Neuregulin-1 regulates LTP at CA1 hippocampal synapses through activation of dopamine D4 receptors

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Neuregulin-1 (NRG-1) is genetically linked with schizophrenia, a neurodevelopmental cognitive disorder characterized by imbalances in glutamatergic and dopaminergic function. NRG-1 regulates numerous neurodevelopmental processes and, in the adult, suppresses or reverses long-term potentiation (LTP) at hippocampal glutamatergic synapses. Here we show that NRG-1 stimulates dopamine release in the hippocampus and reverses early-phase LTP via activation of D4 dopamine receptors (D4R). NRG-1 fails to depotentiate LTP in hippocampal slices treated with the antipsychotic clozapine and other more selective D4R antagonists. Moreover, LTP is not depotentiated in D4R null mice by either NRG-1 or theta-pulse stimuli. Conversely, direct D4R activation mimics NRG-1 and reduces AMPA receptor currents and surface expression. These findings demonstrate that NRG-1 mediates its unique role in counteracting LTP via dopamine signaling and opens future directions to study new aspects of NRG function. The novel functional link between NRG-1, dopamine, and glutamate has important implications for understanding how imbalances in Neuregulin-ErbB signaling can impinge on dopaminergic and glutamatergic function, neurotransmitter pathways associated with schizophrenia.

depotentiation | ErbB receptor | plasticity | schizophrenia | clozapine

The trophic and differentiation factor NRG-1 and its receptors (ErbB2–4) are expressed in the developing nervous system and adult brain, including the hippocampus. NRG-1 is translated as a transmembrane protein and released in an activity-dependent manner (1). Initially, long-term NRG-1 signaling was shown to regulate neuronal expression of neurotransmitter receptor genes for glutamate, acetylcholine, and GABA (2–5). More recently, the tight association of the NRG-1 receptor ErbB4 with glutamate receptors at postsynaptic densities suggested that NRG-1 signaling could regulate synaptic function in a more acute fashion (6, 7). Consistent with this idea, we and others have shown that NRG-1β rapidly regulates glutamatergic (7–11) and cholinergic (12) synaptic function in the hippocampus and prefrontal cortex (PFC).

Long term potentiation (LTP) and long term depression (LTD) at Schaeffer collateral-to-CA1 hippocampal synapses (SC-CA1) are believed to underlie complex cognitive processes such as learning and memory. At this synapse, postsynaptic NMDAR activation and increases in AMPAR excitatory postsynaptic currents (EPSCs) are necessary for LTP induction and expression, respectively. An additional mechanism that contributes to synaptic homeostasis at adult glutamatergic synapses is depotentiation (13, 14). In acute hippocampal slices and in freely moving animals, LTP is reversed (depotentiated) by brief, subthreshold theta pulse stimulation (TPS) if delivered during a labile period shortly after LTP induction (14). In the amygdala, depotentiation correlates with fear extinction and requires AMPAR internalization (15). We recently reported that NRG-1β depotentiates early-phase LTP at hippocampal SC-CA1 synapses, in a time-dependent fashion ($\leq$ 30min following induction), by reverting potentiated AMPA receptor EPSCs back to pre-LTP levels. Moreover, ErbB receptor inhibition blocked both NRG-1β and TPS-mediated depotentiation, indicating that NRG-1/ErbB signaling can modulate homeostasis at glutamatergic synapses (8).

Dopamine (DA) is an important modulator of LTP and LTD at glutamatergic synapses throughout the brain (see 16). Its function has been investigated mostly in the striatum and PFC, areas heavily innervated by dopaminergic fibers. Most studies in the hippocampus (16, 17), which receives diffuse inputs from the ventral tegmental area (VTA), have focused on the role of D1-type DA receptors (D1R and D5R). These receptors are positively coupled to adenylate cyclase and are required for the stabilization of late-phase LTP *in vitro* (18, 19) and *in vivo* (20). However, the role of D2-type receptors (D2R-D4R), the major pharmacological target of antipsychotics, in hippocampal synaptic plasticity remains largely unknown (16).

Mice genetically altered for NRG-1, ErbB4, and NMDAR subunits share certain behavioral abnormalities, such as hyperactivity and impaired sensory inhibition (21, 22). Interestingly, the antipsychotic clozapine reverses or ameliorates these behaviors, suggesting an involvement of DA signaling. These observations, combined with our prior work on the effects of NRG-1β on glutamatergic function, prompted us to investigate a possible functional link between NRG-1β/ErbB signaling, dopaminergic transmission, and regulation of early-phase LTP at SC-CA1 glutamatergic synapses. Here we demonstrate a novel functional link between NRG-1, glutamate, and DA signaling. Given the genetic association of *NRG-1* (21), and its receptor *ErbB4* (23), with schizophrenia, and the clinical pharmacological studies reporting imbalances in glutamatergic and dopaminergic neurotransmission in the disorder, our studies suggest mechanisms by which NRG-1 may contribute to the etiology and pathophysiology underlying schizophrenia.

Results

Neuregulin-1 Elicits a Prolonged Release of DA in the Dorsal Hippocampus. To investigate whether NRG-1 signaling acutely regulates DA levels, we used reverse microdialysis to deliver NRG-1β and simultaneously collected dialysates to measure extracellular DA. Rats were used to ensure proper microdialysis probe placement within the dorsal CA1, and to measure low DA levels with

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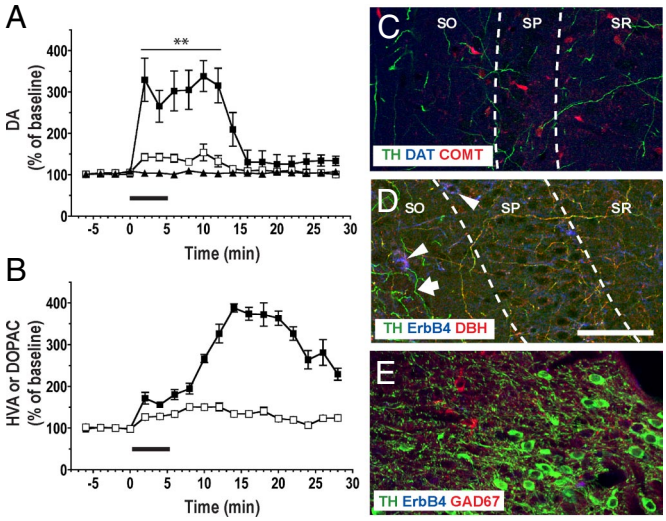

Fig. 1. NRG-1β elicits a rapid and pronounced increase of extracellular DA. Levels of DA and its catabolites were measured by microdialysis (*A* and *B*), and biomarkers of DA metabolism were analyzed by triple immunofluorescence (*C–E*). (*A*) DA levels were measured before, during, and after a 5-min infusion of 1 nM NRG-1β (■). In separate cohorts, PD158780 (10 μM) was continuously applied starting 10 min prior to NRG-1β infusion (□). Controls received ACSF vehicle (▲). (*B*) Time course of extracellular HVA (■) and DOPAC (□) accumulation after NRG-1β infusion. Time=0 indicates the onset of NRG-1β infusion (black bars). Dialysates were collected every 2 min before and after infusion of drugs; basal levels represent the mean of four time points prior to treatment (set at 100%). Points are mean ± SEM (n = 5 per group). **, $P < 0.01$ (2-way ANOVA). (*C*) Triple-immunofluorescence showing expression of COMT (red) and TH (green), but not DAT (blue), in the CA1 region (dashed lines demark pyramidal layer). (*D*) Dopaminergic fibers, defined as TH+ (green, arrow) and DBH−, were identified in *stratum oriens* (SO) of CA1. Additionally, fibers containing norepinephrine, defined as TH+/DBH+ (yellow), were observed also in *strata pyramidale* (SP) and *radiatum* (SR). Some ErbB4+ cells (blue, arrowhead) were in the vicinity of TH+ fibers. (*E*) ErbB4 immunoreactivity (blue) was not detected on either TH+ (green) or GAD67+ (red) neurons in the VTA. [Scale bar: 100 μm (*C* and *D*); 135 μm (*E*).]

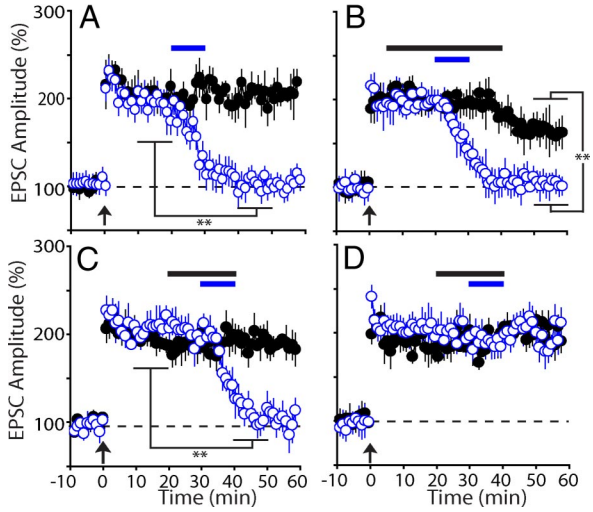

Fig. 2. NRG-1β-induced LTP depotentiation is not dependent on D1/D5Rs and is selectively blocked by D4R antagonists. Depotentiation of LTP by 1 nM NRG-1β (*A*) and effects of selective D1-type (*B*) and D2-type (*C* and *D*) receptor antagonists on NRG-1β-induced depotentiation. The antagonists were perfused onto slices alone (horizontal black bars) or with 1 nM NRG-1β (horizontal blue bars); the bars indicate onset and duration of application. (*A*) LTP was depotentiated rapidly by a 10 min perfusion with 1 nM NRG-1β (open circles), while it was stable for the 1 h recording period in vehicle-treated slices (filled circles). (*B*) The D1/D5R antagonist SCH39166 (100 nM) caused a modest rundown of LTP when applied alone (filled circles), but it did not occlude NRG-1β-mediated depotentiation (open circles). (*C*) The D2/D3R antagonist sulpiride (0.5 μM) did not block NRG-1β-dependent LTP depotentiation (open circles) nor affected LTP expression when applied alone (filled circles). (*D*) The selective D4R antagonist L-745,870 (50 nM) fully inhibited NRG-1β depotentiation (open circles) without affecting LTP expression when applied alone (filled circles). Evoked EPSC were recorded at Vh = −70 mV starting 10 min before LTP induction (baseline), and plotted as mean ± SEM. EPSC amplitudes were normalized to baseline. n = 10 slices for each group. **, $P < 0.01$ (2-way ANOVA).

high temporal resolution [2-min intervals; see supporting information (SI) *Materials and Methods*]. As shown in Fig. 1*A*, 1 nM NRG-1β caused a rapid and dramatic accumulation of extracellular DA within 2 min (340% of baseline), and DA remained at approximately 300% (mean = 309.5 ± 17.1%) for the first 12 min before dropping to approximately 130% (mean = 130.3 ± 17.9%). Pretreatment with the ErbB inhibitor PD158780 for 10 min before NRG-1β infusion dramatically reduced DA release (NRG-1β: 309.5 ± 17.1% vs. NRG-1β+PD158780: 140.4 ± 4.7%, $P < 0.01$, 2-way ANOVA; Fig. 1*A*).

We next analyzed enzymes involved in the clearance of DA from the dorsal hippocampus. Triple-immunofluorescence revealed widespread tyrosine hydroxylase (TH) and catechol-*o*-methyltransferase (COMT) immunoreactivity throughout the dorsal hippocampus, while the dopamine transporter (DAT) was undetectable (Fig. 1*C*; Fig. S1), suggesting DA clearance is mostly achieved by enzymatic degradation and not by re-uptake. Absence of DAT immunoreactivity in hippocampus is specific because the transporter was easily detectable in the amygdala and frontal cortex where expression was low (Fig. S2); similar results were obtained in the mouse (data not shown). Next, we measured by microdialysis homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), the principal DA catabolites (Fig. 1*B*). Extracellular HVA levels rose to 375% of baseline by 14 min after the onset of NRG-1β infusion whereas DOPAC exhibited a minor increase. The offset in time-to-peak between DA and HVA (2 vs. 14 min) is consistent with HVA representing the breakdown product of DA released in response to NRG-1β application. The absence of DAT immunoreactivity and the sharp increase in HVA indicates a major role of COMT in regulating DA turnover and suggests that NRG-1β increases DA release.

The hippocampus receives DA projections mostly from the VTA (24). We used triple immunofluorescence to determine if dopaminergic neurons or their projections to the dorsal hippocampus express ErbB4. As shown in Fig. 1*D*, ErbB4 immunoreactivity was undetectable on dopaminergic fibers (TH^+/DBH^-) in the hippocampus. Moreover, ErbB4 was not detected on either $GAD67^+$ or TH^+ neurons in the VTA (Fig. 1*E*; Fig.S1), consistent with a lack of ErbB4 mRNA expression in the VTA (25). These data suggest that NRG-1β induces DA release in the dorsal hippocampus indirectly by acting either on a local hippocampal circuit or via a polysynaptic loop that includes the VTA (see *Discussion*).

D4R Antagonists Selectively Block NRG-1β Mediated Depotentiation. We investigated the possible role of D1-type and D2-type receptors in mediating the effects of NRG-1β on plasticity at SC-CA1 glutamatergic synapses. As before (8), LTP depotentiation (reversal) was used as our assay because it is a robust and reproducible phenomenon that, unlike LTP induction, is internally controlled by the ability of the slice to produce LTP in the first place. Consistent with our prior studies using field recordings to measure LTP induced by theta burst stimuli (8), 1 nM NRG-1β acutely depotentiated LTP induced by pairing (Fig. 2*A*). By 20–30 min after NRG-1β perfusion, EPSC amplitudes were indistinguishable from pre-LTP levels (pre-LTP: 99.7 ± 11.5% vs. NRG-1β: 100.3 ± 13.1%; $P > 0.05$). NRG-1β concentrations between 0.1–10 nM had

similar effects on depotentiation (Fig. S3), consistent with the work of others (9, 26).

We began by studying the possible role of D1/D5 receptors in NRG-1β-induced depotentiation, since one study suggested their involvement in LTP reversal (27). As reported, we found that the D1/D5R antagonist SCH39166 (100 nM) by itself caused a late and modest rundown of LTP (Fig. 2*B*). However, its effects were temporally different and did not occlude NRG-1β mediated depotentiation, suggesting that NRG-1β functions through a different mechanism (see *Discussion*). Next, we investigated the role of D2-type receptors in mediating the effects of NRG-1β. Semiquantitative real time RT-PCR analyses revealed that all three D2-type receptors (D2R, D3R, and D4R) are expressed in the dorsal hippocampus, including the CA1/CA2 region where our recordings were performed (Fig. S4). Pretreatment of slices with sulpiride (0.5 μM), a D2/D3R antagonist with 100-fold lower affinity for D4R ($K_i > 1$ μM), had no effect on either LTP or NRG-1β-mediated LTP depotentiation (Fig. 2*C*). In stark contrast, pretreatment with L-745,870 (50 nM), a potent and selective D4R inhibitor with low affinities for D2/D3Rs (K_i >1000 nM), completely blocked NRG-1β-mediated LTP reversal (Fig. 2*D*). Potentiated EPSCs before NRG-1β perfusion were 205.5 $\pm$ 16.5% compared with 203.6 $\pm$ 14.8% following treatment ($P > 0.05$). L-745,870 by itself had no effect on LTP expression (Fig. 2*D*). A role of D4Rs in regulating NRG-1β depotentiation was further supported by experiments using clozapine (0.5 μM) and L-741,741 (0.1 μM), two other structurally independent inhibitors (Fig. S5). Consistent with previous studies, none of the above antagonists modified basal EPSC amplitudes (measured at $V_h = -70$mV) or resting potential at the concentrations used.

D4R Mutant Mice Lack NRG-1β- and TPS-Mediated LTP Depotentation. As a complementary approach to determine the requirement for D4R signaling, we investigated LTP depotentiation by NRG-1β and TPS in hippocampal slices from D4R knockout mice (28). As shown in Fig. 3*A*, LTP could be induced at SC-CA1 synapses from D4R null mice and their controls. However, consistent with our pharmacological data using D4R antagonists, 1 nM NRG-1β failed to depotentiate LTP in acute slices from D4R null mice but worked effectively in slices from matching wild-type controls.

TPS reverses LTP at SC-CA1 synapses in an activity-, time-, and ErbB receptor-dependent manner (8, 13, 14), indicating that endogenous NRG-ErbB signaling plays an important role *in vivo* to maintain homeostasis at glutamatergic synapses. We therefore investigated a potential role of D4Rs in TPS-induced depotentiation. Perfusion of wild-type slices with L-745,870 following LTP induction blocked TPS-mediated LTP reversal (Fig. 3*B*). Moreover, TPS were completely ineffective in slices from D4R knockout mice, while they effectively reversed LTP in interleaved experiments using wild-type slices (Fig. 3*C*). Taken together, our results indicate that both ErbB and D4 receptor functions are critical for the depotentiation of LTP by TPS.

D4R Activation Mimics NRG-1β-Mediated LTP Depotentiation. Based on the above results, we asked if the selective activation of D4Rs is sufficient to depotentiate LTP. We found that PD168077 (200 nM), a widely used D4R-selective agonist with low affinities for D2 and D3 receptors, effectively depotentiated LTP (Fig. 4*A*). By 30 min after the onset of PD168077 perfusion, EPSC amplitudes essentially returned to baseline levels (pre-LTP: 101.7 $\pm$ 8.2% vs. PD168077: 105.7 $\pm$ 6.2%; $P > 0.05$). This effect was completely blocked by L-745,870, confirming that the effect is mediated via D4Rs (Fig. 4*B*). In contrast, the selective D2/D3R agonist 7-Hydroxy-DPAT did not depotentiate LTP (Fig. 4*C*). Therefore, activation of D4Rs is sufficient to mimic NRG-1β effects on LTP at SC-CA1 glutamatergic synapses.

We next investigated if LTP reversal by D4R activation is associated with a selective reduction of synaptic AMPAR currents,

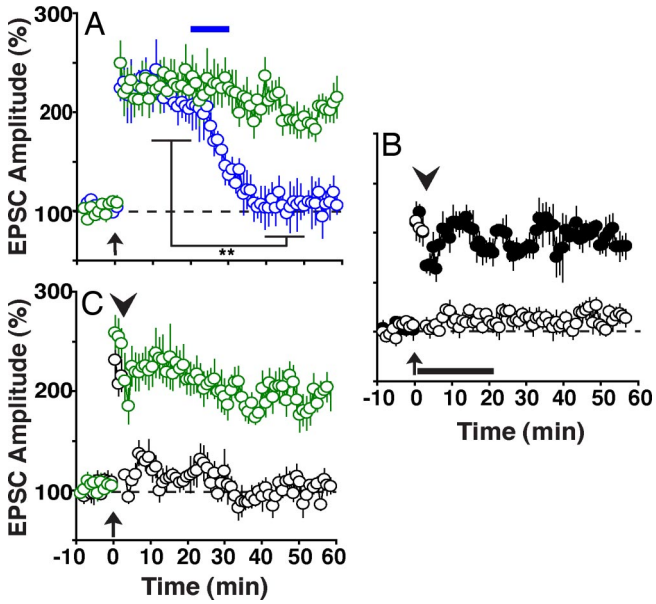

Fig. 3. D4R null mice lack NRG-1β and TPS-mediated LTP depotentation. Effects of 1 nM NRG-1β and TPS on LTP expression in acute slices from D4R mutant mice. (*A*) LTP could be induced in slices from both D4R mutant (green circles) and control WT (blue circles) mice, but 1 nM NRG-1β (blue bar) failed to depotentiate LTP in slices from D4R knockouts ($n = 7$ mice and slices per group). (*B*) TPS (downward arrowhead) depotentiated LTP in vehicle-treated slices from wild-type mice (green circles), but the D4R antagonist L-745,870 (50 nM; black bar) blocked depotentiation by TPS (filled circles; $n = 10$ slices and mice). (*C*) TPS failed to depotentiate LTP in slices from D4R null mice (green circles), whereas they effectively reversed LTP in control slices (open circles) ($n = 5$ mice and 7 slices per group). Experiments using slices from mutant and controls were interleaved, and the genotype was unknown to the experimentor. Measurements and plotting of normalized EPSC are as described in Fig. 2. **, $P < 0.01$ (2-way ANOVA).

as we previously showed for NRG-1β-mediated depotentiation (8). Like NRG-1β, PD168077 selectively reduced the AMPAR component of potentiated EPSCs without affecting NMDAR currents (Fig. 4*D*). Mean AMPAR EPSC amplitudes measured 50 min following LTP induction (corresponding to 20 min after PD168077) were not significantly different from pre-LTP values (baseline: 100 $\pm$ 2.3% vs. 50 min: 110 $\pm$ 5.0%; $P > 0.05$). No significant change in the NMDAR component, measured at +40 mV, was observed (baseline: 100 $\pm$ 5.3%; 20 min: 102.9 $\pm$ 3.1%; 50 min: 98.3 $\pm$ 2.7%; $P > 0.05$).

D4R Activation Induces the Internalization of GluR1-Containing AMPARs. There is general agreement that LTP expression at SC-CA1 synapses results, at least in part, from increased surface expression and synaptic recruitment of GluR1-containing AMPARs. Taking advantage of the absence of functional D4R in cultured hippocampal neurons (29), we investigated if activation of heterologously expressed D4R promoted the internalization of GluR1-containing AMPAR in dissociated neurons treated with glycine to induce chemical LTP (cLTP) (30). Neurons were cotransfected with expression vectors for D4R and GluR1 amino-terminally tagged with pH-sensitive (superecliptic) GFP (seGluR1) to visualize cell surface GluR1 in real time using live cell imaging after cLTP induction (8), which increases seGluR1 surface expression (Fig. S6). As shown in representative frames from live recordings (Fig. 5*A*), D4R activation by PD168077 (100 nM) significantly reduced seGluR1 fluorescence in dendritic segments and spines. Quantitative analysis revealed that PD168077 reduced seGluR1 surface levels to 57 $\pm$ 7.0% after 20 min, relative to pretreatment levels (Fig. 5*C*). The effects of PD168077 were totally blocked by

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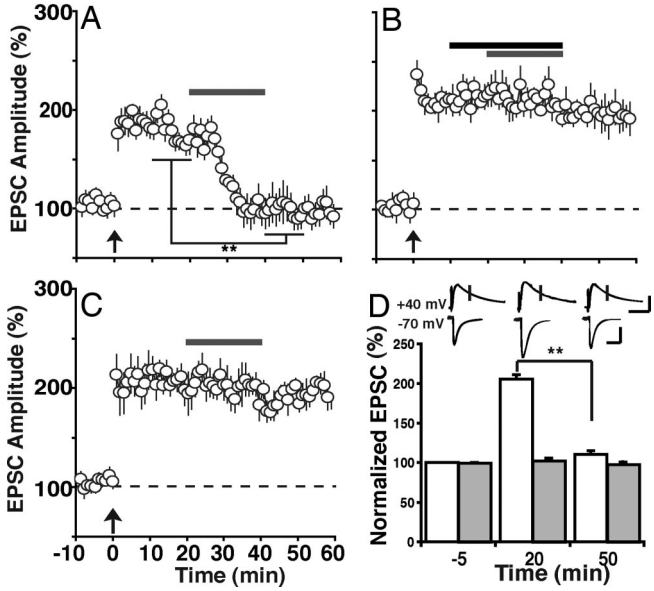

Fig. 4. Stimulation of D4R, but not D2/D3Rs, depotentiates LTP at SC-CA1 glutamatergic synapses. The effects of agonists (gray bars) for D4R (*A* and *B*) and D2/D3R (*C*) on LTP expression were analyzed as described in Fig. 2. (*A*) The D4R agonist PD168077 (200 nM) rapidly depotentiated LTP. (*B*) Depotentiation by PD168077 was blocked by the D4R antagonist L-745,870 (black bar); effects of L-745,870 alone are shown in Fig. 2. (*C*) The D2/D3R agonist 7-hydroxy-DPAT (100 nM) had no effect on LTP expression. (*D*) PD168077 selectively reduced the amplitude of AMPAR EPSCs (open bar), but not NMDAR EPSCs (gray bar), in potentiated slices. AMPAR and NMDAR EPSC amplitudes were measured −5, 20, and 50 min relative to LTP induction and recorded at holding potentials of −70 mV and +40 mV, respectively. NMDAR EPSC amplitudes were measured 50 ms following the stimulus (indicated by tick marks) as explained in *Materials and Methods*. Values represent the mean ± SEM; $n = 8-10$ slices per group; **, $P < 0.01$ (2-way ANOVA).

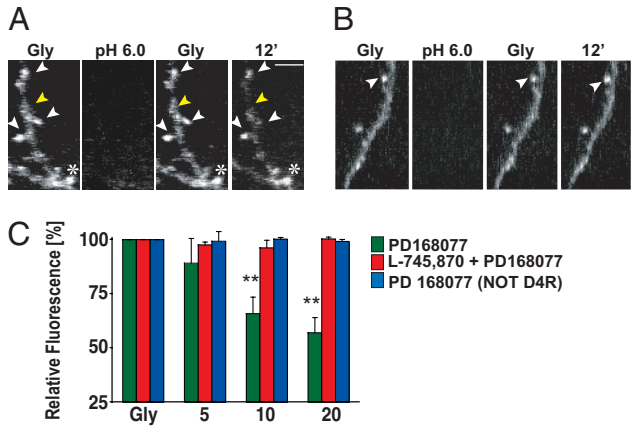

Fig. 5. Activation of D4R promotes the internalization of GluR1-containing AMPARs in dissociated hippocampal neurons. Imaging of surface seGluR1-mediated fluorescence on neuronal processes of cells treated with the D4R agonist PD168077 (100 nM) following cLTP induction. Surface fluorescence in neurons expressing seGluR1 and D4R before and after (*A*) treatment with the agonist PD168077 or (*B*) co-treatment with PD168077 and the D4R antagonist L-745,870 (50 nM). Time of onset of PD168077 perfusion is min = 0. Micrographs are representative examples of surface fluorescence after glycine-induced cLTP (*Gly*) during and after quenching of fluorescence at pH 6.0, and after 12 min of PD168077 perfusion. Spines and dendritic areas (white and yellow arrowheads, respectively) are indicated, and the asterisk marks an area of stable fluorescence confirming the focal plane remained stable during recording. (*C*) Bar graph summarizes data from neurons co-transfected with seGluR1 and D4R and treated with PD168077 (green; ROI = 36, $n = 7$ cells) or treated with PD168077 and L-745,870 (red; ROI = 19, $n = 4$ cells), and neurons transfected only with seGluR1 (not D4R) and treated with PD168077 (blue; ROI = 25, $n = 5$ cells). **, $P < 0.01$ ANOVA (1-way). (Scale bar = 4 μm.)

L-745,870 (Fig. 5 *B* and *C*). In parallel experiments using neurons transfected only with seGluR1 (no D4R) and treated with PD168077, no detectable changes in surface seGluR1 were observed (Fig. 5*C*). Moreover, PD168077 did not cause seGluR1 internalization if applied to neurons before cLTP induction (Fig. S7). Taken together, these results indicate that D4R activation is sufficient to cause internalization of cell-surface AMPARs following cLTP induction, consistent with our electrophysiological experiments showing that PD168077 reduces AMPAR EPSC amplitudes at potentiated synapses.

Discussion

We have shown here that NRG-1β regulates DA levels in the hippocampus, which in turn can reverse LTP at SC-CA1 synapses via the activation of D4Rs. Using pharmacological and genetic approaches we found that stimulation of D4Rs is necessary to depotentiate LTP in response to NRG-1β and theta-patterned stimuli, and sufficient to reverse LTP when stimulated by specific receptor agonists. Like NRG-1β, D4R activation causes a selective reduction of AMPAR currents and internalization of GluR1 receptors. These results introduce two important novel concepts: (1) they demonstrate how NRG-1 functionally links dopaminergic and glutamatergic transmission, and (2) they show that NRG-1 can attenuate LTP via D4Rs. As discussed below, our results suggest that DA has differential functions as a modulator of LTP at hippocampal SC-CA1 synapses depending on the DA receptor subtypes it engages. Moreover, the link between NRG-1, DA release and the regulation of plasticity at glutamatergic synapses by DA could have important implications since imbalances in the dopaminergic and glutamatergic neurotransmission systems have been associated with schizophrenia and other psychiatric disorders.

NRG-1β Regulates Extracellular DA Levels. We (ref. 8; this work) and others (7, 9, 10) have shown that NRG-1β exerts its effects on synaptic plasticity within minutes. By using highly sensitive detection techniques and large sampling sizes (15–18 rats/curve; see *Materials and Methods*), we have been able to measure DA with sufficient temporal resolution to demonstrate that its rapid accumulation (< 2min) following NRG-1β perfusion precedes the effects of NRG-1β on LTP depotentiation. The gradual rise of extracellular HVA following the increase in DA, taken together with the distribution of COMT and DAT in the hippocampus (Fig. 1), suggests that NRG-1β stimulates DA release. The effect of NRG-1β on DA release is not restricted to the hippocampus because it also increases extracellular DA levels in the striatum (D.P. and A.B., unpublished), and consistent with our findings, a transgenic mouse expressing a dominant-negative form of ErbB4 exhibits alterations in dopaminergic function (31).

A direct action of NRG-1β on hippocampal DA axons appears unlikely, as the cell bodies and processes of VTA dopaminergic neurons lack ErbB4 immunoreactivity and the hippocampus itself is void of DA neurons. Rather, the strongest ErbB4 immunoreactivity is detected in *GABA*ergic interneurons (7, 32), and *in situ* hybridization studies showed ErbB4 transcripts at their highest levels in GABAergic neurons in the hippocampus and forebrain (25, 33), while ErbB3 mRNA is found mostly in glia and not in hippocampal or VTA neurons (ref. 25; I. Karavanova and A.B., unpublished work). Thus, the most parsimonious explanation for the acute actions of NRG-1β on DA release is via a local hippocampal circuit involving GABAergic interneurons. Other reported effects of NRG-1β are consistent with this idea. For example, NRG-1β acutely suppresses α7-containing AChR cur-

rents and surface expression in hippocampal interneurons (12) and regulates transmitter release from GABAergic interneurons of the PFC (26).

Downstream Effects of NRG-1 on DA Signaling and Plasticity at Glutamatergic Synapses. In contrast to D1-type receptors whose role in the stabilization of LTP is well established, little is known about D2-type receptor involvement in hippocampal synaptic plasticity (16). Here we show that D4R activation has a suppressive effect on LTP. Our findings suggest that the engagement of D4Rs by NRG-1/ErbB signaling in the hippocampus could serve to counterbalance pro-LTP effects of D1/D5Rs. This role of D4R signaling is consistent with work from our group (ref. 8; this report) and others (6, 7, 9, 34) showing that activation of the NRG-1/ErbB pathway *in vitro* and *in vivo* reduces or suppresses the induction and expression of LTP at SC-CA1 synapses. Only one report suggested a facilatory role of ErbB4 signaling on LTP induction (10), an apparent inconsistency likely to result from differences in age or methodologies used (i.e., adult acute slices vs. organotypic embryonic slices). Our results on the effects of D4R stimulation on glutamatergic function in the hippocampus are also consistent with those observed at neostriatal and PFC excitatory synapses, where stimulation of D2-type receptors reduces AMPAR EPSCs and plasticity (35, 36).

Our data suggest that DA can have temporally distinct modulatory functions during different phases of LTP by engaging D1-type vs. D2-type receptors. While D1/D5R activation selectively stabilizes late-phase LTP (16–20), the suppressive effects of NRG-1β on LTP expression, which are mediated via D4R activation and are time-dependent (<30–40 min; ref. 8), function to reverse early-phase LTP. This time-dependence is consistent with the internalization of GluR1-containing AMPA receptors that are transiently recruited to synapses following potentiation (37) and differs from the D1R/D5R effects that require *de novo* translation of AMPA receptors (16, 17).

Precisely how the spatiotemporal balance of D1-type versus D2-type receptor activity is determined remains an area of intense investigation (38). The dynamics of DA accumulation, as well as the receptor subtype, subcellular localization, interaction with scaffolding proteins and coupling with intracellular signaling pathways all contribute to the cellular response to DA. Hence, the balance of D1-type versus D2-type receptor signaling in response to NRG-1β-mediated DA release, and consequently its functional outcome, could differ between brain regions (i.e., hippocampus, striatum, PFC) and depend on the local distribution of NRG isoforms, ErbB, and DA receptors. How the activities of D1- vs. D2-type receptors are balanced to regulate distinct phases of LTP at SC-CA1 synapses and the precise locus of D4R function are important questions to address in the future, but based on the exceedingly low levels of D4R expression (Fig. S2), these challenging studies will require the development of more sensitive reagents.

Implications of a Link Between NRG-1, DA, and Glutamate for Psychiatric Disorders. Schizophrenia is a complex, highly heritable and developmental disorder, and its manifestation is proposed to result from deficits in functional connectivity and synaptic plasticity. Alterations in dopamine and glutamate neurotransmission have been pharmacologically implicated in the pathophysiology of the disorder (39), and numerous schizophrenia "at risk" genes have been identified by linkage and association analyses (40, 41). Among these genes are *NRG-1* (21, 42, 43) and its receptor *ErbB4* (23). Stefansson, *et al.* focused on the possible biological role of NRG-1 in glutamate hypofunction in their original report (21), based on prior studies showing that NRG-1β regulates NMDAR expression (3), that ErbB4 and NMDARs colocalize at postsynaptic densities (6, 7), and on the observation that NRG-1 and ErbB4 hypomorphic mice (21, 44) share behavioral deficits with NMDAR NR1 hypomorphs (22). Subsequent reports supported a functional interaction between NRG-1/ErbB and glutamatergic transmission and plasticity (7–9, 11), and regulation of hippocampal gamma oscillations (45). However, there is ample evidence of reciprocal interactions between the glutamatergic and dopaminergic systems and the importance of these interactions for cognitive function (46). The observation that clozapine administration can reverse or ameliorate behavioral deficits in NRG-1 and NR1 hypomorphic mice (21, 22) suggests a possible link between NRG-1/ErbB4, glutamate, and DA signaling. Here we have presented for the first time direct evidence that supports a functional link.

Regulation of signal-to-noise ratio at glutamatergic synapses by DA has been proposed as an important mechanism influencing working memory in the PFC (47), which, as explained above, is conceivably regulated by NRG-1. Moreover, D4R polymorphisms have been genetically linked to ADHD (48), a disorder associated with perturbations in DA balance and information processing and with reduced executive functions (35). The fact that ErbB signaling can attenuate or "filter" glutamatergic transmission (11) and plasticity (7–9), acting at least in part through DA, suggests that imbalances in NRG-1/ErbB signaling could impair working memory and executive functions which are affected in schizophrenia, ADHD, and other psychiatric disorders.

Materials and Methods

Materials. NRG-1β_1 EGF-like domain peptide (amino acid 176–246) was from R&D Systems. Antibodies: COMT mAb (BD Biosciences), DAT mAb (Biogenesis), GAD67 (Chemicon), ErbB4 mAb (Thermo Scientific), TH (Novus Biologicals), and secondary Alexa488-conjugated (Molecular Probes), Cy5 and Cy3 (Jackson ImmunoResearch). Neurochemical HPLC standards were purchased from SIGMA, drugs from SIGMA and Tocris, and PD158780 from Calbiochem.

Animals. Animals were housed on a 12–12 h light-dark schedule with access to food and water ad libitum and for analysis were euthanized by CO_2 inhalation and cervical dislocation as approved by the NIH Office of Laboratory Animal Welfare.

Reverse Microdialysis. Microdialysis and neurochemical measurements were performed as described (49, 50). Guide shafts were inserted into the brains of 3–4 month old Fischer 344 male rats at coordinates AP −4.30 mm, L 4.0 mm and DV 1.0 mm with respect to Bregma, the midsagittal sinus and the brain surface (51). Experiments were performed 1 week following implantation of the guide shaft. Microdialysis tubes (effective length 2 mm) were inserted 12 h before sample collection, and ACSF perfused overnight at 0.1 μl/min; the flow rate was increased to 2 μl/min before experiments. Samples (4 μl) were collected at 2 min intervals and stabilized with 1 μl of 0.1 M HCl and 100 mM EDTA. Baseline samples were collected for at least 10 min before delivery of 1 nM NRG-1β, or NRG-1β plus 10 μM PD158780. PD158780 was perfused 10 min before NRG-1β administration to assure complete ErbB receptor blockade. After collection, rats were euthanized, brains fixed in 10% formalin, sectioned at 40 μm and stained with cresyl violet to verify probe placement. Animals with probe misplacement (i.e., outside of CA1 region) were excluded (14 out of 52 total) before measurement of DA and its metabolites. DA, HVA, and DOPAC were measured immediately after collection as described previously (49, 50), by comparing sample peak areas to external standards. Average DA baseline concentration was 0.9 ± 0.04 nM, uncorrected for the 18% recovery rate of microdialysis probes.

Immunofluorescence. Analyses were performed, essentially as described (45), using 4% paraformaldehyde-fixed horizontal 50 μm vibratome sections from adult male C57BL/6J mice. Sections were processed for triple-immunofluorescence in 0.1 M PBS, pH 7.4 in sections permeabilized with 0.25% Triton X-100 and incubated with primary antibodies for 40 h at 40°C. After extensive washes, secondary antibodies were applied for 90 min at room temperature, washed, and sections mounted in Mowiol-DABCO. Sections were analyzed in a Zeiss 510 Meta confocal microscope at 20X magnification.

Electrophysiology. Preparation of acute hippocampal slices (300 μm) and recordings from 4–6 weeks old male C57BL/6J mice (Jackson Laboratories, MA) and D4R mutants (28), backcrossed to C57BL/6J mice for >30 generations, were performed as described (8) (see *SI Materials and Methods*). Briefly, slices were continuously perfused at 2 ml/min (30°C) in a submerged recording chamber with ACSF containing (in mM) 125 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgCl_2$, and 11 glucose. Whole-cell patch clamp recordings were performed with

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glass microelectrodes (6–7 MΩ) filled with internal solution (in mM: 130 Cs-methanesulfonate, 10 Hepes, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 QX-314, 8 NaCl, 10 phosphocreatine, pH7.2 adjusted with CsOH). Schaffer collateral/commissural fibers were stimulated at 0.05 Hz (0.1 msec, 20–40 μA) using a borosilicate two-barrel stimulation silver wire electrode filled with oxygenated ACSF. Baseline EPSCs were set to 40–50% of maximum responses and recorded for at least 10 min after obtaining a stable baseline. For the induction of LTP, a general pairing protocol was used, composed of 100 pulses at 2 Hz and a holding potential of −10 to 0 mV. LTP depotentiation was elicited using theta pulse stimuli (TPS: 5 Hz/1 min) as previously described (14). AMPAR and NMDAR components of evoked EPSCs were measured in whole-cell voltage-clamp mode (Axopatch 700A amplifier, Axon Instruments, CA) using holding potentials of −70 mV and +40 mV, respectively. NMDAR EPSCs were measured 50 ms poststimulus to ensure that the AMPAR component had mostly decayed. The experimenter was blind to all pharmacological treatments, except for a final round of confirmatory experiments. Recorded data were filtered at 3 kHz, sampled at 10 kHz using pClamp, and analyzed with Clampfit (Axon Instruments, CA).

Chemical LTP and Live Imaging. Preparation of neuronal cultures, induction of cLTP and transfection of neurons (DIV 13–15) with superecliptic GFP-tagged GluR1 (seGluR1) have been described (8). Neurons were co-transfected with plasmids expressing seGluR1 and either D4R or an 'empty' CMV-driven vector using Lipofectamine 2000 (Invitrogen), and cLTP induced 48–72 h after transfection (8). After establishing a stable baseline, cultures were perfused for 12–20 min with either PD168077 (100 nM) alone, or PD168077 plus L-745,870 (50 nM); L-745,870 was continuously perfused starting from LTP induction to ensure complete D4R blockade; cLTP induction in the presence of L745,870 was normal. Images were collected and analyzed as described (8), except that images were recorded in a spinning disk scanhead (Ultraview RS; Perkin–Elmer, Wellesley, MA) with an argon-krypton laser (15 mW, 488 nm excitation line) and a cooled CCD camera (Hamamatsu C9100, Bridgewater, NJ). Images were analyzed with MetaMorph (Molecular Devices, Downingtown, PA). Regions of interest (ROI) were defined as 20 μm of neurite taken at least 10 μm from the soma. Values recorded from the first two frames after pH-neutralization were arbitrarily set as 100%.

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