Type III Neuregulin 1 Is Required for Multiple Forms of Excitatory Synaptic Plasticity of Mouse Cortico-Amygdala Circuits

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The amygdala plays an important role in the formation and storage of memories associated with emotional events. The cortical glutamatergic inputs onto pyramidal neurons in the basolateral nucleus of the amygdala (BLA) contribute to this process. As the interaction between neuregulin 1 (Nrg1) and its ErbB receptors has been implicated in the pathological mechanisms of schizophrenia, loss of Nrg1 may disrupt cortical–amygdala neural circuits, resulting in altered processing of salient memories. Here we show that Nrg1 is critical in multiple forms of plasticity of cortical projections to pyramidal neurons of the BLA. The miniature EPSCs in *Nrg1* heterozygous animals have a faster time constant of decay and evoked synaptic currents have a smaller NMDA/AMPA ratio than those recorded in wild-type (WT) littermates. Both high-frequency electrical stimulation of cortical inputs and θ burst stimulation combined with nicotine exposure results in long-lasting potentiation in WT animals. However, the same manipulations have little to no effect on glutamatergic synaptic plasticity in the BLA from *Nrg1* heterozygous mice. Comparison of WT, *Nrg1* heterozygous animals and $\alpha 7$ nicotinic receptor heterozygous mice reveals that the sustained phase of potentiation of glutamatergic transmission after θ burst stimulation with or without nicotine only occurs in the WT mice. Together, these findings support the idea that type III Nrg1 is essential to multiple aspects of the modulation of excitatory plasticity at cortical–BLA synapses.

Introduction

Neuregulin 1 (*Nrg1*) and its signaling partner, the *ErbB4* receptor, have been identified as leading candidate schizophrenia susceptibility genes (Stefansson et al., 2002; Harrison and Weinberger, 2005; Harrison and Law, 2006; Lu et al., 2010; Stefanis et al., 2011; Bae et al., 2012). Nrg1 signaling is important in nervous system development contributing to aspects of neuronal migration, synaptogenesis, gliogenesis, and neuron–glia communication (Taveggia et al., 2005; Lopez-Bendito et al., 2006; Quintes et al., 2010; Ting et al., 2011). In mature animals, alterations in Nrg1/ErbB signaling are manifest in aberrant peripheral myelination, and deficits in synaptic plasticity in hippocampal and cortical slices (Michailov et al., 2004; Kwon et al., 2005; Bjarnadottir et al., 2007; Li et al., 2007; Brinkmann et al., 2008; Chen et al., 2008b; Pitcher et al., 2011; Shamir et al., 2012). Likewise, Nrg1 and ErbB receptor interactions have been shown to affect the

synaptic function of many neuronal transmitter receptors, including glutamate α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, *N*-methyl-D-aspartate (NMDA) receptors, and nicotinic acetylcholine receptors (nAChRs) (Kwon et al., 2005; Bjarnadottir et al., 2007; Hancock et al., 2008; Zhong et al., 2008; Abe et al., 2011; Canetta et al., 2011; Pitcher et al., 2011; Ting et al., 2011; Fenster et al., 2012).

In the hippocampus and neocortex, Nrg1/ErbB4 signaling has a major impact on the formation and function of glutamatergic transmission. Nrg1 activation of ErbB4 in cortical and hippocampal interneurons induces formation of glutamatergic synapses on these interneurons (Fazzari et al., 2010; Ting et al., 2011) and acutely regulates excitatory plasticity of pyramidal neurons (Li et al., 2007; Chen et al., 2010a, 2010b; Fazzari et al., 2010; Pitcher et al., 2011; Bae et al., 2012; Fenster et al., 2012; Shamir et al., 2012).

The type III Nrg1 isoform is also an important regulator of cholinergic signaling. Nrg1 regulates both the expression of AChR genes and targeting of nAChRs to the neuronal cell surface (Sandrock et al., 1997; Yang et al., 1998; Chang and Fischbach, 2006), and the type III Nrg1 isoforms in particular are critical regulators of presynaptic targeting of $\alpha 7$ -containing ($\alpha 7^*$) nAChRs (Chen et al., 2008b; Hancock et al., 2008; Zhong et al., 2008).

The possible role of Nrg1/ErbB signaling in the amygdala has not been explored. Type III Nrg1 is expressed in the basolateral nucleus of the amygdala (BLA) and in cortical neurons that project to the BLA (see below), and ErbB4 is expressed in interneu-

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rons within the BLA as well as highly expressed in the basomedial area (Shamir et al., 2012). The BLA receives extensive cholinergic input from the nucleus basalis of Meynert (Carlsen et al., 1985; Wenk, 1997; Schafer et al., 1998; Muller et al., 2011). Both $\alpha 7^*$ and non $\alpha 7^*$ nAChRs have been shown to contribute to presynaptic modulation of cortical–BLA synapses by exogenously applied nicotine (Jiang and Role, 2008).

To gain insight into the functional significance of Nrg1/ErbB signaling in the BLA, we investigated whether heterozygous deletion of type III *Nrg1* alters cortical–BLA transmission and/or changes the efficacy of cholinergic modulation of these circuits. We found that heterozygous deletion of type III *Nrg1* blocked the induction of LTP at cortical–BLA synapses, whether activated by patterned stimulation alone or by concurrent electrical stimulation paired with nicotine exposure. Parallel studies in mice heterozygous for $\alpha 7^*$ nAChR revealed a direct phenocopy of plasticity deficits in the type III *Nrg1* heterozygous mice, and type III *Nrg1* heterozygotes have reduced $\alpha 7^*$ nAChRs along projections within the BLA. Hence, both type III *Nrg1* and $\alpha 7$ -containing nAChRs appear to be critical in inducing LTP of glutamatergic transmission in BLA.

Materials and Methods

All mouse care and experimental procedures were approved by the Institutional Animal Care Research Advisory Committee of the State University of New York at Stony Brook. Mice of either sex used in these experiments include wild-type (WT) (C57 BL6), *Nrg1*^{Lwrtm1/+} (Wolpowitz et al., 2000) and *Chrna7*^{Baytm1} ($\alpha 7^{+/-}$ or $\alpha 7^{-/-}$) (The Jackson Laboratory). Both mutant lines are maintained on a pure C57 BL6 background. All experiments were performed before weaning.

Slice preparation. Coronal brain slices were prepared from WT mice or from *Nrg1* or $\alpha 7$ mutant mice at postnatal age 14–20 d. Animals were anesthetized with a mixture of ketamine and xylazine (100 mg ketamine and 6 mg xylazine/kg body weight injected i.p.). After decapitation, the brain was transferred quickly into a sucrose-based solution bubbled with 95% O₂ and 5% CO₂ and maintained at ~3°C. This solution contained the following (in mM): sucrose 230, KCl 2.5, MgSO₄ 10, CaCl₂ 0.5, NaH₂PO₄ 1.25, NaHCO₃ 26, and glucose 10. Coronal brain slices (300 μ m) were prepared using a Leica VT1000S vibratome (Leica). Slices were equilibrated with an oxygenated artificial CSF (aCSF) at room temperature (24–26°C) for at least 1 h before transfer to the recording chamber. The slices were continuously superfused with aCSF at a rate of 2 ml/min containing the following (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.25, NaHCO₃ 26, CaCl₂ 2, MgCl₂ 2, and glucose 10 bubbled with 95% O₂ and 5% CO₂ at room temperature.

Electrophysiological recordings. Brain slices were placed on the stage of an upright, infrared-differential interference contrast microscope (BX51WI, Olympus Optical). BLA pyramidal neurons were visualized with a 40 $\times$ water-immersion objective by infrared microscopy (4915 camera, COHU). Patch electrodes with a resistance of 4–6 M Ω were pulled with a laser-based micropipette puller (P-2000, Sutter Instrument). Signals were recorded with a Multi Clamp 700A amplifier and pClamp10 software (Molecular Devices). The pipette solution contains the following (in mM): 130 K-gluconate, 2 KCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 1 ATP, and 0.2 GTP, pH 7.3. To examine the voltage dependence of the evoked PSCs, 130 mM Cs-methane-sulfonate was used instead of K-gluconate. All recordings included in this study are from rapidly activating and strongly accommodating pyramidal neurons (Jiang and Role, 2008). The following criteria were applied for inclusion of recorded cells in the study; failure to meet all of these criteria resulted in exclusion from the sample population: (1) seal resistances maintained throughout the recording period at >5 G Ω ; (2) holding current in whole-cell clamp configuration remained within 10% of the initial value and was $\leq$ 100 pA; and (3) series resistance; R_s measured every 10 min throughout the course of the experiment remained stable (i.e., <10% change from initial value).

To examine transmission at cortical–BLA inputs, EPSCs were evoked by field stimulation with a concentric bipolar stimulation electrode (FHC) placed in the external capsule. A 0.1 Hz single stimulation (dura-

tion, 1 ms; intensity, 10–30 μ A) was delivered via the Master-8 stimulator (A.M.P.I.). For minimal stimulation, the stimulation strength was adjusted to trigger EPSCs in response to stimulation in ~50% of the trials, which was typically equivalent to activation at ~10–15% of the maximum response amplitude. In some experiments, a stronger stimulation was used (20–30% of maximal response as in Tsvetkov et al., 2004). High-frequency stimulation (HFS, 1 s at 100 Hz) 2 ms pulses were delivered 5 times with 10 s interval. The θ burst stimulation pattern (TBS) used delivered four 2 ms pulses of depolarization at 50 Hz, repeated 10 times at a 5 Hz interburst interval. Spontaneous EPSCs in BLA pyramidal neurons were monitored in a voltage-clamp configuration at a holding potential of –60 mV. Bicuculline (10 μ M) was continuously present in aCSF to isolate glutamatergic synaptic transmission. Miniature EPSC currents were recorded in the presence of 1 μ M TTX. Data were filtered at 2 kHz by Multi Clamp 700A and analyzed using Clampfit10 (Axon Instruments) and Mini Analysis 6.0 (Synaptosoft).

Statistical analyses were performed using nonparametric statistical tests as the majority of electrophysiological measures were not normally distributed. Tests included the Kolmogorov–Smirnov (KS), Mann–Whitney (MW), and paired-sample Wilcoxon signed rank test with Origin 9.0 (Originlab). The median values were reported from the entire population tested. Data were considered significantly different when $p < 0.05$. The study is based on >200 recordings from 81 mice, unless otherwise noted; n values reported are numbers of recordings.

Nicotine delivery and doses. Nicotine (500 nM) was applied with a picospritzer (Parker Instrumentation). Pressure (5–10 psi) was applied for 1 min to a 1–2 μ m diameter patch pipette that was positioned ~15 μ m from the soma of the recorded neurons. All experiments with acute nicotine were performed in the presence of 200 nM atropine to preclude muscarinic receptor activation.

Anterograde tracing. P21 mice were anesthetized using isoflurane, and holes were drilled in the skull using a 0.7 mm drill bit (Fine Science Tools) 1.54 mm anterior and 0.45 mm lateral to bregma. A 25 gauge Hamilton syringe was then inserted at a depth of 1.5 mm from the dural surface and 1 μ l of 10% biotin dextran amine (BDA; Invitrogen) conjugate was injected manually over 3 min, and the needle was withdrawn after an additional 2 min to prevent reflux. The skin above the skull was closed with tissue adhesive (3M vetbond), and the animals were placed on a heating pad until they recovered from the anesthesia. The animals were administered analgesic, every day until death 5 d after injection via transcardial perfusion.

Immunofluorescence. For immunofluorescent analysis, P21–P28 mice were transcardially perfused with 4% PFA in PBS. After perfusion the brains were isolated, postfixed with 4% PFA, cryoprotected in 30% sucrose for 2 d, and stored at –80°C in OCT until sectioned. For $\alpha 7$ nAChR surface expression, frozen sections (15 μ m) were blocked for 1 h with 5% normal donkey serum and incubated for 1 h with α -bungarotoxin conjugated to biotin XX (α -BgTx-biotin XX; 1:1000; Invitrogen). Antigen retrieval was performed to detect CTIP-2 (Abcam, 1:500) by incubating sections in sodium citrate buffer, pH 6, for 15 min at 100°C followed by blocking step, as stated below. To detect type III *Nrg1*, BDA and CTIP-2 sections (15 μ m) were blocked and permeabilized with 5% normal donkey serum containing 0.1% Triton X-100 (Sigma) for 1 h at room temperature followed by overnight incubation at 4°C with sheep anti-CRD (1:100; for full description and characterization of this antibody, see Yang et al., 1998; Chen et al., 2010a; Hancock et al., 2011) or a 1 h incubation with streptavidin 680 to detect type III *Nrg1* and BDA, respectively. After washes, total $\alpha 7^*$ nAChR expression was detected by incubating the slices for 1 h with α -BgTx-biotin XX (Invitrogen) or α -BgTx conjugated to Alexa488 (α -BgTx-488; Invitrogen) at a 1:1000 dilution. Sections were washed and incubated for 1 h at room temperature with respective secondary antibodies.

Results

Previously, we demonstrated that presynaptic nAChRs play an important role in modulating cortical–amygdala synapses (Jiang and Role, 2008) and that type III *Nrg1* is a key regulator of presynaptic targeting of $\alpha 7^*$ nAChRs in neurons in culture (Hancock

et al., 2008; Zhong et al., 2008). In this study, we sought to determine whether plasticity and presynaptic nicotinic modulation of cortical–BLA synapses are affected in mice heterozygous for a mutation in the *type III Nrg1* gene.

The results presented are from >200 total recordings from 81 mice. Because the *type III Nrg1* knock-out mice die at birth, only heterozygotes for *type III Nrg1* (HET) were used in the electrophysiological studies. Only records that passed all recording criteria as outlined in Materials and Methods were included in the analyses.

Type III Nrg1 is expressed in cortical areas that innervate the BLA: partial deletion of Nrg1 alters the kinetics of miniature EPSCs (mEPSCs)

We examined the expression of type III Nrg1 in the cingulate cortex. Immunostaining with specific antibodies against type III Nrg1 (Yang et al., 1998; Chen et al., 2010a, 2010b; Hancock et al., 2011) and CTIP-2, a transcription factor expressed by deep layer subcortical projection neurons (Arlotta et al., 2005; Molyneux et al., 2005; Chen et al., 2008a; McKenna et al., 2011) revealed type III Nrg1 expression in cortical pyramidal neurons (Fig. 1A) and demonstrated a pronounced reduction in type III Nrg1 expression in these same type of cortical neurons from *type III Nrg1* heterozygous animals (Fig. 1B).

As Nrg1 has been reported to regulate the levels and function of many synaptic receptors, including AMPA- and NMDA-type glutamate receptors, we began our electrophysiological analyses by assessing the profile of baseline synaptic activity in the BLA, comparing WT mice with mice that are heterozygous for *type III Nrg1* (HET). To compare miniature (TTX-resistant) glutamatergic synaptic currents in BLA of WT versus *Nrg1* HET mice, we included both bicuculline (10 μ M) and TTX (1 μ M) in the aCSF. Analysis of a large number of TTX-resistant events recorded from both WT and *Nrg1* HET revealed no difference in mEPSC frequency (Fig. 1C,D; $p = 0.87$, KS test, WT, $n = 14$, HET, $n = 8$; inset, box plot of mEPSC frequency, $p = 0.59$, MW test). Although the amplitudes of mEPSCs in the BLA of *Nrg1* HET mice were somewhat larger than those recorded in WT littermates, the difference does not reach statistical significance (Fig. 1E; $p = 0.12$, KS test, inset, box plot of amplitude data, $p = 0.32$, MW test). In contrast, comparison of the decay kinetics of the miniature synaptic currents revealed a significantly faster time constant in the *Nrg1* HET than in WT, consistent with briefer open time kinetics of the underlying glutamate gated channels (Fig. 1F,G; $p = 0.02$, MW test, WT, $n = 14$; HET, $n = 8$).

Differences in evoked glutamatergic transmission at cortical–BLA synapses of WT versus *type III Nrg1* HET mice

Although the baseline glutamatergic transmission appeared similar between WT and *Nrg1* HET with respect to mEPSC frequency and amplitude, the differences in the kinetic profile of the mEPSCs prompted us to examine the potential effect of deletion of one allele of *Nrg1* on evoked glutamatergic transmission in more detail. To assess the relative contribution of AMPA versus NMDA receptor types to evoked glutamatergic synaptic transmission via cortical inputs, we recorded synaptic currents over a range of holding potentials (Fig. 2A; from -90 mV to $+50$ mV, step = $+20$ mV, the resting membrane potential holding at -60 mV) while stimulating the external capsule fibers. The NMDA to AMPA ratio was calculated using the amplitude of NMDA synaptic currents holding at $+50$ mV divided by the amplitude of AMPA synaptic currents holding at -70 mV. The synaptic current NMDA to AMPA ratio in the WT mice is significantly higher

Figure 1. Nrg1 expression in cingulate cortex–BLA circuit: heterozygous deletion of *type III Nrg1* alters properties of miniature EPSCs in BLA pyramidal neurons. **A**, Representative confocal image of Nrg-1 (red) and CTIP-2 expression in the cingulate cortex. Scale bar, 100 μ m. Left inset, CTIP-2-expressing subcortical projection neurons (green) express type III Nrg1 in layer V of the cingulate. Right inset, The left inset merged with DAPI (dark blue) CTIP-2-positive nuclei (cyan) are pyramidal neuron; CTIP-2-negative nuclei (dark blue) are interneurons. Scale bar, 20 μ m. **B**, Immunofluorescent micrographs of cingulate cortex labeled with type III Nrg1 antibody (red) and DAPI (blue) in WT (left) and in the *type III Nrg1* (+/−) mouse. Scale bar, 30 μ m. **C**, Top, Sample traces of mEPSCs recorded in a BLA pyramidal neuron in WT acute slice at a holding potential of -60 mV (1 μ M TTX, 10 μ M bicuculline, and 200 nM atropine). Calibration: 5 pA, 100 ms. Bottom, Sample traces of mEPSCs recorded in a comparable BLA pyramidal neuron in an acute slice prepared from a *Nrg1* HET recording conditions in **C** are otherwise identical. **D**, Cumulative distribution of the mEPSCs interevent interval times from 22 separate experiments in WT ($n = 14$) and *Nrg1* HET ($n = 8$) neurons; acute slice with recording conditions as in **D**. There is no significant difference in the interevent intervals, indicating that WT and *Nrg1* HET BLA neurons have similar mEPSC frequency (solid black line, WT; dotted red line, *Nrg1* HET; $p = 0.87$, KS test). Inset, Box plot of mEPSC frequencies from the population data ($p = 0.59$, MW test). The horizontal lines in each box (starting from the lower bar) indicate the range of the data that fall within the 25th, 50th, and 75th percentile values, respectively. The whiskers indicate the 5th and 95th percentile values. The square symbol within the box represents the mean of the population. **E**, Cumulative distribution of the mEPSC amplitudes from WT and *Nrg1* HET animals. The cumulative distribution of mEPSC amplitudes shows that *Nrg1* HET animals have somewhat larger amplitudes, although this difference was not statistically significant (solid black line, WT; dotted red line, *Nrg1* HET; WT, $n = 14$; HET, $n = 8$; $p = 0.12$, KS test). Inset, Box plot of mEPSC amplitudes from the population data ($p = 0.32$, MW test). **F**, Left, Digital averaged mEPSC recorded in representative WT BLA pyramidal neuron (black) and a representative *Nrg1* HET neuron (red; number of events averaged: WT, $n = 78$; HET, $n = 95$). *Nrg1* HET mEPSC has somewhat larger amplitude and significantly faster time constant of decay than WT. Calibration: 2 pA, 5 ms. Right, averaged WT trace was scaled to the same amplitude as *Nrg1* HET for comparison of decay time course. **G**, Pooled tau decay time constant (single exponential fit) for WT and *Nrg1* HET mEPSCs. The decay time constant for *Nrg1* HET mEPSC is significantly faster than that of WT (tau WT = 13.7 ± 0.8 ms, $n = 14$ neurons; 1289 events vs tau HET, red; 10.2 ± 1.2 ms, $n = 8$ neurons, 846 events; $p = 0.02$, MW test). * $p < 0.05$.

than it is in the *Nrg1* HET mice (Fig. 2*B*; $p = 0.038$, MW test; WT, $n = 7$; and HET, $n = 6$). Examination of the I versus V relationship for each of the underlying glutamate receptor-mediated currents was then performed (Fig. 2*C,D*). This analysis revealed that the NMDA receptor-mediated synaptic currents were not significantly different in *Nrg1* HET and WT BLA pyramidal neurons, with the exception of a single holding potential (Fig. 2*C*; at $V_h -90$ mV, $p = 0.35$; -70 mV, $p = 0.83$; -50 mV, $p = 1$; -30 mV, $p = 0.13$; -10 mV, $p = 0.01$; $+10$ mV, $p = 0.43$; $+30$ mV, $p = 0.94$; $+50$ mV, $p = 0.94$; MW test). However, such studies also revealed that the amplitude of AMPA receptor-mediated synaptic currents was significantly greater in *Nrg1* HET compared with WT mice at all hyperpolarizing holding potentials (Fig. 2*D*; WT = 7, HET = 6 at $V_h -90$ mV, $p = 0.004$; -70 mV, $p = 0.01$; -50 mV, $p = 0.005$; -30 mV, $p = 0.015$; -10 mV, $p = 0.007$; $+10$ mV, $p = 0.70$; $+30$ mV, $p = 0.25$; $+50$ mV, $p = 0.12$; MW test).

We next sought to assess whether the observed differences in the AMPA receptor-mediated synaptic currents in WT versus *Nrg1* HET mice might alter the overall input versus output relationship of cortical–BLA synapses in WT compared with *Nrg1* HET. To examine the input/output relationship, we increased the stimulation intensity from 10 to 200 μ A (by 10 μ A per step) and measured the amplitude of the evoked synaptic currents in both WT and *Nrg1* HET animals. The points at which the response was 10% and 90% of the maximum amplitude are compared by genotype in Figure 2*E, F*. At lower stimulation, the evoked synaptic currents are equivalent in WT and *Nrg1* HET (Fig. 2*E,F*; low stimulation, $p = 0.89$, MW test; WT, $n = 10$; HET, $n = 8$). With increased stimulation intensity, the amplitude of the evoked currents in the *Nrg1* HET animals was somewhat larger than in WT siblings, although the trend is not statistically significant (Fig. 2*E,F*; high stimulation, $p = 0.56$, MW test). Together, these data are consistent with the idea that, whereas the net postsynaptic responses are not significantly different, the relative contribution of the postsynaptic AMPAR- versus NMDAR-mediated responses in *Nrg1* HET mice are greater than that of the WT.

High-frequency burst stimulation of cortical inputs elicits LTP at cortical–BLA synapses in WT, but not in type III *Nrg1* HET, mice

Prior studies of evoked synaptic transmission have focused on the cortical projections to pyramidal neurons in the BLA

Figure 2. Heterozygous deletion of type III *Nrg1* alters the properties of evoked EPSCs at cortical–BLA pyramidal neuron synapses. *A*, Sample traces of evoked EPSCs from cortical–BLA inputs with a 20 mV step depolarization from -90 mV to $+50$ mV ($V_h = -60$ mV). The amplitude of AMPA receptor-mediated currents was measured at the peak (indicated by the dotted line labeled A). The amplitude of NMDA receptor-mediated currents was measured 50 ms after the EPSC peak (indicated by the dotted line labeled N). Calibration: 25 pA, 20 ms. *B*, NMDA to AMPA ratio for WT versus *Nrg1* HET. The NMDA to AMPA ratio was calculated as the amplitude of the NMDA receptor-mediated component measured at $V_h = +50$ mV divided by the amplitude of the AMPA receptor component at $V_h = -70$ mV. The WT NMDA to AMPA ratio is significantly higher than that recorded in *Nrg1* HET animals (WT: 1.48 ± 0.35 , $n = 7$; HET: 0.53 ± 0.04 , $n = 6$; $p = 0.04$, MW test). * $p < 0.05$. *C*, Plots of I versus V relationship for NMDA-receptor-mediated component of cortical–BLA evoked EPSCs. The amplitude of the NMDA component was measured in *A* with steps from the holding potential as indicated. There is no significant difference between the NMDA I versus V for WT and *Nrg1* HET animals at all but one holding potential (WT, $n = 7$; HET, $n = 6$; at $V_h -90$ mV, $p = 0.35$; -70 mV, $p = 0.83$; -50 mV, $p = 1$; -30 mV, $p = 0.13$; -10 mV, $p = 0.01$; $+10$ mV, $p = 0.43$; $+30$ mV, $p = 0.94$; $+50$ mV, $p = 0.94$; MW test). *D*, Plots of I versus V relationship for AMPA-receptor-mediated component of cortical–BLA evoked EPSCs. The amplitude of the AMPA component was measured as indicated in *A* with steps in the holding potential. *Nrg1* HET animals have significantly larger amplitude of AMPA receptor-mediated currents than WT at comparable hyperpolarized, but not at depolarized, holding potentials (WT, $n = 7$; HET, $n = 6$; at $V_h -90$ mV, $p = 0.004$; -70 mV, $p = 0.01$; -50 mV, $p = 0.005$; -30 mV, $p = 0.015$; -10 mV, $p = 0.007$; $+10$ mV, $p = 0.7$; $+30$ mV, $p = 0.25$; $+50$ mV, $p = 0.12$; MW test). *E*, Examination of input/output characteristics of evoked EPSCs at WT versus HET cortical–BLA synapses. Top, Evoked EPSC traces from cortical–BLA synapse in WT; representative EPSCs at 10% versus 90% of maximal stimulation. Calibration: 50 pA, 200 ms. Bottom, Pooled data of responses to 10% (left) versus 90% (right) stimulation intensity in WT ($n = 10$). *F*, Evoked EPSCs recorded in *Nrg1* HET BLA ($n = 8$) at 10% and 90% stimulation intensity. There are no statistically significant differences between WT and *Nrg1* HET at either the lower (WT, $n = 10$; HET, $n = 8$; $p = 0.89$, MW test) or higher stimulation intensities ($p = 0.56$, MW test), although a trend toward somewhat larger evoked EPSCs at higher stimulation intensities in the type III *Nrg1* HET is noted. Calibration: 50 pA, 200 ms.

Figure 3. High-frequency burst stimulation reliably elicits LTP in cortical–BLA circuits of WT mice. **A**, Evoked EPSCs during control conditions (0.1 Hz, minimum stimulation conditions ~50% failures, EPSC amplitude ~10–15% maximum response amplitude), and at 5 min and at 30 min after HFS burst (100 Hz, 1 s, 5 times with 10 s interval). Five superimposed traces are shown for each condition. The 10 μ M bicuculline was included throughout. Calibration: 10 pA, 50 ms. **B**, Plot of evoked EPSC amplitude before and after HFS from a representative recording. In this example, both the number of stimuli that evoked successful EPSCs was increased and the increased amplitude of the EPSCs lasted for $\geq$ 30 min. **C1**, The amplitude histogram distribution of control responses from a representative recording. **C2**, The amplitude histogram distribution after HFS. After HFS, there are significantly fewer failures, and the amplitude distribution reveals fewer small and more large amplitude events than under the control conditions (i.e., pre-HFS; minimal stimulus conditions). **D**, Pooled data of evoked EPSC amplitudes versus time in WT (average of 3 sequential responses, including failures; 9 pairs exhibited LTP after HFS). The post-HFS amplitudes are significantly increased in WT compared with the pre-HFS control. **E**, Left, Summary data of EPSC potency for all experiments in WT mice. Evoked EPSC amplitudes (potency) of all positive (i.e., nonzero) EPSCs evoked with the minimal stimulus paradigm under control conditions versus after HFS. The overall effect of HFS was statistically significant ($n = 9$, $p = 0.02$, paired sample Wilcoxon signed rank test $*p < 0.05$). Right, Dot link plot of the changes in potency in each experiment, comparing control conditions to after HFS; 7 of 9 pairs showed an increased potency. **F**, Left, Summary data of EPSC success probabilities for all experiments in WT mice. The overall effect of HFS was statistically significant ($n = 9$, $p = 0.004$, paired sample Wilcoxon signed rank test $*p < 0.05$). Right, Dot link plot of the changes in success probability in each experiment comparing control conditions to after HFS; all 9 pairs showed an increased P_s .

because of their role in emotional memory (Jiang and Role, 2008). The plasticity of cortical glutamatergic inputs in WT and *Nrg1* HET mice was compared by direct extracellular stimulation of the incoming cortical fiber tracts within the external capsule and concomitant recording of stimulus-evoked glutamatergic EPSCs (eEPSCs) in BLA pyramidal neurons before and after patterned stimulation of the cortical tract. Assay of eEPSCs elicited by a minimal stimulation protocol (i.e., stimulation intensity adjusted such that ~50% of electrical stimuli elicited eEPSCs; see Materials and Methods) before and after a single high-frequency burst revealed robust and long-lasting potentiation of evoked glutamatergic transmission in WT mice (Fig. 3). The onset of potentiation typically began 1–3 min after HFS and persisted for

at least 30 min (Fig. 3). Representative recordings and analysis of evoked transmission before and after a high-frequency burst are shown in Figure 3A–C. Analyses of these data revealed that plasticity is manifest in both increased amplitude and an increase in the fraction of successful single evoked responses (i.e., nonfailures) following the patterned stimulation (Fig. 3B,C). A histogram of all of the evoked responses in this representative cell reveals that a single HFS burst elicits a right shift of the amplitude peaks and a reduced number of failures in response to subsequent 0.1 Hz stimulation (Fig. 3C1,C2). Most cortical–BLA synapses in the WT animals demonstrated this long-lasting potentiation of glutamatergic transmission (Fig. 3D–F; $n = 9$). HFS significantly increased the mean amplitude of eEPSCs (Fig. 3D,E; $p = 0.017$, paired sample Wilcoxon signed rank test, $n = 9$). The observed success probability of eEPSCs was also significantly increased (Fig. 3F; $p = 0.003$, paired sample Wilcoxon signed rank test, $n = 9$). In sum, the HFS LTP was associated with increased potency of evoked EPSCs (Figure 3E; 7 of 9 tested) and increased success probability (P_s , Fig. 3F; 9 of 9 tested).

In contrast to the robust and consistent effect of a single burst of HFS on long-lasting synaptic plasticity of cortical–BLA synapses in WT animals, we were able to evoke LTP at only a few cortical–BLA synapses in *Nrg1* HET mice using the same stimulation protocol ($n = 2$ of 9). The overall mean amplitude was not changed by HFS (Fig. 4D). As shown in Figure 4A, some short-term post-tetanic potentiation (PTP) was recorded after HFS of cortical BLA synapses in the *Nrg1* HET, but sustained changes in transmission were not observed in most recorded cells (Fig. 4A–C). Neither the potency (Fig. 4E; nonzero events, $p = 0.42$, paired sample Wilcoxon signed rank test, $n = 9$) nor the success rate of eEPSCs was significantly affected by HFS burst (Fig. 4F; $p = 0.49$, paired sample Wilcoxon signed rank test, $n = 9$). These observations are consistent with the fact that type III *Nrg1* is essential to the induction of LTP at cortical–BLA glutamatergic synapses.

Type III *Nrg1* is capable of back-signaling (Bao et al., 2003; Hancock et al., 2008; Zhong et al., 2008; Canetta et al., 2011). Because the LTP induction is impaired in the *type III Nrg1* HET animals, we tested whether acute stimulation of back-signaling (stimulated by adding recombinant ErbB4 extracellular domain proteins, sErbB) rescued the plasticity at cortical–BLA synapses. Brain slices from *Nrg1* HET animals were incubated with 10 nM soluble ErbB4 for at least 1 h. Evoked EPSCs (evoked at ~25% of maximum response) were measured before and after HFS. Under these conditions, HFS consistently induced potentiation at WT but not *Nrg1* HET synapses (Fig. 4G; WT, $n = 6$; *Nrg1* HET, $n = 7$; $p = 0.003$, MW test). Preincubation with soluble ErbB4 restored HFS induced potentiation in 3 of 9 tested *Nrg1* HET neurons (Fig. 4G; *Nrg1* HET, $n = 7$; HET + sErbB, $n = 3$; $p = 0.04$, MW test). This rescue is most apparent when one considers the responses of individual cells (Fig. 4G, right); HFS induced potentiation in all WT neurons tested (6 of 6), 14% of *Nrg1* HET neurons (1 of 7), and 33% of *Nrg1* HET neurons from slices pretreated with soluble ErbB4 (3 of 9). Thus, type III *Nrg1* back-signaling appears to contribute to HFS elicited LTP at these cortical–BLA glutamatergic synapses.

The observed lack of potentiation in *Nrg1* HET animals could result from a fundamental impairment on the underlying mechanisms of potentiation or reflect a change in the threshold necessary to induce potentiation. To address this question, we increased the strength of stimuli used to evoke EPSCs to 20–25% of maximal response (Tsvetkov et al., 2004). The increase in stimulus did not change the extent of potentiation in WT (Fig. 5A; 10% MAX, $n = 9$; 25% MAX, $n = 6$; $p = 0.60$, MW test) or *Nrg1*

Figure 4. High-frequency burst stimulation failed to elicit LTP in cortical–BLA circuits in *Nrg1* HET mice. **A**, Three overlapping sample traces of evoked EPSC during control conditions (pre-HFS; minimal stimulation conditions as delineated in Fig. 3 legend), at 5 min and at 30 min after a high-frequency burst in a representative BLA pyramidal neuron from *Nrg1* HET animals. HFS elicited only a brief PTP of transmission. During the brief PTP of ~5 min after HFS, there is an increase of success rate and amplitude. There is typically no effect of HFS after the brief PTP in the *Nrg1* HET animals. Calibration: 10 pA, 50 ms. **B**, Plot of evoked EPSC amplitude before and after HFS from a representative experiment in *Nrg1* HET mice. **C1**, The amplitude histogram distribution of control conditions from a representative experiment in *Nrg1* HET mice. **C2**, The amplitude histogram distribution after HFS from the same representative experiment in *Nrg1* HET mice. **D**, Pooled data of evoked EPSC amplitudes versus time (average of 3 sequential responses, including failures; 7 of 9 pairs displayed no LTP after HFS in *Nrg1* HET). The post-HFS amplitudes are not significantly increased. **E**, Left, Summary data of EPSC potency for all experiments in *Nrg1* HET mice. Evoked EPSC amplitudes (potency) of all positive (i.e., nonzero) EPSCs evoked with the minimal stimulus paradigm under control conditions versus after HFS in *Nrg1* HET mice. The overall effect of HFS was not statistically significant ($n = 9$, $p = 0.43$, paired sample Wilcoxon signed rank test). NS, Not significant. Right, Dot link plot of the changes in amplitude in individual experiments comparing the amplitude of evoked responses under control conditions with post-HFS in *Nrg1* HET mice. **F**, Left, Summary data of EPSC success probabilities for all experiments in *Nrg1* HET mice. The overall effect of HFS was not statistically significant ($n = 9$, $p = 0.50$, paired sample Wilcoxon signed rank test). NS, Not significant. Right, Dot link plot of the changes in success probability in each experiment in *Nrg1* HET mice, comparing control conditions with post-HFS. NS, Not significant. **G**, Left, The extent of potentiation is represented as the ratio of post-HFS (>30 min) to pre-HFS (control) amplitude for each experiment. Under the stimulation conditions used (pre-HFS: 20–25% of maximum response amplitude), there was a significant increase in EPSC amplitude after HFS in all WT mice tested (range, 1.35–1.65 HFS/CON ratio, 6 of 6). In contrast, the HFS/CON ratio was significantly decreased in *Nrg1* HET animals (WT, $n = 6$; *Nrg1* HET, $n = 7$; $p = 0.003$, MW test, $*p < 0.05$). Treatment of slices from *Nrg1* HET mice with soluble ErbB4 elicited significantly rescues the extent of the potentiation elicited by HFS (*Nrg1* HET, $n = 7$; *Nrg1* HET treated with soluble extracellular domain of ErbB4, $n = 3$; $p = 0.04$, MW test, $*p < 0.05$) in one-third of tested cells (3 of 9). Right, Bar plot of the percentage of total experiments in which the HFS/CON ratio > 1 under different conditions: WT, 100% (6 of 6); *Nrg1* HET, 14.3% (1 of 7); and *Nrg1* HET treated with soluble extracellular domain of ErbB4, 33.3% (3 of 9). Soluble ErbB4 significantly increased the percentage of neurons in which the HFS/CON ratio was increased compared with *Nrg1* HET controls.

Figure 5. HFS-induced synaptic plasticity is independent of stimulus strength in both WT and *Nrg1* HET mice. Comparison of WT (**A**) and *Nrg1* HET (**B**) mice before and after HFS burst stimulation when the baseline stimulation was adjusted to elicit ~10% of the maximal response amplitude or ~25% of the maximal response amplitude. The extent of potentiation (or lack thereof in the case of the *Nrg1* HET) was not significantly different for the two stimulation paradigms tested. **A**, WT LTP was reliably elicited in both conditions. There is no significant difference in the potentiation ratio (10% MAX, $n = 9$; 25% MAX, $n = 6$; $p = 0.60$, MW test). NS, Not significant. **B**, *Nrg1* HET. There is no significant difference in the potentiation ratio (10% MAX, $n = 9$; 25% MAX, $n = 6$; $p = 0.38$, MW test). NS, Not significant.

HET animals (Fig. 5B; 10% MAX, $n = 9$; 25% MAX, $n = 6$; $p = 0.38$, MW test).

Type III *Nrg1* is required for nAChR-induced facilitation of glutamatergic transmission in BLA

We examined the expression of type III *Nrg1* and $\alpha 7^*$ nAChRs in the cingulate cortex and the amygdala. Immunostaining with a type III *Nrg1*-specific antibody (Yang et al., 1998; Chen et al., 2010a, 2010b; Hancock et al., 2011) revealed expression in cortical pyramidal neurons (Fig. 6A). Costaining with α -BgTx, which specifically binds to $\alpha 7^*$ nAChRs in the CNS, demonstrated co-expression of $\alpha 7^*$ nAChRs in deep layer cortical neurons (Fig. 6A). To determine whether cortical neurons that express $\alpha 7^*$ nAChRs project to the BLA, we injected the cingulate cortex with the anterograde tracer BDA (see Materials and Methods) and, after 5 d, stained sections of the external capsule and the BLA with α -BgTx. Figure 6B shows labeling of BDA⁺ cortical projections in both the EC and the BLA with α -BgTx, confirming that cortical neurons that innervate BLA express $\alpha 7^*$ nAChRs along their axons.

As type III *Nrg1* has been implicated in the regulation of both the expression and targeting of nAChRs in other CNS and PNS neurons, we tested whether heterozygous deletion of type III *Nrg1* altered nicotine-induced plasticity of glutamatergic transmission at synapses in the BLA (Jiang and Role, 2008). In our previous study, we have found that a brief exposure to nicotine (2 min, 500 nM) facilitated glutamatergic transmission to pyramidal neurons in the BLA of WT mice. In the presence of TTX (1 μ M) to monitor miniature synaptic currents, nicotine elicited a robust increase in the frequency of glutamatergic miniature EPSCs in ~50% of neurons tested in WT mice ($n = 17$). The effects of nicotine were reflected in a rapid (within seconds) and significant increase in mEPSC frequency (Fig. 6C,E,I; WT control vs WT + nicotine, $p < 0.001$, paired sample Wilcoxon signed rank test, $n = 17$), without a detectable effect on mEPSC amplitude (Fig. 6C,G,J; WT control vs WT nicotine, $p = 0.16$, paired sample Wilcoxon signed rank test). Intervent intervals of mEPSCs, but not of mEPSC amplitude, were significantly different in control versus nicotine conditions in the WT (Fig. 6E; $p = 0.003$, KS test, $n = 17$; Fig. 6G; $p = 0.97$, KS test, $n = 17$). Together, these data are consistent with the fact that a presynaptic mechanism contributes to the nicotine-induced facilitation of

Figure 6. Nicotine elicits a robust facilitation of glutamatergic transmission in WT but not in *Nrg1* HET mice. **A**, Representative confocal fluorescence image from cingulate cortex layer 5 pyramidal neurons after staining with an antibody to type III *Nrg1* (red) and biotinylated α -BgTx (green). Pyramidal neuron cell bodies in the cingulate cortex express both type III *Nrg1* and the BgTx target, consistent with their expression of $\alpha 7$ -containing nicotinic acetylcholine receptors ($\alpha 7^*$ nAChR). **B**, Left, Schematic diagrams (adapted from Paxinos and Franklin, 2004), illustrating the injection of BDA into layer 5 of cingulate cortex and the location of micrographs of the labeled projections within the external capsule (EC; **B**, right top) and labeled axons within the BLA (**B**, right bottom). Fluorescent images of cingulate cortical projections within the EC and BLA, where BDA fibers are labeled in dark blue and BgTx labeled in green, show multiple areas of overlap (in cyan; white arrowheads) consistent with $\alpha 7^*$ nAChRs being localized along cingulate projections and at sites of termination within the BLA. Scale bars: **A**, 5 μ m; **B** (right), 5 μ m. **C**, Top, Sample traces of mEPSCs recorded in a representative BLA neuron in acute slice from WT mice at a holding potential (V_h) of -60 mV (1 μ M TTX,

glutamatergic transmission at cortical–BLA synapses, as previously described.

To test whether nicotinic modulation of glutamatergic transmission was also observed in *type III Nrg1* heterozygous animals, we first assessed mEPSCs in recordings from BLA pyramidal neurons in *Nrg1* HET mice ($n = 9$). As delineated above, baseline glutamatergic synaptic input to BLA pyramidal neurons in *Nrg1* HET animals was not significantly different from that observed in their WT littermates (Fig. 1C). Neither the frequency nor the amplitude of glutamatergic mEPSCs in *Nrg1* HET animals was significantly different from those recorded in WT littermates (Fig. 1D,E), although the time constant or synaptic current decay is significantly faster in *Nrg1* HET compared with WT littermate control.

In contrast to baseline synaptic activity, the nicotinic modulation of mEPSCs

10 μ M bicuculline, and 200 nM atropine). Calibration: 5 pA, 100 ms. Bottom, Sample traces of mEPSCs after brief application of nicotine (500 nM, 60 s). Calibration: 5 pA, 100 ms. **D**, Sample traces of mEPSCs recorded in a representative BLA neuron in acute slice from *Nrg1* HET mice. All conditions and scales as in **A**. **E**, Cumulative distribution of the mEPSC interevent intervals in WT mice ($n = 17$) demonstrating the facilitatory effect of acute nicotine on spontaneous glutamatergic synaptic transmission. The cumulative plot is significantly left shifted from the control distribution after brief exposure to nicotine. Such a left shift is indicative of an increased mEPSC frequency and consistent with previous reports that nicotine causes an increase in the probability of glutamate release at synapses on BLA pyramidal neurons (solid line, WT control; dotted line, WT + nicotine) (see Jiang and Role, 2008). WT control versus WT + nicotine are significantly different ($n = 17$, $p = 0.003$, KS test). **F**, Cumulative distribution of the mEPSC interevent intervals recorded in *Nrg1* HET mice ($n = 9$). The cumulative distribution of mEPSC interevent interval times was not significantly different between *Nrg1* HET and *Nrg1* HET + nicotine, indicating similar mEPSC frequency (solid line, HET control; dotted line, HET + nicotine; $n = 9$, $p = 0.40$, KS test). **G**, **H**, Cumulative distribution of mEPSC amplitude in WT and *Nrg1* HET mice: acute nicotine exposure was without effect on the amplitude of mEPSCs in both WT and *Nrg1* HET mice (**G**, solid line, WT control; dotted line, WT + nicotine; $n = 17$, $p = 0.97$, KS test; **H**, solid line, HET control; dotted line, HET + nicotine; $n = 9$, $p = 0.07$, KS test). **I**, Population data of mEPSC frequency WT versus *Nrg1* HET. Nicotine significantly increased the mEPSC frequency in WT but not *Nrg1* HET mice (WT, $n = 17$, $p < 0.0001$; HET, $n = 9$; $p = 1$; paired sample Wilcoxon signed rank test, $*p < 0.05$). Treatment of slices from *Nrg1* HET mice with soluble ErbB4 elicited significant rescue of the extent of the facilitation induced by acute nicotine in 3 of 11 cells (HET Con, $n = 9$; HET Nic + sErbB, $n = 3$; $p = 0.02$, MW test). $*p < 0.05$. **J**, Population data of mEPSC amplitudes. Nicotine had no effect on mEPSC amplitude in either WT or *Nrg1* HET BLA pyramidal neurons (WT, $n = 17$, $p = 0.16$; HET, $n = 9$, $p = 0.65$; paired sample Wilcoxon signed rank test). Treatment with soluble ErbB4 did not significantly affect the mEPSC amplitude (HET Con, $n = 9$; HET Nic + sErbB, $n = 3$; $p = 0.06$, MW test).

recorded in BLA pyramidal neurons in *Nrg1* HET mice was significantly different from WT. Unlike their WT littermates, treatment with nicotine was without effect on mEPSC frequency or amplitude at 100% of synapses tested in the BLA from *Nrg1* HET animals (Fig. 6*D,F,H*). The cumulative distribution plots of mEPSC interevent intervals (Fig. 6*F*; $p = 0.4$, KS test, $n = 9$) and amplitude (Fig. 6*H*; $p = 0.07$, KS test, $n = 9$) showed no significant difference before and after treatment with nicotine. Box plot analysis of the pooled data reveal the same lack of effect of nicotine on mEPSC frequency or amplitude in the *Nrg1* HET (Fig. 6*I*; HET control vs HET nicotine, $p = 1$, paired sample Wilcoxon signed rank test, $n = 9$; Fig. 6*J*; HET control and HET nicotine, $p = 0.65$, paired sample Wilcoxon signed rank test, $n = 9$). Our findings demonstrate that the heterozygous deletion of *type III Nrg1* eliminates nicotine-mediated modulation of mEPSCs in the BLA.

To examine whether acute stimulation of type III Nrg1 back-signaling could rescue nicotinic facilitation in the *Nrg1* HET animals, amygdala slices from *Nrg1* HET animals were treated with soluble ErbB4-ECD. ErbB4-ECD treatment rescued nicotinic facilitation of mEPSC frequency in 3 of 11 cells (Fig. 6*I*; HET Con, $n = 9$; HET Nic + sErbB4, $n = 3$; $p = 0.02$, MW test), without affecting mEPSC amplitude (Fig. 6*J*; HET Con, HET Nic + sErbB4, $p = 0.06$, MW test). These results are consistent with a key role of type III Nrg1 in regulating levels of a subset of presynaptic nAChRs, presumably the $\alpha 7^*$ nAChRs (Zhong et al., 2008).

Nicotine facilitates TBS elicited LTP of cortical–BLA synaptic transmission in WT mice

Prior studies of cortical–BLA circuits demonstrated that TBS of cortical inputs typically elicits short-term enhancement of excitatory synaptic plasticity in BLA pyramidal neurons (Jiang and Role, 2008). However, when the TBS stimulation of cortical input is combined with a single exposure to nicotine, there is a significant decrease of the threshold for activation of LTP (Fig. 7*A1–A3*) (Jiang and Role, 2008). Figure 7 summarizes findings from examination of WT littermates of *Nrg1* HET mice, documenting the short-term facilitation elicited by TBS of cortical inputs to the BLA (Fig. 7*A3*; control and TBS, $p < 0.001$, paired sample Wilcoxon signed rank test, $n = 16$). Likewise, concurrent activation of cortical inputs with TBS and application of a single dose of nicotine (500 nM) typically elicited LTP, with near-doubling of the eEPSC amplitudes for 30–60 min in WT mice and in WT littermates of *Nrg1* HETs (Jiang and Role, 2008) (Fig. 7*A3*; TBS + Nic, $p < 0.001$; 30 min after T + N, $p < 0.001$, paired sample Wilcoxon signed rank test, $n = 16$). These findings demonstrate that cholinergic signaling at WT cortical–BLA glutamatergic synapses can play a critical role in the induction and/or maintenance of LTP.

Nicotine does not induce LTP after TBS in type III Nrg1 heterozygous mice

We next tested whether the levels of expression of type III Nrg1 influenced the cholinergic modulation of TBS-induced synaptic plasticity in cortical–BLA circuits. To test this, we examined excitatory synaptic transmission at cortical–BLA synapses from *Nrg1* HET mice before and after TBS and in the presence or absence of concomitantly applied nicotine (500 nM). As in the WT littermates, most pyramidal neurons responded to TBS with a short-term change in the amplitude of eEPSCs in *Nrg1* HET animals (Fig. 7*B1–B3*; control and TBS, $p = 0.04$, paired sample Wilcoxon signed rank test, $n = 8$). However, in contrast to our

Figure 7. Nicotine lowers the threshold for TBS induced LTP of cortical–BLA synapses in WT mice, but not in mice heterozygous for either *Nrg1* or the $\alpha 7$ nAChR subunit. **A1**, Representative evoked EPSC responses to 0.1 Hz stimulation of a WT neuron during control conditions (1, control), immediately after TBS (2, TBS), 5 min after TBS (3, 5 min after TBS), immediately after concurrent administration of nicotine and TBS (4, TBS + Nic), and 30 min after TBS + nicotine (5, 30 min after T + N). Sample records shown were taken at the time points as indicated in **B** (from left to right). Calibration: 20 pA, 25 ms. **A2**, Plot of evoked EPSC amplitudes recorded from representative BLA pyramidal neuron with 0.1 Hz stimulation of cortical input (average of 3 sequential responses). Arrows indicate the time of delivery of TBS alone (first arrow) and the time of delivery of TBS + nicotine (second arrow; + Nic). The traces are labeled to indicate the 1–5 corresponding time and conditions of the recording as shown in **A**. **A3**, The averaged evoked EPSC amplitude for all WT neurons recorded ($n = 16$). TBS itself elicits brief potentiation (TBS, $p < 0.0001$, paired sample Wilcoxon signed rank test). After 5 min, the amplitudes drop back to control level (5 min after TBS, $p = 0.23$). Exposure to nicotine converts this brief potentiation to sustained potentiation induced by TBS (TBS + Nic, $p < 0.0001$; 30 min after T + N, $p < 0.0001$, paired sample Wilcoxon signed rank test). * $p < 0.05$. **B1**, Representative evoked EPSC traces from *Nrg1* HET examined with the same protocol as in **A**. Calibration: 10 pA, 25 ms. **B2**, Plot of evoked EPSC amplitudes versus time in *Nrg1* HET (average of 3 sequential responses). TBS alone (first arrow) elicits brief potentiation as in WT. In contrast, TBS + nicotine (second arrow + Nic) is without effect in *Nrg1* HET. **B3**, The averaged evoked EPSC amplitude for all *Nrg1* HET neurons recorded ($n = 8$). TBS itself elicits a brief potentiation (TBS, $p = 0.04$; 5 min after TBS, $p = 0.84$; paired sample Wilcoxon signed rank test). Nicotine is without effect when paired with TBS in *Nrg1* HET (TBS + Nic, $p = 0.15$; 30 min after T + N, $p = 0.64$, paired sample Wilcoxon signed rank test). * $p < 0.05$. **C1**, Representative evoked EPSC responses from $\alpha 7$ HET using the same experimental protocol as in **A**. Calibration: 10 pA, 20 ms. **C2**, Plot of EPSC amplitudes recorded before and after TBS $\pm$ nicotine in an $\alpha 7$ HET (average of 3 sequential responses). Nicotine is without effect on EPSCs with or without prior TBS in the $\alpha 7$ HET mice. **C3**, The averaged evoked EPSC amplitudes for all the neurons recorded in the $\alpha 7$ HET ($n = 7$). TBS does not elicit significant potentiation whether or not paired with nicotine application (TBS, $p = 0.38$, 5 min after TBS, $p = 0.22$; TBS + Nic, $p = 0.81$; 30 min after T + N, $p = 0.23$, paired sample Wilcoxon signed rank test).

Figure 8. Role of $\alpha 7^*$ nAChRs in type III Nrg1-dependent cortical–BLA synaptic plasticity. **A, B**, An $\alpha 7^*$ nAChR selective antagonist, MLA, administered at 10 nM, significantly attenuated the extent of LTP induction in WT animals. The effect of MLA was tested by comparison of the post-HFS to pre-HFS (control) amplitude ratios (**A**) and by the number of experiments in which the HFS/CON ratio was > 1 (**B**). A total of 10 nM MLA significantly reduced HFS LTP (WT, $n = 6$; WT + MLA, $n = 8$; $p = 0.01$, MW test). **C**, Confocal images of α -BgTx (red) to detect surface and total $\alpha 7^*$ nAChR expression in the BLA of WT, Nrg1 HET, $\alpha 7$ HET, and $\alpha 7$ KO mice. Scale bar, 5 μ m. Arrowheads indicate $\alpha 7^*$ nAChR on fibers. * $\alpha 7^*$ nAChR⁺ principal pyramidal-like cells within the BLA. **D**, Semiquantitative measurements of $\alpha 7$ surface expression (left) in the BLA of WT, Nrg1 HET, and $\alpha 7$ HET mice where the data were plotted as a ratio of the pixel volume of the surface expression over the pixel volume of total expression (right). Error bars represent SEM. * $p < 0.001$ (left panel of surface labeling). * $p < 0.01$ (right panel of surface/total ratio) by ANOVA followed by Bonferroni's *post hoc* test ($n = 3$ animals per genotype; 2 image fields per animal were analyzed and normalized to $\alpha 7$ KO background levels).

findings in WT mice, pairing of TBS with nicotine had no significant additional effect on either the extent or the duration of enhanced glutamatergic transmission in Nrg1 HET mice (Fig. 7B1–B3; TBS + Nic, $p = 0.15$; 10 min after T + N, $p = 0.64$, paired sample Wilcoxon signed rank test, $n = 8$). Figure 7B1, B2 shows representative tracings and time course of the effects of TBS $\pm$ nicotine; Figure 7B3 contains the population data ($n = 8$). These data demonstrate that modulation of TBS-induced synaptic plasticity by nAChR activation is deficient in type III Nrg1 heterozygous animals.

$\alpha 7$ HET mice phenocopy the type III Nrg1 HET mice with respect to the effects of TBS and its modulation by nicotine

Several prior studies demonstrate a prominent role for Nrg1 signaling in the regulation of expression and targeting of neuronal nAChRs (Hancock et al., 2008; Zhong et al., 2008). In particular, type III Nrg1 back-signaling has been shown to be linked to the regulation of targeted insertion of $\alpha 7$ -containing nAChRs ($\alpha 7^*$ nAChRs) along axons (Hancock et al., 2008). To test whether the loss of nAChR modulation of synaptic transmission in Nrg1 HETs was the result, at least in part, of decreased levels of $\alpha 7$, we repeated a subset of the plasticity studies in mice heterozygous for $\alpha 7$ deletion ($\alpha 7$ HET). In particular, as summarized in Figure 7C, TBS of glutamatergic inputs to BLA in $\alpha 7$ HETs failed to elicit long-lasting synaptic potentiation whether or not the TBS stimulation was coupled with nicotine application (Fig. 7C3; TBS, $p = 0.38$; TBS + Nic, $p = 0.81$; 10 min after T + N, $p = 0.22$, paired sample Wilcoxon signed rank test, $n = 7$ experiments, 4 mice).

Only a subset of pyramidal neurons ($n = 1$ of 7 neurons) showed even a brief increase in the amplitude of eEPSCs after TBS or combined TBS and nicotine (Fig. 7C1, C2). These data suggest that the activation of $\alpha 7$ -containing nicotinic receptors is required for the induction of LTP of glutamatergic transmission at synapses onto pyramidal neurons in the BLA.

Block of $\alpha 7$ receptor attenuates LTP induction in WT animals

To determine whether $\alpha 7^*$ nAChRs contribute to HFS induced potentiation of cortical–BLA synapses in WT mice, we repeated the HFS analysis in WT slices in the absence and presence of the $\alpha 7$ -selective nAChR antagonist, methyllycaconitine (MLA). Consistent with the results presented above, HFS elicited reliable LTP in WT neurons (100%, 6 of 6 cells). In contrast, 10 nM of MLA attenuated LTP induction in WT animals. In the presence of MLA, HFS elicited LTP in only 25% (2 of 8) cells (Fig. 8A, B; WT, $n = 6$; WT + 10 MLA, $n = 8$; $p = 0.01$, MW test). These findings support a crucial role for $\alpha 7^*$ nAChRs in mediating LTP at significant subset of cortical–BLA synapses.

As noted above, we have previously demonstrated that presynaptic targeting of $\alpha 7^*$ nAChRs is regulated by type III Nrg1 back-signaling (Hancock et al., 2008; Zhong et al., 2008), that cortical pyramidal neurons that innervate the BLA express both type III Nrg1 and $\alpha 7^*$ nAChRs (Fig. 6A), and that acute stimulation of type III Nrg1 back-signaling rescues HFS induced LTP at a subset of synapses in type III Nrg1 HET animals (Fig. 4G). These findings raise the possibility that one critical target of Nrg1 signaling that is affected in the Nrg1 HET animals is presynaptic $\alpha 7^*$ nAChRs. To examine this in more detail, we stained sections of BLA with α -BgTx both before (to label surface receptors) and after permeabilization (for labeling of surface and intracellular $\alpha 7^*$ nAChRs; Fig. 8C). Both surface and total α -BgTx-stained fibers were visible in WT BLA. No staining of slices from $\alpha 7$ knock-out animals was seen under either condition. Surface $\alpha 7^*$ nAChR and the surface/total ratio of $\alpha 7^*$ nAChR were reduced in type III Nrg1 HET (by $\sim 70\%$ and 60% , respectively; total $\alpha 7^*$ nAChR levels were decreased slightly in the Nrg1 HET). Both surface and total levels of $\alpha 7^*$ nAChR were reduced in the $\alpha 7$ HET BLA (by $\sim 80\%$ and 70% , respectively) so that the surface/total ratio of α -BgTx was not significantly changed (Fig. 8C, D; WT, $n = 6$; Nrg1 HET, $n = 6$; $p < 0.01$; $\alpha 7$ HET, $n = 3$; $p = 0.11$). These results are consistent with the conclusions that targeting of $\alpha 7^*$ nAChRs to presynaptic sites is regulated by type III Nrg1 and that the presynaptic $\alpha 7^*$ nAChRs are a crucial contributor to plasticity of cortical–BLA synapses.

Discussion

The major findings of this study are that mice with only one functional allele of the type III Nrg1 gene lack multiple aspects of excitatory synaptic plasticity in cortical–BLA circuits. Specifically, in type III Nrg1 heterozygotes, cortical–BLA synapses failed

to potentiate in response to either HFS (Fig. 3 vs Fig. 4) or TBS in combination with brief exposure to low concentrations of nicotine, conditions that reliably induce sustained potentiation of synaptic transmission in WT littermates (Fig. 7) (Jiang and Role, 2008). This latter result was phenocopied in $\alpha 7^*$ nAChR heterozygotes (Fig. 7). In addition to these dramatic changes in plasticity, more subtle differences between *type III Nrg1* heterozygotes and WT mice were seen in terms of baseline synaptic transmission. Most notably, we found a decrease in NMDA/AMPA ratio, largely driven by increased AMPA receptor-mediated synaptic current amplitude (Fig. 2), an accelerated rate of decay of mEPSCs (Fig. 1), and a failure of nicotine to increase mEPSC frequency (Fig. 6).

These studies add the cortical–BLA synapse to a growing roster of circuits in which the strength of Nrg1/ErbB4 signaling plays a critical role in modulating activity-dependent plasticity (Bao et al., 2003, 2004; Li et al., 2007; Buonanno et al., 2008; Neddens et al., 2009; Chen et al., 2010b; Shamir et al., 2012). However, the current study identifies a unique role for type III Nrg1 in circuit modulation. In general, acute stimulation of ErbB signaling in the hippocampus inhibits or reverses LTP, a result that is supported by genetic deletion of *ErbB4*. Genetic disruption of *Nrg1* (affecting all isoforms) creates a more complex picture: Bjarnadottir et al. (2007) reported deficits in TBS, but not tetanus-induced hippocampal LTP in *Nrg1* heterozygous animals, and increased paired pulse facilitation, differences that were reversed by low, but not high, concentrations of exogenous Nrg1 peptide. In the BLA, we examined the ability of either a HFS or a θ burst stimulus to elicit LTP. The HFS paradigm probes the effects of a single high-frequency burst of activity on subsequent synaptic responses; θ burst patterned stimulation and its modulation by nicotine assess more fine-tuned deficits in synaptic plasticity and the possible role of changes in presynaptic modulation. We found that *type III Nrg1* heterozygotes fail to develop LTP in response to either tetanus or a θ burst + nicotine protocol, raising the likelihood that reducing type III Nrg1 affects the modulation of these circuits by a different mechanism than proposed for the effects of Nrg1–ErbB4 signaling in cortex and hippocampus (Pitcher et al., 2008; Chen et al., 2010b).

Our results clearly demonstrate that normal levels of type III Nrg1 are required for plasticity at cortical–amygdala synapses. Acute stimulation of type III Nrg1 back-signaling restored plasticity at a subset of these synapses in *type III Nrg1* HET BLA (Fig. 4G). Based on these rescue experiments, we conclude that ongoing type III Nrg1 signaling in the BLA is essential for potentiation of cortical–BLA synapses. It is important to note that type III Nrg1 is expressed both in the cortical neurons that project to the BLA (Figs. 1A and Fig. 6A), and in a subset of BLA neurons (our unpublished observations). As a result, lack of type III Nrg1 back-signaling in either presynaptic inputs, postsynaptic BLA neurons, or both, could contribute to the changes in cortical–BLA synaptic transmission observed in the *type III Nrg1* HETs.

Cortical amygdala synapses in $\alpha 7^*$ nAChR heterozygotes fail to potentiate in response to combined θ burst and nicotine stimulation (Fig. 7); the $\alpha 7^*$ nAChR selective antagonist MLA blocks potentiation at these synapses (Fig. 8), and there is a substantial reduction in surface $\alpha 7^*$ nAChRs in the BLA of *type III Nrg1* heterozygotes (Fig. 8). Type III Nrg1 regulates the level of expression (Yang et al., 1998; Mathew et al., 2007) and the presynaptic targeting (Hancock et al., 2008; Zhong et al., 2008) of $\alpha 7^*$ nAChRs. As such, decreased expression and/or altered loss of sufficient functional presynaptic $\alpha 7^*$ nAChRs might be one factor

accounting for the failure of nicotine to either increase spontaneous activity or to effectively synergize with patterned stimulation in the BLA. Given the similarities in nicotine responsiveness of cortical–BLA synapses and the surface $\alpha 7^*$ nAChR levels between the *type III Nrg1* HETs and the $\alpha 7$ nAChR HETs (Figs. 7, 8), as well as the requirement for $\alpha 7^*$ nAChR for cortical–BLA plasticity (Fig. 8A,B), we propose that type III Nrg1 back-signaling is required for presynaptic targeting of $\alpha 7^*$ nAChRs to cortical–BLA synapses.

Acute stimulation of type III Nrg1 back-signaling in BLA slices partially rescued cortical–BLA synaptic plasticity and nicotine responsiveness at a subset of synapses tested. At present, we do not know why the rest of the synapses tested did not detectably respond to soluble ErbB4 treatment. One possibility is that type III Nrg1 back-signaling from distal projections back to the cortical cell soma (which are not present in the acute slice preparation) is also required at these synapses (Bao et al., 2003, 2004). Maintenance of WT levels of presynaptic $\alpha 7^*$ nAChRs has been shown to involve multiple mechanisms of downstream type III Nrg1 back-signaling; back-signaling through type III Nrg1 can elicit a local recruitment of preexisting internal $\alpha 7^*$ nAChRs to the cell surface and/or a protein synthesis-dependent targeting of α^* nAChRs to axons (Hancock et al., 2008; Zhong et al., 2008). Failure to rescue plasticity and nicotine responses at some cortical–BLA synapses might indicate a requirement for the latter mechanism. A second possibility is that there is insufficient presynaptic type III Nrg1 at the cortical–BLA synapses to support a full response to acute soluble ErbB4 stimulation (as in Canetta et al., 2011). The amount of type III Nrg1 protein within layer 5 cortical neurons appears to be reduced substantially in the *Nrg1* HET animals (Fig. 1A), consistent with a reduced efficacy of back-signaling in these cortical projections. A third possibility is that lack of type III Nrg1—ErbB4 forward signaling also contributes to the deficit in cortical–BLA synaptic plasticity in the *type III Nrg1* heterozygotes. ErbB4 is expressed on a small population of GABAergic interneurons within the BLA (Shamir et al., 2012), a population of neurons that plays an important role in the overall activity within the BLA (Ehrlich et al., 2009). Preliminary analyses of cortical–amygdala synaptic plasticity in ErbB4 knock-out animals reveal a phenotype intermediate between WT and the *type III Nrg1* heterozygotes.

Although much of our focus in this study is on the presynaptic role of type III Nrg1 and $\alpha 7^*$ nAChRs at cortical–BLA synapses, we also observed alterations of several characteristic of the postsynaptic BLA neurons, including altered mEPSC decay kinetics and altered NMDA/AMPA ratios. At present, we do not know whether these postsynaptic changes result from altered type III Nrg1 back-signaling within the BLA pyramidal neurons (a significant fraction of which express type III Nrg1), or are secondary to either alterations in cortical inputs or alterations in ErbB4-expressing GABAergic interneurons.

The possible functional interaction between type III Nrg1 and $\alpha 7^*$ nAChR is especially intriguing given the significant evidence from genetic studies implicating both *NRG1* and *CHRNA7* as schizophrenia susceptibility genes (Freedman et al., 2001; Stefansson et al., 2002; Harrison and Weinberger, 2005; Leonard and Freedman, 2006). Activation of $\alpha 7^*$ nAChRs by acetylcholine might be an important regulator of cortical–BLA and other cortical–limbic circuits that are affected in schizophrenia patients.

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