

Presynaptic Type III Neuregulin1-ErbB signaling targets $\alpha 7$ nicotinic acetylcholine receptors to axons

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Type III Neuregulin1 (Nrg1) isoforms are membrane-tethered proteins capable of participating in bidirectional juxtacrine signaling. Neuronal nicotinic acetylcholine receptors (nAChRs), which can modulate the release of a rich array of neurotransmitters, are differentially targeted to presynaptic sites. We demonstrate that Type III Nrg1 back signaling regulates the surface expression of $\alpha 7$ nAChRs along axons of sensory neurons. Stimulation of Type III Nrg1 back signaling induces an increase in axonal surface $\alpha 7$ nAChRs, which results from a re-

distribution of preexisting intracellular pools of $\alpha 7$ rather than from increased protein synthesis. We also demonstrate that Type III Nrg1 back signaling activates a phosphatidylinositol 3-kinase signaling pathway and that activation of this pathway is required for the insertion of preexisting $\alpha 7$ nAChRs into the axonal plasma membrane. These findings, in conjunction with prior results establishing that Type III Nrg1 back signaling controls gene transcription, demonstrate that Type III Nrg1 back signaling can regulate both short-and long-term changes in neuronal function.

Introduction

The Neuregulin1 (Nrg1) gene encodes an extremely important and diverse family of proteins that signal by binding to the ErbB family of receptor tyrosine kinases. Nrg1-ErbB signaling regulates neural development, glial growth, myelination, and the maintenance of synaptic connections in both the peripheral and central nervous system (Michailov et al., 2004; Taveggia et al., 2005; Chen et al., 2006; Lopez-Bendito et al., 2006). Nrg1 activation of ErbB signaling regulates the levels of several ion channels, including subtypes of neuronal nicotinic acetylcholine receptors (nAChRs; Yang et al., 1998; Liu et al., 2001; Kawai et al., 2002; Chang and Fischbach, 2006), *N*-methyl-d-aspartic acid receptors (Ozaki et al., 1997; Gu et al., 2005), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Kwon et al., 2005; Li et al., 2007), and γ -amino butyric acid_A

receptors (Rieff et al., 1999; Okada and Corfas, 2004). Nrg1 has been implicated as a schizophrenia susceptibility gene, and the potential relationship between Nrg1 and circuits affected in schizophrenia underscores the importance of determining the molecular mechanisms of action of Nrg1 signaling in the developing and adult nervous systems (Stefansson et al., 2002).

The Nrg1 gene encodes over a dozen different protein products. Multiple promoters and alternative splicing leads to the synthesis of three (and perhaps as many as six) classes of proteins differing in their amino-terminal domains, as well as in the EGF-like domain (α or β), and/or in the length of the C termini (Falls, 2003). These differences in primary sequence translate into subtle and dramatic differences in signaling strategies. Although Types I and II Nrg1 are either directly secreted or released after constitutive cleavage, Type III Nrg1 remains membrane tethered as a result of its unique N-terminal transmembrane domain (Wang et al., 2001; Taveggia et al., 2005). Thus, Types I and II Nrg1 can participate in paracrine signaling, whereas Type III Nrg1 predominantly signals in a juxtacrine manner. Additionally, it has been shown that Type III Nrg1 also functions as a receptor, in that Type III Nrg1-ErbB interaction results in signaling within the Type III Nrg1-expressing cell (Bao et al., 2003, 2004).

The integrity of a neuron-neuron synapse is determined by several molecular interactions between pre- and postsynaptic cells. Throughout the central and peripheral nervous system,

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Abbreviations used in this paper: AFI, average fluorescence intensity; Akt inh., Akt inhibitor; a.u., arbitrary units; CHX, cycloheximide; DRG, dorsal root ganglia; E, embryonic day; ECD, extracellular domain; nAChR, nicotinic acetylcholine receptor; NF, neurofilament; Nrg1, Neuregulin1; PAO, phenylarsine oxide; PIP₃, phosphatidylinositol 3,4,5 trisphosphate; PtdIns 3K, phosphatidylinositol 3-kinase; vGlut1, vesicular glutamate transporter 1; WM, wortmannin; WT, wild type.

The online version of this paper contains supplemental material.

nAChRs function as cation-selective ligand-gated ion channels and play important roles in synaptic signaling (Berg and Conroy, 2002). There is increasing evidence for the importance of nAChRs in the modulation of transmitter release from pre-synaptic terminals (McGehee et al., 1995; MacDermott et al., 1999), neuronal survival (Dajas-Bailador et al., 2000; Mechawar et al., 2004), and postsynaptic signaling (Zhang et al., 1996; Zhang and Berg, 2007). Individual nAChR channels have unique biological functions that are determined by the pentameric combination of 11 distinct subunits: $\alpha 2-\alpha 7$, $\alpha 9$, $\alpha 10$, and $\beta 2-\beta 4$ (Rosenberg et al., 2002). Of particular interest are the $\alpha 7^*$ nAChRs ($\alpha 7$ -containing nAChRs) because of their high permeability to calcium and abundant expression in both mammalian and avian peripheral nervous systems (Roth and Berg, 2003; Fucile et al., 2005).

The Type III isoform of Nrg1 was identified as the nerve-derived factor responsible for increasing acetylcholine-evoked currents in neurons and was shown to regulate expression of neuronal nAChR subunit mRNAs, including that of $\alpha 7$ (Yang et al., 1998). More recent findings in our laboratory reveal a requirement for presynaptic Type III Nrg1 in potentiation of glutamatergic transmission by nicotine in a central sensory motor-gating circuit (Du, C., C. Zhong, M. Hancock, D.A. Talmage, and L.W. Role. 2004. Society for Neuroscience 34th Annual Meeting; unpublished data). These results inspired us to investigate the molecular mechanisms underlying presynaptic Type III Nrg1's cell-autonomous effect on functional $\alpha 7^*$ nAChRs along axonal projections.

In this paper, we show that presynaptic Type III Nrg1 is required for normal levels of presynaptically targeted $\alpha 7^*$ nAChRs in primary sensory neurons. Sensory neurons from Type III Nrg1 mutant mice exhibit deficits of the surface expression of $\alpha 7^*$ nAChRs. Acute stimulation of Type III Nrg1 back signaling increases the number of $\alpha 7^*$ nAChRs on sensory axons from wild-type (WT), but not from homozygous Type III Nrg1, mutant embryos. These increases in axonal surface $\alpha 7^*$ nAChRs result from redistribution of preexisting intracellular pools of $\alpha 7$ rather than from increased $\alpha 7$ protein synthesis. Increases in surface pools of $\alpha 7^*$ nAChR are accomplished, at least in part, by local activation of a phosphatidylinositol 3-kinase (PtdIns 3K) signaling pathway, which in turn stimulates the membrane insertion of intracellular $\alpha 7^*$ nAChRs.

Results

Reduction of Type III Nrg1 expression results in decreased axonal $\alpha 7$ nAChR surface expression

Subsets of primary sensory neurons within the dorsal root ganglia (DRG) express Type III Nrg1 and $\alpha 7$ nAChR subunit mRNAs (Boyd et al., 1991; Wolpowitz et al., 2000; Genzen et al., 2001; Taveggia et al., 2005). In the following experiments, we examined the expression of $\alpha 7^*$ nAChRs and Type III Nrg1 on the surface of axons from sensory neurons of WT or Type III Nrg1 $^{−/−}$ mouse embryos. Embryonic neurons were used for these experiments because mutant mice lacking Type III Nrg1 die at birth (Wolpowitz et al., 2000).

To label surface $\alpha 7^*$ nAChRs, primary sensory neurons were labeled with α BgTx-488 (Alexa 488-conjugated α -bungarotoxin) before fixation and permeabilization (Fig. 1 A). α -BgTx is an effective reagent for probing $\alpha 7^*$ nAChRs because of its high affinity for $\alpha 7^*$ nAChRs (Ravdin and Berg 1979). After fixation and permeabilization, we applied antibodies recognizing the cysteine-rich domain of Type III Nrg1 (red) or neurofilament (NF) proteins (blue; Fig. 1 A; Yang et al., 1998). In neurons from WT mice, axons were studded with distinct puncta of α BgTx-488 and Type III Nrg1 along the same sensory axons. Pretreatment with 5 μ M methyllycaconitine or 1 μ M of nicotine before addition of α BgTx-488 blocked surface labeling of $\alpha 7^*$ nAChRs, confirming the specificity of surface α BgTx-488 labeling (Fig. 1 D). We repeated this analysis using sensory neurons isolated from Type III Nrg1 $^{−/−}$ embryos. Labeling with α BgTx-488 identified discrete clusters of $\alpha 7^*$ nAChRs on the axonal surface of mutant neurons (Fig. 1 A). However, when we quantified the number of these α BgTx-488 clusters we found that the mutants had $\sim 50\%$ fewer clusters than WT neurons (WT, 6.47 ± 0.69 clusters/100 μ m; Type III Nrg1 $^{−/−}$, 3.22 ± 0.59 clusters/100 μ m; Fig. 1 B). Additionally, we found that the mutants had a 35% reduction in mean cluster area relative to WT neurons (WT, $0.26 \pm 0.02 \mu\text{m}^2$; Type III Nrg1 $^{−/−}$, $0.17 \pm 0.02 \mu\text{m}^2$; Fig. 1 C).

Although there is a difference in $\alpha 7^*$ nAChR clusters expressed along axons of Type III Nrg1 $^{−/−}$ sensory neurons, we did not detect an overall reduction of total $\alpha 7$ protein in mutant neurons (Fig. 1 E). This result, coupled with our findings in Fig. 1 (A–C), indicates that Type III Nrg1 specifically affects the surface expression of $\alpha 7^*$ nAChRs. The decrease in surface $\alpha 7^*$ nAChRs along axons of Type III Nrg1 $^{−/−}$ neurons did not result from general deficits in endocytic recycling because these neurons retained WT rates of transferrin receptor recycling (Fig. S1, A and B, available at <http://www.jcb.org/cgi/content/full/jcb.200710037/DC1>). We next looked to see whether Type III Nrg1 $^{−/−}$ neurons have defects in axonal targeting of presynaptic proteins. We did not detect differences in the axonal targeting of synaptophysin or vesicular glutamate transporter 1 (vGlut1) along mutant axons (Fig. S1, C and D).

To demonstrate that the α BgTx-488 surface binding measurements reflected the presence of functional $\alpha 7^*$ nAChRs, we quantified internal concentrations of Ca^{2+} along sensory axons after a 1-min application of 1 μ M of nicotine. In response to nicotine, we detected an increase in internal concentrations of Ca^{2+} within 1 min along axons of WT neurons (Fig. 1, F and G). When this experiment was repeated in the presence of 1 μ M α BgTx, we did not detect an increase in intracellular concentrations of Ca^{2+} , indicating that the nicotine-induced changes were mediated by functional $\alpha 7^*$ nAChRs. Nicotine application did not increase internal Ca^{2+} concentrations in Type III Nrg1 $^{−/−}$ axons (Fig. 1 G), a result which is consistent with a significant decrease in surface $\alpha 7^*$ nAChRs in these neurons.

Type III Nrg1 back signaling increases surface expression of $\alpha 7^*$ nAChR clusters

Type III Nrg1 functions as a membrane-tethered bidirectional signaling molecule (Bao et al., 2003, 2004). To test the possibility that Type III Nrg1, acting as a receptor, contributes to the

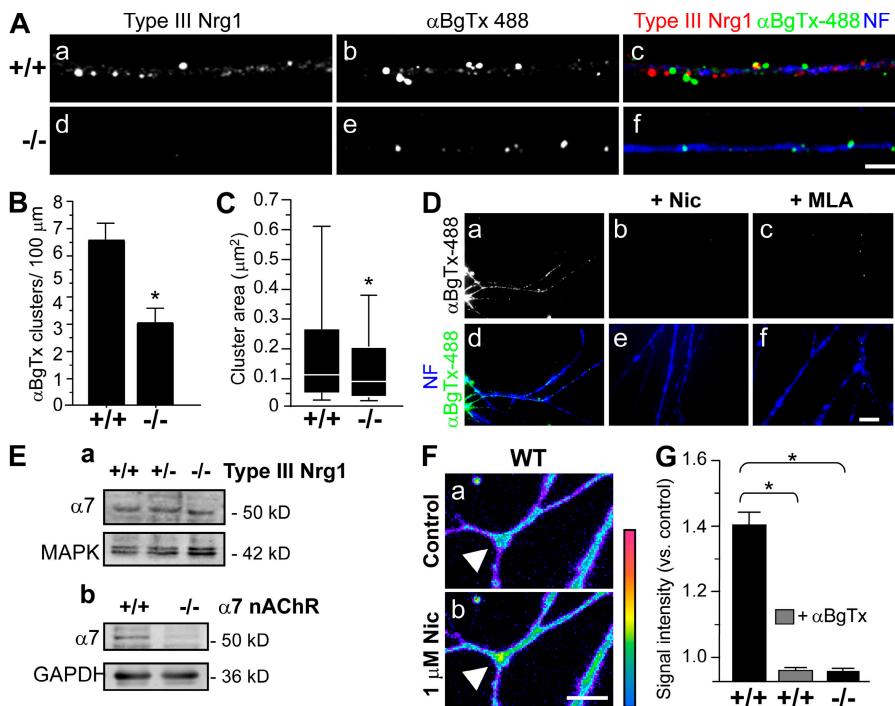


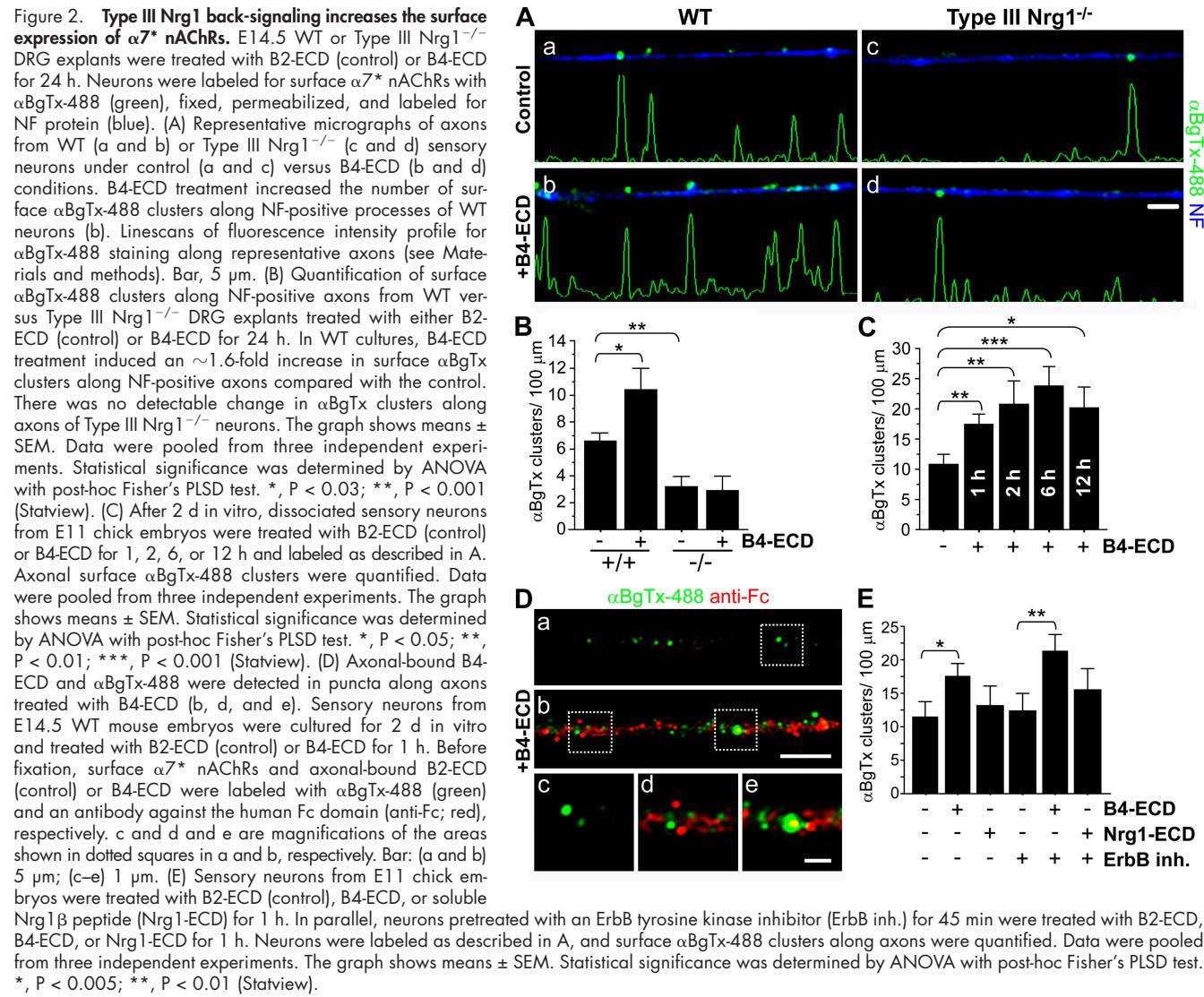
Figure 1. Decreased surface expression of $\alpha 7^*$ nAChRs along axons of Type III Nrg1 $^{-/-}$ sensory neurons. Sensory ganglia were extirpated from embryonic day (E) 14.5 WT or Type III Nrg1 $^{-/-}$ embryos and plated as explants. After 2 d in vitro, neurons were labeled for surface $\alpha 7^*$ nAChRs with α BgTx-488 (green). Neurons were fixed, permeabilized, and stained for Type III Nrg1 (red) and NF protein (blue). (A) Representative micrographs of α BgTx-488 clusters along NF-positive axons of WT and Type III Nrg1 $^{-/-}$ sensory neurons at 2 d in vitro. Type III Nrg1 staining was detected along WT (a) but not Type III Nrg1 $^{-/-}$ (d) axons. Fewer surface α BgTx-488 clusters were detected along mutant axons (e) as compared with WT (b). Confocal images were acquired from a 100 \times oil objective. Bar, 5 μm . (B) Quantification of surface α BgTx-488 clusters per 100 μm of axonal length revealed an \sim 50% reduction of clusters along mutant axons. The graph shows means \pm SEM. Data were pooled from three independent experiments. Statistical significance determined by ANOVA. *, P < 0.001 (Statview). (C) Quantification of the surface α BgTx-488 cluster area. Loss of Type III Nrg1 expression resulted in an \sim 35% reduction of α BgTx-488 cluster area. Data pooled from three independent experiments were analyzed using nonparametric statistics and presented as box plots (see Materials and methods). Statistical significance was determined by the Kolmogorov-Smirnov Test. *, P < 0.0001 (Statview). (D) Pretreatment of sensory neurons with 1 μM of nicotine (Nic; b and e) or 5 μM methyllycaconitine (MLA; c and f) prevents surface α BgTx-488 labeling along axons. Bar, 10 μm . (E) Immunoblot analysis of total $\alpha 7$ subunit protein levels in sensory neurons from E14.5 WT, Type III Nrg1 $^{+/-}$, and Type III Nrg1 $^{-/-}$ embryos cultured for 2 d in vitro (a). MAPK1/2 probing in the bottom panel shows equal lysate loading. Immunoblots of total $\alpha 7$ subunit protein levels in brain extracts from WT or $\alpha 7$ nAChR $^{-/-}$ embryos are also shown (b). Glyceraldehyde 3-phosphate dehydrogenase probing in the bottom panel shows equal lysate loading (b). (F) In WT sensory neurons, nicotine application (1 μM for 1 min) resulted in an increased internal concentration of Ca^{2+} (indicated in pseudo color; described in Materials and methods). The white arrowhead highlights an axonal region affected by nicotine. Bar, 10 μm . (G) Changes in internal concentration of Ca^{2+} in response to application of nicotine in WT and mutant axons are plotted. Note that 100 nM α BgTx completely eliminated the response to nicotine in WT axons and that sensory axons from Type III Nrg1 $^{-/-}$ animals did not respond to nicotine. Data are from two independent experiments. The graph shows means \pm SEM. Statistical significance was determined by ANOVA with post-hoc Fisher's PLSD test. *, P < 0.0001 (Statview).

regulation of $\alpha 7^*$ nAChR levels on sensory axons, we treated WT or Type III Nrg1 $^{-/-}$ sensory neurons with 2 nM of either the extracellular domain (ECD) of ErbB2 (B2-ECD; control) or ErbB4 (B4-ECD) for 24 h (Fig. 2, A and B). B4-ECD, but not B2-ECD, binds with high affinity to the EGF-like domain of Nrg1 (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200710037/DC1>; Fitzpatrick et al., 1998; Bao et al., 2003). We visualized $\alpha 7^*$ nAChRs present on the surface of sensory axons by labeling with α BgTx-488 (green) before fixation (Fig. 2 A). Treating WT sensory neurons with B4-ECD for 24 h led to an increase in the number of α BgTx-488 clusters on the axonal surface (from 6.47 ± 0.69 clusters/100 μm in control cultures to 10.38 ± 1.59 clusters/100 μm ; Fig. 2 B). When we repeated the experiment using sensory neurons from Type III Nrg1 $^{-/-}$ embryos, there was no significant change in the number of α BgTx-488 clusters on mutant axons (from 3.07 ± 0.72 clusters/100 μm in control cultures to 2.74 ± 1.14 clusters/100 μm in B4-ECD treated). This effect did not result from general changes in the levels of surface proteins, as we did not detect a change in the axonal surface levels of TrkA in response to a 24-h B4-ECD treatment (Fig. S1, E and F).

We next asked how soon after B4-ECD treatment the increase in surface $\alpha 7^*$ nAChRs was seen. In sensory neurons treated with B4-ECD, we detected an increase in the surface expression of $\alpha 7^*$ nAChR clusters along axons within 1 h,

with the maximal response occurring after 6 h (Fig. 2 C) and sustained for 24 h (Figs. 1 B, 2 B, and 3 A). Many of the surface $\alpha 7^*$ nAChR clusters induced by B4-ECD treatment were localized in close proximity to membrane-bound B4-ECD (Fig. 2 D).

Prior studies have shown that activation of ErbB tyrosine kinase signaling can alter the surface expression of $\alpha 7^*$ nAChRs in neurons (Liu et al., 2001; Kawai et al., 2002; Chang and Fischbach, 2006). To verify that the increase in surface clusters we detected in response to B4-ECD treatment was mediated by Type III Nrg1 acting as a receptor, we assessed the response of sensory neurons treated with B4-ECD in the presence of 2 μM ErbB inh. (inhibitors of ErbB tyrosine kinase activity; PD 168393 and PD 158780) for 1 h (Fig. 2 E). We also assessed the effect on $\alpha 7^*$ nAChRs of acute activation of ErbB signaling by treating neurons with recombinant Nrg1 β peptide (Nrg1-ECD, 10 ng/ml). In response to B4-ECD treatment, we detected an increase in surface $\alpha 7^*$ nAChR clusters along axons in the presence of ErbB inh. (ErbB inh., 12.31 ± 2.43 clusters/100- μm axonal length; and ErbB inh. + B4-ECD, 20.94 ± 2.37 clusters/100- μm axonal length; Fig. 2 E). Conversely, we did not detect an increase in axonal surface $\alpha 7^*$ nAChR clusters in response to Nrg1-ECD treatment (B2-ECD [control], 11.30 ± 1.74 clusters/100- μm axonal length; and Nrg1-ECD, 13.20 ± 2.72 clusters/100- μm axonal length; Fig. 2 E).



Collectively, these results indicate that in sensory neurons, Type III Nrg1, acting as a receptor for B4-ECD, regulates the surface expression of $\alpha 7^*$ nAChR, whereas activation of ErbB tyrosine kinases does not.

Stimulation of Type III Nrg1 back signaling increases surface expression of $\alpha 7^*$ nAChRs in the absence of new protein synthesis

Stimulation of Type III Nrg1 back signaling elicits a transcriptional response mediated by the Nrg1 intracellular domain (Bao et al., 2003, 2004). To determine if Type III Nrg1-dependent regulation of axonal $\alpha 7^*$ nAChR levels resulted from Nrg1 intracellular domain-induced synthesis of $\alpha 7$ and/or changes in $\alpha 7^*$ nAChR surface expression along axons, we compared measures of total (surface and internal) neuronal 125 I- α BgTx binding (Fig. 3 A) and total immunoreactive $\alpha 7$ nAChR protein (Fig. 3 B) in sensory neurons treated with B2-ECD (control) or B4-ECD for 24 h. We also asked whether B4-ECD treatment increased surface $\alpha 7^*$ nAChR levels in the presence of a protein synthesis inhibitor (Fig. 3, C–E).

Surface $\alpha 7^*$ nAChRs were quantified by 125 I- α BgTx labeling in live in vitro preparations of chick sensory neurons and, in parallel, the total pool of $\alpha 7^*$ nAChRs was quantified by 125 I- α BgTx binding after permeabilization with 0.5% saponin (Fig. 3 A). Under basal conditions, \sim 12% of the total $\alpha 7^*$ nAChRs were on the surface, which is similar to prior results emphasizing the large internal pools of nAChRs in neurons (Cho et al., 2005). After a 24-h treatment with B4-ECD, we observed an \sim 2.7-fold increase in surface 125 I- α BgTx binding. In B4-ECD-stimulated neurons, \sim 30% of the $\alpha 7^*$ nAChRs were at the surface, but the total pool of 125 I- α BgTx-labeled sites was not changed. These results are consistent with Type III Nrg1 back signaling inducing a relocalization of $\alpha 7^*$ nAChRs from preexisting internal pools to clusters along the axonal surface. Results on increased surface versus total $\alpha 7^*$ nAChRs were confirmed by immunoblot assay (Fig. 3 B). These results indicate that in sensory neurons, Type III Nrg1, acting as a receptor, can increase axonal surface $\alpha 7^*$ nAChRs without significantly increasing the size of the total $\alpha 7^*$ nAChR pool.

To extend our examination of the idea that Type III Nrg1 back signaling stimulates insertion of preexisting $\alpha 7^*$ nAChRs

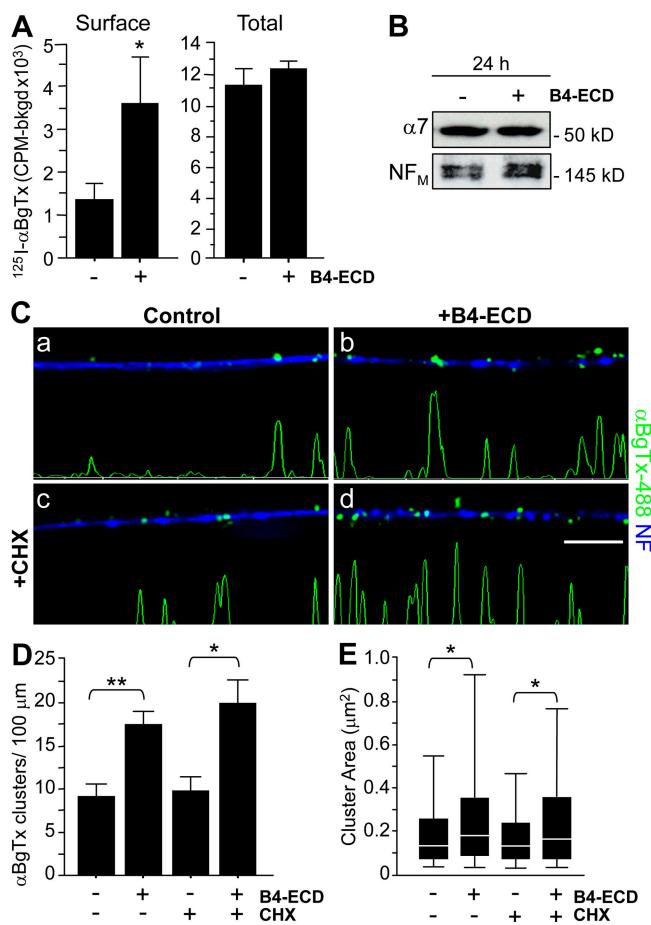


Figure 3. Type III Nrg1 back signaling increases the surface expression of $\alpha 7^*$ nAChRs in the absence of protein synthesis. Dissociated sensory neurons from E11 chick embryos were cultured for 2 d in vitro and treated with either B2-ECD (control) or B4-ECD for 1 or 24 h. (A) Quantification of surface or total pools of $\alpha 7^*$ nAChR by ^{125}I - αBgTx radiolabeling in sensory neurons treated with either B2-ECD (control) or B4-ECD for 24 h. In response to a 24-h B4-ECD treatment, we detected an ~ 2.7 -fold increase in surface ^{125}I - αBgTx binding compared with control conditions (B2-ECD [control], $1,339.15 \pm 329.77$ cpm; and B4-ECD, $3,562.81 \pm 1,111.19$ cpm). B4-ECD treatment did not induce a change in total ^{125}I - αBgTx binding as compared with the control (B2-ECD [control], $11,159.74 \pm 1,059.79$ cpm; and B4-ECD, $12,258.85 \pm 580.11$ cpm). The graph shows means \pm SEM. Data were pooled from three independent experiments with greater than or equal to three wells per condition per experiment. Statistical significance was determined by ANOVA. *, $P < 0.05$ (Statview). (B) Immunoblot analysis of total $\alpha 7$ subunit protein in sensory neurons treated with B2-ECD (control) or B4-ECD treatment for 24 h. In response to B4-ECD treatment, we did not detect a difference in total $\alpha 7$ subunit protein. NF_M probing in bottom panel shows equivalent lysate loading. (C) Sensory neurons were treated with B2-ECD (control) or B4-ECD for 1 h. In parallel, neurons pretreated with CHX for 45 min were treated with B2-ECD or B4-ECD for 1 h. Neurons were labeled with $\alpha\text{BgTx-488}$ (green), fixed, permeabilized, and colabeled for NF protein (blue). CHX treatment (c and d) did not affect either the basal number of $\alpha\text{BgTx-488}$ clusters on control neurons (c) or the response to B4-ECD (d). Linescans of fluorescence intensity profiles of $\alpha\text{BgTx-488}$ along representative axons (see Materials and methods) are shown. Bar, 5 μm . (D) Quantification of surface $\alpha\text{BgTx-488}$ clusters along NF-labeled axons. B4-ECD treatment induced an ~ 1.9 -fold increase in surface $\alpha\text{BgTx-488}$ clusters along axons, and B4-ECD treatment in the presence of CHX induced an ~ 2.1 -fold increase. Data were pooled from three independent experiments. The graph shows means \pm SEM. Statistical significance was determined by ANOVA with post-hoc Fisher's PLSD test. *, $P = 0.01$; **, $P < 0.0001$ (Statview). (E) Quantification of surface $\alpha\text{BgTx-488}$ cluster area. B4-ECD treatment in the presence or absence of CHX induced an increase in $\alpha\text{BgTx-488}$ cluster area. Data pooled from three independent

into the axonal membrane, we repeated the B4-ECD stimulation of dispersed sensory neurons in the presence of the protein synthesis inhibitor cycloheximide (CHX; Fig. 3, C–E). Neurons were pretreated with 10 $\mu\text{g}/\text{ml}$ CHX for 45 min and then stimulated for an additional hour with either B2-ECD (control) or B4-ECD. Neither the B4-ECD-induced increase in the number of surface $\alpha\text{BgTx-488}$ clusters (Fig. 3 D) nor the increase in cluster area (Fig. 3 E) were affected by the CHX pretreatment (CHX, 9.72 ± 1.65 clusters/100- μm axonal length; CHX + B4-ECD, 19.96 ± 2.83 clusters/100- μm axonal length; CHX, $0.23 \pm 0.02 \mu\text{m}^2$; and CHX + B4-ECD, $0.32 \pm 0.01 \mu\text{m}^2$). Thus, the increase in surface $\alpha 7^*$ nAChRs resulted from a redistribution of preexisting receptors rather than from new receptor synthesis.

Type III Nrg1 back signaling increases surface expression of $\alpha 7^*$ nAChRs in the absence of endocytosis

Prior studies have shown that endocytosis can alter the surface expression of neurotransmitter receptors (Man et al., 2007; Xia et al., 2007). Using a pharmacological inhibitor of endocytosis, phenylarsine oxide (PAO; Hertel et al., 1985), we investigated whether B4-ECD-mediated increase in the surface expression of $\alpha 7^*$ nAChR clusters was caused by alterations in receptor endocytosis. We applied PAO to sensory neurons for 45 min before B2-ECD (control) or B4-ECD treatment. Although PAO did not significantly alter the number of surface $\alpha 7^*$ nAChR clusters along axons under control conditions, it significantly increased the area of the clusters (from $0.2 \pm 0.01 \mu\text{m}^2$ under control conditions to $0.29 \pm 0.03 \mu\text{m}^2$ after PAO treatment; Fig. 4 C). After B4-ECD treatment for 1 h, we detected an increase in both the number and area of surface $\alpha 7^*$ nAChR clusters along axons when endocytosis was blocked (Fig. 4 B, PAO, 16.03 ± 2.69 clusters/100- μm axonal length; and PAO + B4-ECD, 29.77 ± 3.41 clusters/100- μm axonal length; Fig. 4 C, PAO, $0.29 \pm 0.03 \mu\text{m}^2$; and PAO + B4-ECD, $0.34 \pm 0.04 \mu\text{m}^2$). Based on our findings, we conclude that the increase in surface $\alpha 7^*$ nAChRs results from an increase in membrane insertion rather than a decrease in endocytosis.

Type III Nrg1 back signaling activates a PtdIns 3K signaling pathway

Our main objective in the following experiments was to test for signaling pathways activated downstream of Type III Nrg1 that might direct membrane targeting of $\alpha 7^*$ nAChRs. Because of the substantial evidence that PtdIns 3K signaling is a major regulator of membrane protein trafficking (Tengholm and Meyer, 2002; Man et al., 2003; Viard et al., 2004; Chae et al., 2005), we asked whether stimulation of Type III Nrg1 back signaling activated PtdIns 3K signaling. Sensory neurons were stimulated with B2-ECD (control) or B4-ECD for 5 min and then labeled with an antibody recognizing the PtdIns 3K product, phosphatidylinositol

experiments were analyzed using nonparametric statistics and presented as box plots (see Materials and methods). Statistical significance was determined by the Kolmogorov-Smirnov Test. *, $P \leq 0.0001$ (Statview).

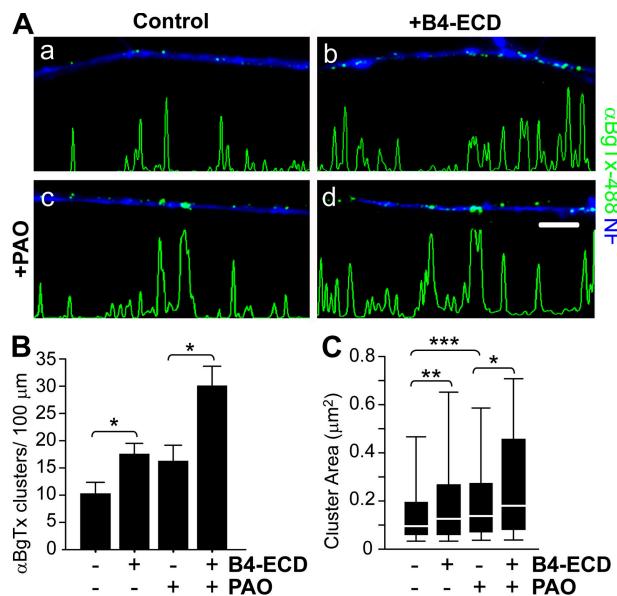


Figure 4. Type III Nrg1 back signaling increases $\alpha 7^*$ nAChRs cluster area in the absence of endocytosis. Dissociated sensory neurons from E11 chick embryos were treated with B2-ECD (control) or B4-ECD for 1 h. In parallel, neurons were treated with PAO for 45 min and treated with B2-ECD or B4-ECD for an additional hour. Neurons were labeled with α BgTx-488 (green), fixed, permeabilized, and colabeled for NF protein (blue). (A) Representative micrographs of α BgTx-488 staining along NF-positive axons. B4-ECD treatment increased surface α BgTx-488 clusters in the presence of PAO (d). Linescans of fluorescence intensity profiles of α BgTx-488 along representative axons (see Materials and methods) are shown. Bar, 5 μ m. (B) Quantification of surface α BgTx-488 clusters along NF-labeled axons. B4-ECD treatment in the presence and absence of PAO induced \sim 1.7 and 1.9 increases in surface α BgTx-488 clusters along axons, respectively. Data were pooled from two independent experiments. The graph shows means \pm SEM. Statistical significance was determined by ANOVA with post-hoc Fisher's PLSD test. *, P < 0.03 (Statview). (C) Quantification of surface α BgTx-488 cluster area. B4-ECD treatment in the presence or absence of PAO induced an increase in α BgTx-488 cluster area. Data pooled from two independent experiments were analyzed using nonparametric statistics and presented as box plots (see Materials and methods). Statistical significance was determined by the Kolmogorov-Smirnov Test. *, P = 0.03; **, P = 0.0001; ***, P < 0.0001 (Statview).

3,4,5 trisphosphate (PIP₃; Fig. 5 A). In neurons treated with B4-ECD, we detected an increase in PIP₃ staining along tau-positive axons. This response was comparable to that seen after acute treatment with 100 ng/ml NGF and was completely blocked in cultures pretreated with 500 nM of the PtdIns 3K inhibitor wortmannin (WM).

As an additional test of whether Type III Nrg1 back signaling activates a PtdIns 3K signaling pathway, we measured the phosphorylation status of the PtdIns 3K effector kinase Akt in WT and Type III Nrg1^{−/−} sensory neurons (Fig. 5, B–D; and Fig. S3, A–D, available at <http://www.jcb.org/cgi/content/full/jcb.200710037/DC1>). After a 10-min B4-ECD treatment, we detected phospho-Akt in puncta along B4-ECD-bound axons of WT neurons; however, we did not detect a change in phospho-Akt in axons from mutant explants (Fig. 5, C and D). The response to B4-ECD treatment was selective for the neurons and for the PtdIns 3K–Akt pathway. There was no activation of Akt in nonneuronal cells (Fig. S3 A) and there was no activation of MAPK in neurons (Fig. S3 E). Additionally, B4-ECD treatment was able to induce activation of Akt when ErbB kinase activity

(Fig. S3 C) and NGF/TrkA signaling (Fig. S3 D) were blocked. Thus, we conclude that Type III Nrg1 back signaling rapidly and locally activates PtdIns 3K signaling.

PtdIns 3K signaling activated by Type III Nrg1 back signaling is required for increased $\alpha 7^*$ nAChR surface expression

We tested whether PtdIns 3K activation is required for Type III Nrg1-mediated changes in $\alpha 7^*$ nAChR surface expression (Fig. 6). We treated dispersed sensory neurons with either B2-ECD (control) or B4-ECD for 1 h in the presence of 500 nM of the PtdIns 3K inhibitor WM. Surface $\alpha 7^*$ nAChR clusters were labeled with α BgTx-488 (green), after which neurons were fixed and immunolabeled with an antibody against NF protein (blue; Fig. 6 A). In response to the B4-ECD treatment, we detected a significant increase in the number (Fig. 6 B) and area (Fig. 6 C) of α BgTx-488 clusters along NF-positive processes. WM treatment alone did not affect the number of α BgTx-488 clusters but did inhibit the response to B4-ECD, preventing the increase in the number of axonal surface $\alpha 7^*$ nAChRs (Fig. 6 B, B2-ECD [control], 9.08 \pm 1.53 clusters/100- μ m axonal length; B4-ECD, 17.41 \pm 1.48 clusters/100- μ m axonal length; WM, 9.58 \pm 2.17 clusters/100- μ m axonal length; and WM + B4-ECD, 10.75 \pm 1.27 clusters/100- μ m axonal length). Pretreatment with 5 μ M of a small molecule inhibitor of Akt kinase activity (Akt inhibitor [Akt inh.]) also prevented the response to B4-ECD (Fig. 6 B, Akt inh., 10.33 \pm 1.42 clusters/100- μ m axonal length; and Akt inh. + B4-ECD, 8.48 \pm 1.58 clusters/100- μ m axonal length). Both WM and Akt inh. prevented the increase in surface α BgTx cluster area in response to B4-ECD treatment (Fig. 6 C, B2-ECD [control], 0.25 \pm 0.02 μ m²; B4-ECD, 0.35 \pm 0.02 μ m²; WM, 0.22 \pm 0.02 μ m²; WM + B4-ECD, 0.23 \pm 0.02 μ m²; Akt inh., 0.22 \pm 0.02 μ m²; and Akt inh. + B4-ECD, 0.25 \pm 0.02 μ m²). We conclude that Type III Nrg1 back signaling activates PtdIns 3K signaling and that PtdIns 3K/Akt activities are required for Type III Nrg1-induced targeting of $\alpha 7^*$ nAChRs to the axonal surface.

Discussion

Nrg1-ErbB signaling plays an important role in synaptic plasticity, in part by regulating the levels of pre- and postsynaptic receptors and ion channels (Ozaki et al., 1997; Huang et al., 2000; Chae et al., 2005; Gu et al., 2005; Kwon et al., 2005; Bjarnadottir et al., 2007; Li et al., 2007; Role and Talmage, 2007; Woo et al., 2007), including nAChRs containing the $\alpha 7$ subunit (Yang et al., 1998; Liu et al., 2001; Kawai et al., 2002; Chang and Fischbach, 2006). In this study we demonstrate that Type III Nrg1, acting as a receptor for ErbB4, controls the insertion of $\alpha 7^*$ nAChRs into axonal membranes. Type III Nrg1 accomplishes this by activating a local PtdIns 3K signaling pathway. These findings expand the repertoire of identified mechanisms by which Type III Nrg1 bidirectional signaling contributes to the establishment and the maturation of functional presynaptic terminals (Bao et al., 2003, 2004). Our major findings supporting this conclusion are the following: genetic disruption of Type III Nrg1 affects surface $\alpha 7^*$ nAChR levels;

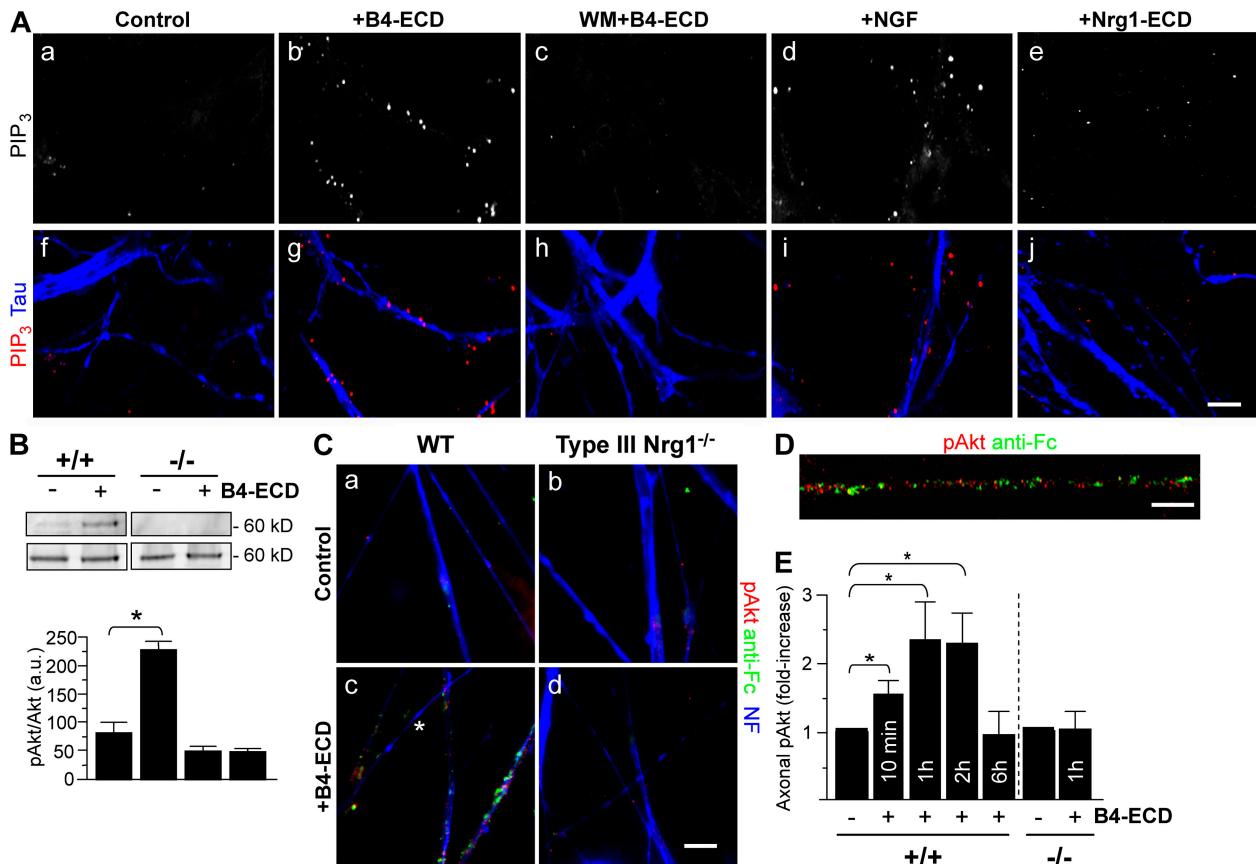


Figure 5. Type III Nrg1 back signaling activates the PtdIns 3K signaling pathway. (A) Dissociated sensory neurons from E11 chick embryos were treated for 5 min with B2-ECD (control), B4-ECD, 50 ng/ml NGF, or 10 ng/ml of soluble Nrg1 β peptide (Nrg1-ECD). In parallel, neurons were treated with WM for 45 min before B4-ECD stimulation (WM + B4-ECD). Neurons were fixed, permeabilized, and costained for PIP₃ (red) and tau protein (blue) to label axons. Both B4-ECD (g) and NGF (i) treatment induced puncta of PIP₃ along tau-positive axons. Neither B4-ECD stimulation in the presence of WM (c and h) nor that of Nrg1-ECD (e and j) induced an increase in PIP₃. Confocal images were obtained with a 40 \times oil objective. Bar, 10 μ m. (B) Immunoblot analysis of phospho-Akt (Ser 473) in WT or Type III Nrg1^{-/-} sensory neurons treated with either B2-ECD (control) or B4-ECD for 10 min. In WT neurons, B4-ECD treatment induced an approximately threefold increase in phospho-Akt, whereas no response was detected in mutant neurons. Total Akt in the bottom panel shows equal lysate loading. The bar graph represents phospho-Akt normalized to total Akt immunoreactive bands. Data are representative of three independent experiments. The graph shows means \pm SEM. Statistical significance was determined by ANOVA with post-hoc Fisher's PLSD test. *, P < 0.002 (Statview). (C and D) E14.5 WT (a and b) or Type III Nrg1^{-/-} (c and d) DRG explants were treated with B2-ECD (control) or B4-ECD for 10 min. Surface-bound B4-ECD or B2-ECD were labeled with an antibody against the human Fc domain (anti-Fc; green) before fixation. Neurons were fixed, permeabilized, and stained for phospho-Akt (red) and NF protein (blue). B4-ECD treatment increased phospho-Akt along Fc-positive axons of WT neurons (b and D) but did not do so along axons of mutant neurons (d). Note the close proximity of anti-FC and phospho-Akt puncta in the high-power micrograph shown in e. The asterisk denotes an axon negative for both anti-Fc and phospho-Akt immunolabeling (c). A 63 \times oil objective was used (a-d). Confocal imaging was obtained with a 100 \times oil objective (D). Bar: (a-d) 10 μ m; (D) 5 μ m. (E) Quantification of the average fluorescence intensity (AFI) of phospho-Akt along axons of WT or Type III Nrg1^{-/-} sensory neurons treated with B2-ECD (control) or B4-ECD for 10 min or 1, 2, or 6 h (see Materials and methods). Along WT axons, B4-ECD treatment induced increases in phospho-Akt. Along axons of mutant neurons, we did not detect an increase in phospho-Akt in response to B4-ECD treatment. The graph shows means \pm SEM. Data are from three independent experiments. Statistical significance was determined by ANOVA. *, P < 0.02.

Type III Nrg1 functioning as a receptor can regulate insertion of preformed $\alpha 7^*$ nAChRs into axonal surfaces; and Type III Nrg1 activates an axonal PtdIns 3K signaling pathway that mediates increased levels of surface $\alpha 7^*$ nAChRs.

Prior work has shown that presynaptic Type III Nrg1 is critical for establishing stable interactions with postsynaptic partners and, ultimately, for the survival of neuromuscular synapses as well as for glia development and myelination (Wolpowitz et al., 2000; Michailov et al., 2004; Taveggia et al., 2005; Chen et al., 2006). Our studies demonstrate that Type III Nrg1 acts as a presynaptic signaling receptor. Activation of presynaptic Type III Nrg1 signaling, either by postsynaptic ErbB4 (as shown in Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200710037/DC1>), by the ErbB3 present on glia, or after depolarization (Bao et al., 2003, 2004), locally regulates the levels of presynaptic $\alpha 7^*$ nAChR surface expression. As a result, Type III Nrg1 back signaling, by regulating presynaptic $\alpha 7^*$ nAChRs, alters the magnitude of acetylcholine modulation of neurotransmitter release (MacDermott et al., 1999; Jones and Wonnacott, 2004).

full/jcb.200710037/DC1), by the ErbB3 present on glia, or after depolarization (Bao et al., 2003, 2004), locally regulates the levels of presynaptic $\alpha 7^*$ nAChR surface expression. As a result, Type III Nrg1 back signaling, by regulating presynaptic $\alpha 7^*$ nAChRs, alters the magnitude of acetylcholine modulation of neurotransmitter release (MacDermott et al., 1999; Jones and Wonnacott, 2004).

We demonstrate that stimulating Type III Nrg1 activates a PtdIns 3K signaling pathway and that PtdIns 3K and Akt activity are required for increased surface expression of $\alpha 7^*$ nAChRs. At present, we do not know the molecular details of how Type III Nrg1 communicates with the PtdIns 3K signaling pathway or what Akt substrates regulate $\alpha 7^*$ nAChR trafficking.

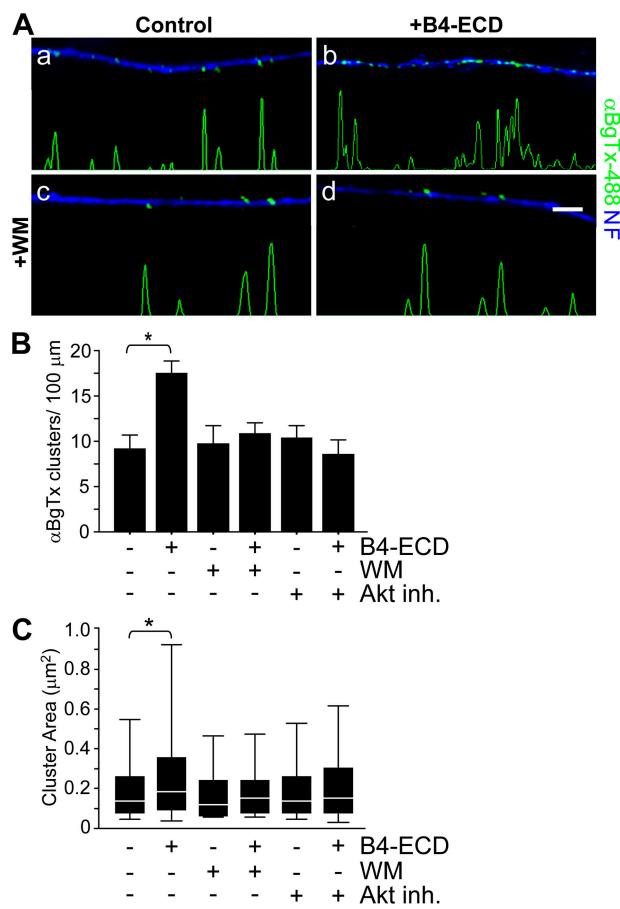


Figure 6. PtdIns 3K–Akt signaling activated by Type III Nrg1 back signaling is required for increased $\alpha 7^*$ nAChR surface expression. Dissociated sensory neurons from E11 chick embryos were treated with B2-ECD (control) or B4-ECD for 1 h. In parallel, neurons were pretreated with WM or an Akt inh. for 45 min before treatment with B2-ECD or B4-ECD for an additional hour. Neurons were labeled for surface $\alpha 7^*$ nAChRs with α BgTx-488 (green), fixed, permeabilized, and costained for NF protein (blue). (A) Representative micrographs of α BgTx-488 staining along NF-positive axons. B4-ECD treatment increased surface α BgTx-488 clusters (b), which did not occur in the presence of WM (d). Linescans of fluorescence intensity profiles of α BgTx-488 along representative axons (see Materials and methods) are shown. Bar, 5 μ m. (B) Quantification of surface α BgTx-488 clusters along sensory neuron axons represented in A. B4-ECD treatment induced an \sim 1.9-fold increase of surface α BgTx-488 clusters but not in the presence of WM or Akt inh. Data were pooled from three independent experiments. The graph shows means \pm SEM. Statistical significance was determined by ANOVA with post-hoc Fisher's PLSD test. *, $P < 0.0001$ (Statview). (C) Quantification of surface α BgTx-488 cluster area. B4-ECD treatment induced an increase in α BgTx-488 cluster area but not in the presence of WM or Akt inh. Data pooled from three independent experiments were analyzed using nonparametric statistics and presented as box plots (see Materials and methods). Statistical significance determined by the Kolmogorov-Smirnov Test. *, $P = 0.0001$ (Statview).

The intracellular domains of Type III Nrg1 lack known functional motifs that could mediate protein–protein interactions.

PtdIns 3K and its product PIP₃ are ubiquitous signaling molecules that link cell surface receptors to intracellular downstream effectors. PtdIns 3K signaling regulates the trafficking of several cell surface proteins, such as glucose transporter 4 (Tengholm and Meyer, 2002), excitatory amino acid carrier 1 glutamate transporter (Davis et al., 1998), calcium channels (Viard et al., 2004), and alpha-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptors (Man et al., 2003). An increased understanding of the molecular mechanisms that regulate trafficking of these proteins is certain to inform our further studies on presynaptic $\alpha 7^*$ nAChR targeting.

We have used α BgTx binding to identify both surface and internal pools of $\alpha 7^*$ nAChRs. α BgTx binding requires at least partial assembly of $\alpha 7$ subunits into pentamers (Mitra et al., 2001). Because we do not see effects of Type III Nrg1 back signaling on α BgTx binding in permeabilized cells, it is unlikely that the early stages of assembling $\alpha 7$ subunits into functional receptors are targeted by back signaling. Assembly of $\alpha 7^*$ nAChRs and trafficking of $\alpha 7^*$ nAChRs through the exocytic pathway can be influenced by synaptic scaffolding proteins such as PICK1 (Baer et al., 2007), reversible palmitoylation (of $\alpha 7^*$ nAChRs by itself; Drisdel et al., 2004), tyrosine residues on unidentified proteins (Cho et al., 2005), and chronic nicotine (Marks et al., 1992). Any of these mechanisms might represent convergent targets with Type III Nrg1–PtdIns 3K signaling for influencing the rate of ER/Golgi to plasma membrane trafficking of $\alpha 7^*$ nAChRs.

Targeting nAChRs to presynaptic locales is an important part of establishing a plastic synapse. The $\alpha 7^*$ nAChRs in particular play a critical role in sustained modulation of neurotransmitter release (McGehee et al., 1995; Girod et al., 2000). Deficits in Type III Nrg1 signaling compromise the ability of nicotine to elicit sustained glutamatergic transmission at ventral hippocampal to nucleus accumbens synapses (Du, C., C. Zhong, M. Hancock, D.A. Talmage, and L.W. Role. 2004. Society for Neuroscience 34th Annual Meeting; unpublished data) and, based on the results presented here, we would predict a similar effect on nicotine/acetylcholine modulation of glutamate release from primary sensory afferents in the dorsal spinal cord (Genzen and McGehee, 2003). From the present data, we cannot say whether the requirement for Type III Nrg1 signaling is limited to developmental synaptogenesis or whether it continues to affect presynaptic $\alpha 7^*$ nAChR targeting to established synapses, contributing to the maintenance of synaptic plasticity throughout the lifespan. If Type III Nrg1 signaling does continue to regulate presynaptic levels of $\alpha 7^*$ nAChR or other receptors, then modulation of Type III Nrg1 signaling in sensory axons could contribute to adaptive physiological changes or to pathological changes in sensory transduction (Birmingham-McDonogh et al., 1997; Kerber et al., 2003).

There are interesting parallels between our findings and pathophysiological studies. Increasing evidence from genetic linkage studies has identified both the *NRG1* (Stefansson et al., 2002; Harrison and Law, 2006) and the $\alpha 7$ nAChR gene *CHRNA7* (Freedman et al., 2001; Leonard and Freedman, 2006) as susceptibility genes for schizophrenia. Postmortem studies have demonstrated decreased levels of α BgTx binding in the hippocampus of schizophrenic patients (Freedman et al., 1995; Breese et al., 2000), and decreased levels of $\alpha 7$ mRNA and α BgTx binding in dorsal lateral prefrontal cortex of schizophrenic brain are associated with risk alleles at the *NRG1* locus (Mathew et al., 2007). It is worth noting that these risk alleles at the *NRG1* locus also have been associated with increased expression of Type I and IV Nrg1 (Law et al., 2006). This raises

the possibility that excess levels of soluble Nrg1 peptide disturbs the balance of bidirectional Nrg1-ErbB signaling that serves to ensure normal levels of functional $\alpha 7^*$ nAChRs.

Materials and methods

Animals and cell culture

DRG explants from E14.5 WT or Type III Nrg1^{-/-} mouse embryos (Wolpowitz et al., 2000) were dissected and cleaned with forceps to remove connective tissue. DRG were plated on glass coverslips (precoated with 1 mg/ml poly-D-lysine and 100 μ g/ml laminin) and cultured in MEM (Invitrogen) supplemented with 10% FBS, 4 mg/ml glucose, 2 mM L-glutamine, 50 U/ml each of penicillin and streptomycin (Invitrogen), and 40 ng/ml β -NGF (Harlan Bioproducts for Science) at 37°C for 2 d. DRG from E11 chick embryos were dispersed and cultured in DME media (Invitrogen) supplemented with 5% chick embryonic extract, 10% horse serum, 2 mM L-glutamine, 50 U/ml penicillin and streptomycin (Invitrogen), and 50 ng/ml β -NGF (Harlan Bioproducts for Science) at 37°C for 2 d. For PIP₃ and phospho-Akt labeling and immunoblotting, culture media was replaced with serum-free media 4 h before soluble factor addition. For chick cultures, serum-free media consisted of DME, 1% BSA (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), insulin-transferrin-selenium supplement (Sigma-Aldrich) and 50 U/ml each of penicillin and streptomycin. For mouse cultures, serum-free media consisted of MEM, 1% BSA, 4 mg/ml glucose, 2 mM L-glutamine, insulin-transferrin-selenium supplement, and 50 U/ml each of penicillin and streptomycin.

Reagents

Where indicated, the following reagents were applied to the cells: 2 nM of soluble B2-ECD or B4-ECD (Bao et al., 2003), 50 ng/ml of recombinant HRG1- β 1 EGF-like domain (R&D Systems), 50 ng/ml β -NGF (R&D Systems), 10 μ g/ml CHX (Sigma-Aldrich), 5 μ M phenylarsine oxide (Sigma-Aldrich), 500 nM WM (EMD), 5 μ M Akt Inh. V (EMD), 2 μ M each of PD 158780 and PD 168393 (EMD), and 200 nM TrkA inhibitor (EMD). Inhibitors were added to the media for 45 min before soluble factor addition.

Immunofluorescence

Cells were fixed with 4% PFA for 20 min at RT, permeabilized with 0.2% Triton X-100 (where indicated) for 5 min at RT, blocked with 10% NDS, and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-Type III Nrg1 (1:500; Yang et al., 1998), anti-NF (1:500; Sternberger Monoclonals, Inc.), anti- τ au (1:250; Sigma-Aldrich), anti-human IgG (Fc-specific; 1:200; Sigma-Aldrich), anti-PIP₃ (1:50; Echelon Biosciences Inc.), anti-phospho-Akt (1:200; Cell Signaling Technology), anti-synaptophysin (1:500; Millipore); anti-vGlut1 (1:200; Synaptic Systems GmbH), anti-TrkA (1:5,000; gift from L.F. Reichardt, University of California, San Francisco, San Francisco, CA), anti-MAP2 (1:500; Millipore), and anti-phospho MAPK (1:1,000; Sigma-Aldrich). Cells were washed and incubated in secondary antibodies conjugated to Alexa 488 (1:500; Invitrogen), Alexa 594 (1:500; Invitrogen), or anti-mannobioside carbohydrate antibodies (1:50; Jackson ImmunoResearch Laboratories) for 1 h at RT. To label PIP₃, slips were treated with Avidin/Biotin Blocking kit (Vector Laboratories) before incubation in anti-PIP₃. The primary antibody signal was amplified by sequential binding with a biotinylated anti-mouse secondary (1:200; Jackson ImmunoResearch Laboratories) and NeutrAvidin-rhodamine red (1:200; Invitrogen). Slips were mounted using VectaShield (Vector Laboratories), and images were captured using a microscope (Axio Imager; Carl Zeiss, Inc.) equipped with Plan-Apochromat objectives (20x with 0.8 NA or 63x oil with 1.4 NA), a charge-coupled device camera (Hamamatsu), and Metamorph software (Version 6.3r5; MDS Analytical Technologies). Confocal images were captured with an NLO Multiphoton (LSM 510; Carl Zeiss, Inc.) on a microscope (Axioskop2 FS; Carl Zeiss, Inc.) equipped with Plan-Neofluar objectives (40 or 100x oil with 1.3 NA) and a charge-coupled device camera. Brightness and contrast were adjusted using Photoshop software (Version 8.0; Adobe).

To label surface $\alpha 7^*$ nAChRs, live sensory neurons were incubated in α BgTx-488 (1:500; Invitrogen) for 15–20 min at 37°C. Cells were fixed and incubated in rabbit anti-Alexa 488 (1:250; Invitrogen) overnight at 4°C, followed by secondary antibody incubation. Surface cluster number and area were measured along NF-positive processes (≥ 10 μ m from soma) using Metamorph software. The lengths of axonal processes were measured by manually tracing NF-positive processes. Control cultures in each experiment were used to define the threshold for measuring clusters at 50%

maximum intensity and greater than or equal to four contiguous pixels (Kawai et al., 2002). To determine nonspecific binding, cultures were treated with 1 μ M of nicotine or 5 μ M methyllycaconitine before labeling. For each experiment, nonspecific labeling was $\leq 12\%$ and was subtracted from all counts. Linescans with widths of 10 contiguous pixels were obtained using Metamorph software.

To quantify Akt activation along axons, neurons were stained with antibodies against phospho-Akt and NF to label axons. 15–20 axons per condition were analyzed in each experiment. The AFI of phospho-Akt along NF-positive processes was measured using MetaMorph software. The AFI of two to three randomly selected regions for each condition were measured as background AFI and subtracted from all measurements. To compare results between independent experiments, the mean AFI from different conditions in each independent experiment was normalized to the control AFI and is expressed as fold increase.

Ca²⁺ imaging

After 3 d in vitro, DRG explants were rinsed with HBSS, loaded with Fluo-3 Ca²⁺ binding dye (Invitrogen) dissolved in HBSS for 30 min at 37°C, and rinsed for 30 min at 37°C in HBSS. The coverslip was placed on a perfusion-equipped stage and perfused at a rate of 0.5 ml/min with HBSS containing 2 μ M tetrodotoxin, 10 μ M bicucullin, 50 μ M D(-)-2-amino-5-phosphono-valeric acid and 20 μ M CNQX (Sigma-Aldrich). Images were captured every 5 s using a spinning disc confocal microscope (DSU; Olympus) equipped with a UPlanSApo objective (60x water with 1.4 NA), electron-multiplying charge-coupled device camera (Hamamatsu), and Slidebook software (Version 4; Olympus). After acquiring images for 1 min, 1 μ M of nicotine was focally applied by pressure ejection for 60 s and the explants were imaged for another 4 min. After a 1- μ M nicotine application, fluorescence intensity within defined axonal regions was quantified with MetaMorph software.

¹²⁵I- α BgTx binding assay

After 2 d in vitro, dispersed chick sensory neurons were treated with 2 nM of soluble B2-ECD or B4-ECD for 24 h. 6 nM ¹²⁵I- α BgTx (2,000 Ci/ μ mol; GE Healthcare) was added directly to the media for the final 20 min. To measure total ¹²⁵I- α BgTx binding, cultures were rinsed three times with PBS and permeabilized with 0.5% saponin in 0.2% FAF-BSA/PBS for 30 min before incubation with 6 nM ¹²⁵I- α BgTx in 0.2% FAF-BSA/PBS for 2 h on ice. For both surface and total labeling, nonspecific binding was assessed by including 1 μ M α BgTx (Sigma-Aldrich) during the labeling with ¹²⁵I- α BgTx. After labeling, cells were washed and solubilized in 1 N NaOH, and bound ¹²⁵I- α BgTx was quantified with a γ counter. Nonspecific binding was subtracted.

Immunoblotting

Neurons were lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with 10 mM DTT, protease inhibitors (Sigma-Aldrich), and phosphatase inhibitors (Thermo Fisher Scientific). Lysates (phospho-Akt, 25 μ g; $\alpha 7$, 40 μ g) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose filters. Filters were blocked in 5% milk in 0.1% Tween-20 TBS solution at RT for 2 h before overnight incubation in primary antibody solutions in 5% BSA or milk in Tween-20 TBS solution at 4°C [anti- $\alpha 7$ nAChR subunit (1:500; Santa Cruz Biotechnology, Inc.), anti-MAPK (1:5,000; Millipore), anti-glyceraldehyde 3-phosphate dehydrogenase (1:10,000; Millipore), anti-NF M (1:7,500; Millipore), anti-phospho-Akt (1:500; Cell Signaling Technology), and anti-Akt (1:1,000; Cell Signaling Technology)]. Detection was performed using Alexa Fluor 680 (Invitrogen) or IRDye-800 (Rockland Immunochemicals) secondary antibodies and an Odyssey Infrared Imaging System (Version 2.1; LI-COR Biosciences).

Transferrin recycling

After 2–4 d in vitro, WT or Type III Nrg1^{-/-} sensory neurons were serum starved for 25 min in serum-free MEM containing 0.5% BSA. Neurons were incubated with 10 μ g/ml transferrin-Alexa 488 (Invitrogen) for 15 min in serum-free media, after which cells were rinsed and chased in complete media supplemented with 1 mg/ml of unlabeled transferrin (Sigma-Aldrich). During the chase period, live images of neurons were captured at 0.5–1- μ m intervals every 2–4 min over a >20-min period using a spinning disc confocal microscope (DSU; Olympus) equipped with a UPlanSApo objective (60x water with 1.4 NA), electron-multiplying charge-coupled device camera, and Slidebook software. The AFI of transferrin-Alexa 488 in collapsed z series was measured using MetaMorph software. The AFI of two to three randomly selected regions for each condition was measured as background AFI and subtracted. The AFI from each time point was normalized to the AFI at $t = 0$ and is expressed as percentage of initial AFI.

Statistical significance

For normally distributed data, statistical significance was evaluated by ANOVA with a post-hoc Fischer's PLSD test for multiple comparisons (Stat-view; Adept Scientific). Non-normally distributed data were analyzed using nonparametric methods and are presented using box plots. The boxes include data points within the middle 50%. The bottom marks the twenty-fifth percentile, the middle line the fiftieth percentile, and the top the seventy-fifth percentile. Vertical lines mark the fifth and ninety-fifth percentiles (see Devay et al. [1999] for a detailed description of data presentation). Statistical significance was evaluated by the Kolmogorov-Smirnov test.

Online supplemental material

In Fig. S1, we show that endocytic recycling of transferrin (A and B) and the trafficking of presynaptic proteins vGlut1 and synaptophysin (C and D) are not impaired in Type III Nrg1^{-/-} sensory neurons. Additionally, we show that activation of Type III Nrg1 back-signaling does not induce an increase in the surface expression of TrkA along axons (E and F). Fig. S2 shows soluble ErbB4-ECD (B4-ECD) bound to Type III Nrg1 puncta along WT axons only (A). B4-ECD treatment induces an increase in the axonal surface expression of $\alpha 7^* nAChRs$ in a dose-dependent manner (B). Fig. S3 shows that Type III Nrg1 back signaling activates Akt in neurons and not nonneuronal cells (A). Inhibition of ErbB (B) or TrkA (C) kinase activity does not prevent the B4-ECD-induced response. We also show that B4-ECD treatment does not activate MAPK in neurons (D). Fig. S4 consists of a diagram summarizing a mechanism by which Type III Nrg1 back-signaling regulates the levels of $\alpha 7^* nAChRs$ along axons. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200710037/DC1>.

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