibit dendritic growth (Wills and Greenberg, unpublished data). However, the finding that NgR1 regulates both dendritic and synaptic growth suggests that NgR1 signaling may couple these processes to coordinate neuronal development.

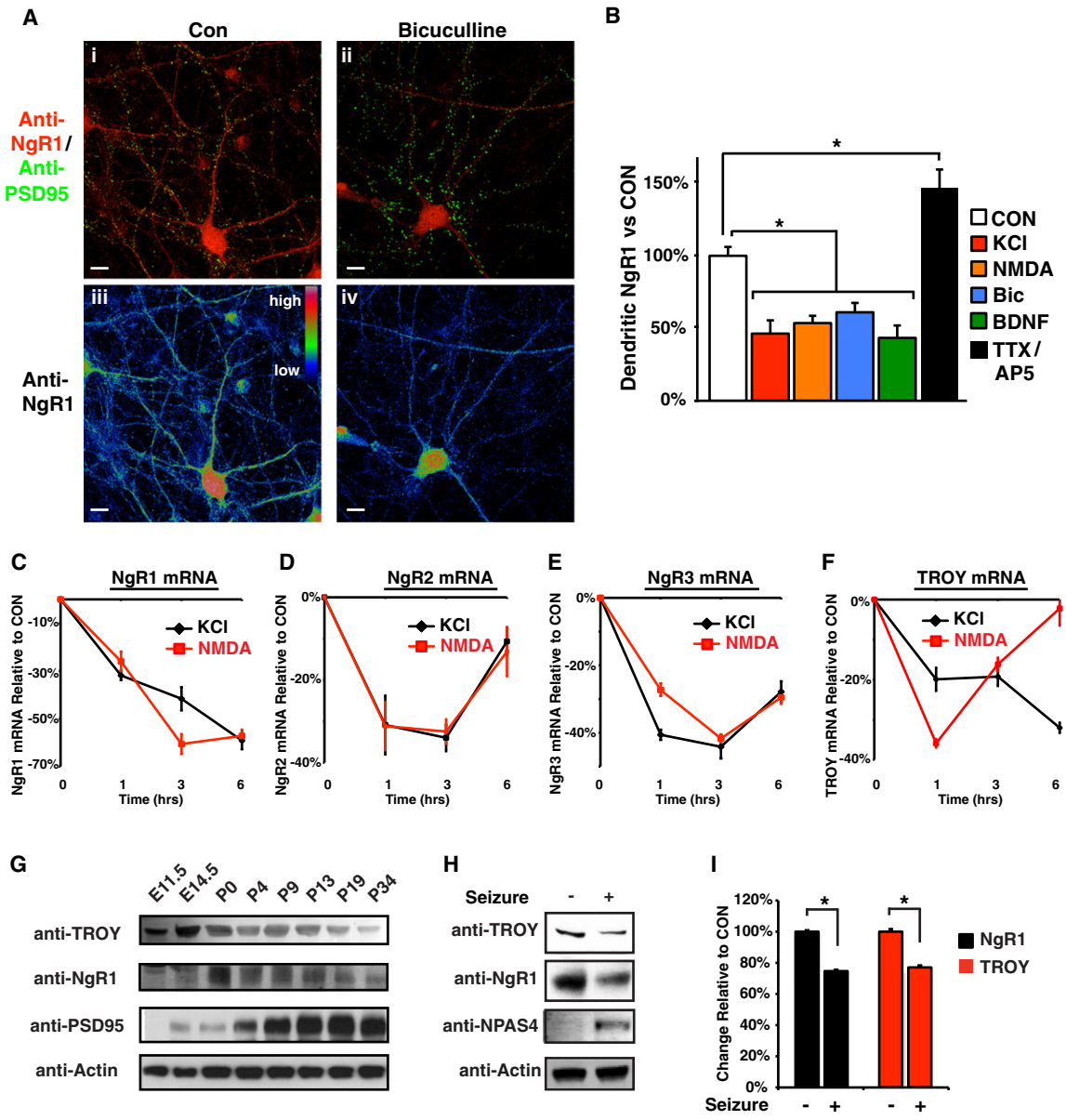

Figure 8. The NgR1 Family and Coreceptor TROY Are Downregulated by Neuronal Activity

(A and B) Expression of NgR1 protein on dendrites is downregulated by increased neuronal activity. (A) Cultured hippocampal neurons were treated with vehicle (CON) or bicuculline (5 μM, 12 hr). Subsequently, cells were fixed and immunolabeled with antibodies against NgR1 and PSD95. Representative images are shown at top (i and ii). At bottom (iii and iv), anti-NgR1 signal has been converted into a heat map (using Rainbow RGB setting in Image J software). Scale bar is 10 μm. (B) Cultured hippocampal neurons were transfected with GFP, treated with compounds for 12 hr and then immunolabeled with antibodies against NgR1. Treatments were vehicle (CON; white bar), KCl (55 mM, red bar), NMDA (30 μM, orange bar), bicuculline (5 μM, blue bar), BDNF (50 nM, green bar), or a combination of tetrodotoxin (TTX, 1 μM) and APV (100 μM, black bar). Data are expressed as percentage change in the number of NgR1 puncta expressed on the dendrites of GFP-transfected neurons relative to mock treatment (CON). * indicates $p < 0.05$, one-way ANOVA with pairwise comparison by Bonferroni post hoc test. Data are mean ± SEM from three experiments; total numbers of neurons analyzed (n) range from 25 to 50 cells per condition. Similar results were observed examining NgR1 protein on the cell surface (see Figure S8A).

(C–F) Neuronal activity causes a downregulation in the mRNA for NgR1, NgR2, NgR3 and TROY. Cultures of hippocampal neurons were either mock-treated (CON) or treated with KCl (55 mM, black bar) or NMDA (30 μM, red bar). RNA was collected at the indicated times, and quantitative RT-PCR was performed for NgR1 (C), NgR2 (D), NgR3 (E), and TROY (F). Message levels were normalized to actin and expressed as percent difference relative to control. Data represent mean ± SEM from four to five experiments. Changes in the mRNA expression of NgR1 and NgR3 were significantly different from control at all time points. NgR2 mRNA was significantly different from control at the 1 hr and 3 hr time points. TROY mRNA was significantly different from control in all but the NMDA 6 hr time point. * indicates $p < 0.01$, repeated-measures ANOVA with pairwise comparisons Bonferroni post hoc test.

(G) TROY protein levels are developmentally downregulated. Protein lysates were prepared from the hippocampi of mice from different developmental ages (E11.5 to P34) and subjected to immunoblot analysis with antibodies directed against TROY, NgR1, PSD95, or actin.

Though our studies were focused on elucidating the developmental function of the NgRs, expression of this family of proteins continues into adulthood, and so it is interesting to speculate that NgR may continue to limit dendritic growth and synapse number in the mature brain. If so, NgR1's dendritic function may be important to consider in the context of neural damage caused by, e.g., injury or stroke, where, it has been suggested, NgR1-mediated inhibition of axonal outgrowth impairs recovery of motor function (Lee et al., 2004; Harvey et al., 2009). Our findings are notable because they raise the possibility that enhanced functional connectivity observed upon blocking NgR1 may be due, at least in part, to increased dendritic growth and elevated synapse formation in postsynaptic neurons.

In addition to its role under pathological circumstances such as injury, it is now clear that Nogo (Delekate et al., 2011) and NgR1 (Lee et al., 2008) have functions in the regulation of neural plasticity. Neural activity causes the downregulation of NgR1, and expression of ectopic NgR1 in the forebrain inhibits memory consolidation (Karlén et al., 2009). These findings imply that NgR1 limits neural connectivity, and in keeping with this idea, mice lacking NgR1 have an abnormal critical period in which ocular dominance plasticity continues abnormally into adulthood (McGee et al., 2005). While these findings suggest that NgR1 constrains plasticity in the brain, it was not known how NgR1 mediates these effects. Results from our study raise the possibility that NgRs limit synaptic plasticity by restricting excitatory synapse development. We speculate that the NgR family functions to limit structural changes in circuitry, from initial circuit formation in the newborn mouse to the closure of the critical period, as well as in the formation of long-term memories and the ability to recover from neural injury. In so doing, the NgR family may ensure wiring fidelity within neural circuits.

EXPERIMENTAL PROCEDURES

Animal Husbandry and Colony Management

NgR1$^{-/-}$ mice have been previously described (Zheng et al., 2005). *NgR2*$^{-/-}$ and *NgR3*$^{-/-}$ mice were obtained from Lexicon Genetics. GFPM mice were obtained from Joshua Sanes (Feng et al., 2000). For more details concerning mouse crosses, genotyping, and knockout validation see Supplemental Information.

DNA Constructs

Details of DNA constructs can be found in the Supplemental Information.

Western Blotting and GEF Pull-Down Assays

For western blotting, hippocampal cultures were collected and homogenized in RIPA buffer. Samples were boiled for 5 min in SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. RBD pull-down assays were conducted according to the manufacturer's suggestions (Upstate Cell Signaling Solutions). For details, see Supplemental Information.

Immunocytochemistry

For immunocytochemistry, neurons were fixed and incubated with the indicated antibodies, as previously described (Tolias et al., 2005). For cell-surface staining of NgR1, anti-NgR1 antibody (1 μg/ml) was added to 14 DIV cultured neurons for 1 hr at 37°C, washed, and fixed as above. For details, see Supplemental Information.

Primary Neuron Cell Cultures

To obtain hippocampal neurons from mutant mice or littermate controls, single-embryo dissections of E16 mouse embryos were performed as previously described (Tolias et al., 2005). Rat hippocampal neurons were prepared from E18 Long-Evans rat embryos (Charles River), as previously described (Xia et al., 1996). Dissociated hippocampal neurons were transfected using the Lipofection method (Invitrogen) according to the manufacturer's suggestions. For details, see Supplemental Information.

Heterologous Cell Culture and Lentiviral Production

HEK293T cells were cultured in DMEM with10% fetal bovine serum and transfected using the calcium phosphate method (Xia et al., 1996). Lentiviruses were produced by co-transfection of HEK293T cells with pLenti-Lox plasmids together with the helper plasmids Δ8.9 and VSV-G, as previously described (Lois et al., 2002). For details, see Supplemental Information. AP binding studies were carried out in COS cells transfected with GFP alone (CON), WTNgR1, WTNgR2, or WTNgR3 expression constructs. TROY-fc (R&D Systems) was conjugated with anti-fc-AP protein (Venkatesh et al., 2005), then incubated with COS cells for 75 min, washed, fixed, and stained to identify AP activity using BCIP/NBT.

Organotypic Slice Culture

Transverse slices (350 μm) of P5-7 hippocampus were prepared and cultured essentially as described in Stoppini et al. (1991). Slices prepared under sterile conditions were cultured on nylon inserts (0.4 μm pore size, Millicell) in 6-well dishes containing 0.75 ml of antibiotic-free medium (20% horse serum/MEM) and incubated in 5% CO_2 at 37°C. Slice cultures were transfected using a Helios Gene Gun (Biorad) at 8 DIV. Slices were fixed at 13 DIV in 2.5% paraformaldehyde and 4% sucrose and processed for immunohistochemistry.

Confocal Image Analysis and Quantification

All imaging analysis experiments were carried using a Zeiss LSM5 Pascal confocal microscope. For details see Supplemental Information. For live imaging experiments, organotypic rat hippocampal slice cultures were prepared at P5, biolistically transfected with shCON or shNgR1 RNAi constructs at 4 DIV, and cultured for three days (7 DIV) before imaging commenced. Spine-density measurements were carried out in Metamorph. For details, see Supplemental Information.

Electron Microscopy and Analysis

EM analysis was carried out on P18 animals, as described in detail in the Supplemental Information.

Electrophysiology

Electrophysiology was performed using standard methods (see Supplemental Information).

Immunohistochemistry

For immunohistochemistry, P18 mice were fixed with 4% paraformaldehyde in PBS by intracardial perfusion. Brains were sectioned coronally with a vibratome at 100 μm. Immunohistochemistry was performed on slice cultures

(H) TROY and NgR1 protein levels are downregulated by kainate-induced seizure. Protein lysates were prepared from the hippocampi of two control and two kainite-seized mice. Lysates were subjected to immnoblot analysis with antibodies directed against TROY, NgR1, NPAS4, and actin.
(I) Quantification of TROY and NgR1 protein levels following kainate-induced seizure. Integrated intensities of TROY and NgR1 were measured by infrared fluorescence detection. Data represent mean ± SEM from two independent experiments and are expressed as change relative to control. * indicates a $p < 0.05$ with a Student's t test. Similar results were observed when examining NgR1 and TROY protein levels following mouse exposure to an enriched environment (see Figures S8C and S8D).

directly on the nylon culture membrane. See Supplemental Information for details.

RT-PCR

RT-PCR was carried out using standard methodologies. See Supplemental Information for details.

Seizures and Enriched Environment

Seizures were induced for 3 hr in adult C57B6 mice by intraperitoneal injection of kainic acid (Ocean Produce International) at a dose of 25 mg/kg before isolation of the hippocampus. For enriched environment experiments, 6-week-old CD1 male mice were either placed in standard laboratory cages or in cages containing a variety of rodent toys of various shapes and colors (PETCO) for zero to six hours prior to isolation of the hippocampus. Hippocampal tissue was lysed in RIPA lysis buffer and total protein was quantified by BCA assay (Pierce).

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.11.029.

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