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ORIGINAL ARTICLE

ErbB4 signaling in dopaminergic axonal projections increases extracellular dopamine levels and regulates spatial/working memory behaviors

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Genetic variants of Neuregulin 1 (NRG1) and its neuronal tyrosine kinase receptor ErbB4 are associated with risk for schizophrenia, a neurodevelopmental disorder characterized by excitatory/inhibitory imbalance and dopamine (DA) dysfunction. To date, most ErbB4 studies have focused on GABAergic interneurons in the hippocampus and neocortex, particularly fast-spiking parvalbumin-positive (PV+) basket cells. However, NRG has also been shown to modulate DA levels, suggesting a role for ErbB4 signaling in dopaminergic neuron function. Here we report that ErbB4 in midbrain DAergic axonal projections regulates extracellular DA levels and relevant behaviors. Mice lacking ErbB4 in tyrosine hydroxylase-positive (TH+) neurons, but not in PV+ GABAergic interneurons, exhibit different regional imbalances of basal DA levels and fail to increase DA in response to local NRG1 infusion into the dorsal hippocampus, medial prefrontal cortex and dorsal striatum measured by reverse microdialysis. Using Lund Human Mesencephalic (LUHMES) cells, we show that NRG/ErbB signaling increases extracellular DA levels, at least in part, by reducing DA transporter (DAT)-dependent uptake. Interestingly, TH-Cre;ErbB4$^{f/f}$ mice manifest deficits in learning, spatial and working memory-related behaviors, but not in numerous other behaviors altered in PV-Cre;ErbB4$^{f/f}$ mice. Importantly, microinjection of a Cre-inducible ErbB4 virus (AAV-ErbB4.DIO) into the mesencephalon of TH-Cre;ErbB4$^{f/f}$ mice, which selectively restores ErbB4 expression in DAergic neurons, rescues DA dysfunction and ameliorates behavioral deficits. Our results indicate that direct NRG/ErbB4 signaling in DAergic axonal projections modulates DA homeostasis, and that NRG/ErbB4 signaling in both GABAergic interneurons and DA neurons contribute to the modulation of behaviors relevant to psychiatric disorders.

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INTRODUCTION

Neuregulin (NRG) and its cognate neuronal receptor tyrosine kinase ErbB4 are genetically associated with increased risk for schizophrenia and its endophenotyes.[1–4] Moreover, disease-associated intronic and splice variants for ErbB4,[5] and altered NRG1, NRG3 and ErbB4 levels[6–8] in the brains of schizophrenic patients have been reported. Remarkably, mice with mutations in *nrg1*, *nrg2*, *nrg3* and *erbb4* display numerous behavioral abnormalities resembling psychiatric symptoms in affected individuals.[9–16] Experiments in rodents suggest that NRG/ErbB4 signaling modulates several neurotransmitter systems including GABA, glutamate, acetylcholine and dopamine (DA; see refs 3,17).

As ErbB4 is highly expressed in GABAergic parvalbumin-positive (PV+) basket cells but absent from glutamatergic neurons,[18–20] and this interneuron subtype is selectively affected in the dorsal prefrontal cortex (DLPFC) of schizophrenia patients where it modulates neuronal network activity underlying cognition,[21,22] most of the earlier studies on NRG/ErbB4 signaling focused on its direct effects in GABAergic interneurons[18,23–25] or its indirect effects on excitatory glutamatergic neurons.[26–28] However, although ErbB4 is also prominently expressed in subcortical areas in the rodent and primate brain, including the substantia nigra compacta and the ventral tegmental area, as reported by us[29,30] and others,[31,32] little is known about its functions in these areas and its interactions with other systems, circuitry and behaviors relevant to psychiatric disorders.

In support of a potential role of ErbB4 in the direct modulation of DAergic neurons, acute delivery of NRG1 by reverse microdialysis into the dorsal hippocampus (hereafter denoted 'hippocampus') rapidly increases extracellular DA levels and reverses LTP at Schaffer collateral-CA1 glutamatergic synapses via activation of D4 receptors.[33] Moreover, acute activation as well as chronic disruption of NRG/ErbB signaling impact DAergic function and metabolism.[34–40] For example, systemic perinatal exposure to NRG1[41] or direct activation of NRG1/ErbB4 signaling in slices[38] increases spike bursting and spontaneous firing, and increases metabotropic glutamate receptor 1-activated currents of mesencephalic DA neurons, respectively. Also, rodents neonatally injected with NRG1 or a pan-ErbB inhibitor exhibit augmented DA

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levels in the nigro-cortico-striatal system in adulthood.[35,37] Interestingly, we recently found that adult NRG2 knockout (KO) mice exhibit a marked imbalance of extracellular DA and its metabolites in the medial prefrontal cortex (mPFC) and striatum. Furthermore, NRG2-deficient mice exhibit augmented GluN2B-containing NMDA receptor synaptic currents at hippocampal glutamatergic synapses,[13] supporting the notion that reciprocal crosstalk between the DAergic system and NMDA receptor trafficking contributes to the modulation of synaptic plasticity at excitatory synapses,[42] and that DA is involved in the development of excitatory/inhibitory (E/I) balance during adolescence.[43]

Taken together, this evidence therefore suggests that NRG/ErbB signaling regulates homeostasis of extracellular DA levels either by directly modulating DAergic neurons, conceivably via mechanisms involving the DA transporter (DAT) or catechol-*O*-methyltransferase, by indirectly modulating neuronal circuits through GABAergic interneurons,[44] or both. The major aims of the present study were to determine if and how ErbB4 signaling in mesencephalic DAergic neurons is necessary to acutely regulate extracellular DA levels, and to determine if chronic ErbB4 ablation in monoaminergic neurons affects behaviors relevant to psychiatric disorders.

MATERIALS AND METHODS

(see Supplementary Information for details).

Animals

TH-Cre;ErbB4$^{f/f}$ and PV-Cre;ErbB4$^{f/f}$ conditional mutant mice, and their littermate ErbB4$^{f/f}$ controls, have been described previously.[45–47] Wild-type C57BL/6J (The Jackson Laboratory, Bar Harbor, ME, USA) were used for mRNA *in situ* hybridization. Embryonic day-15 fetuses from C57BL/6J and ErbB4 null KO mice,[48] hereafter denoted ErbB4-KO, were used for primary cell culture. All procedures were reviewed and approved by the NIH Animal Care and Use Committee.

RNA *in situ* hybridization and immunohistochemistry

Co-expression of TH and ErbB4 mRNA was analyzed by double-fluorescence *in situ* hybridization using RNAscope.[49] Immunohistochemical analysis of ErbB4, DAT, Tau and Ankyrin-G in primary cultures was performed as previously described.[20]

In vivo microdialysis and DA measurements

Local delivery of recombinant NRG1β (GenScript, Piscataway, NJ, USA) encompassing the EGF-like domain (hereafter denoted NRG1) was done by reverse microdialysis.[33] Samples were collected every 15 min into 5 µl 100 mM HCl+1 mM EDTA. Extracellular DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in hippocampus, mPFC and striatum by microdialysis, followed by HPLC-electrochemical detection as previously described.[13] Quantification of extracellular DA was confirmed in independent samples by liquid chromatography, followed by mass spectrometry (Brains On-Line, San Francisco, CA, USA). Probe location was evaluated for each mouse *post hoc*, and only samples with proper probe placement were included in the analysis (Supplementary Figure S5).

In vitro DA measurements and uptake using LUHMES cells

Cells were expanded and differentiated for 6 days as described before.[50] The effects of NRG1 on extracellular DA levels, as well as on [^{3}H]-Methyl-4-phenylpyridinium (MPP^+)/[^{3}H]-DA uptake, were measured in absence or presence of the ErbB kinase inhibitor PD158780 (10 µM) and the DAT-selective inhibitor GBR12935 (100 nM). Extracellular DA content in Lund Human Mesencephalic (LUHMES) media was determined by HPLC as described above. [^{3}H]-MPP^+/[^{3}H]-DA uptake assays were performed as described previously.[51] In brief, cells were incubated for 20 min with NRG1 and/or PD158780 in DMEM/F12 at 37 °C before the addition of 20 nM [^{3}H]-DA (30.0 Ci mmol^{-1}; Perkin Elmer) or [^{3}H]-MPP^+ (79.8 Ci mmol^{-1}; Perkin Elmer). After 10 min, cells were washed with ice-cold phosphate-buffered saline and lysed with 1% sodium dodecyl sulfate. Non-specific uptake was determined with 0.1 mM mazindol (Sigma-Aldrich, St. Louis, MO, USA). The accumulated labeled substrate was measured with a LSC6000 counter (Beckman Coulter, Brea, CA, USA).

Behavioral tests

Cohorts (>5) of adult male mice (3–5 months old) were initially screened for general health, reflexes, as well as sensory and neurological functions before use in behavioral tests, as recommended.[52] Tests were run in the following order: open field, elevated plus maze, pre-pulse inhibition (PPI) of acoustic startle, and either fear conditioning or amphetamine challenge, as described previously.[13,15] Independent cohorts were used for the T-maze, Y-maze and Barnes maze tests.

Rescue of midbrain ErbB4 expression in TH-Cre;ErbB4$^{f/f}$ KO mice

Expression of ErbB4 in mesencephalic DA neurons was selectively rescued by stereotaxic bilateral microinjection (0.5 µl/hemisphere) of adult TH-Cre;ErbB4$^{f/f}$ KO mice with an adeno-associated virus (AAV) harboring a double-floxed Cre-inducible ErbB4 (AAV-ErbB4.DIO); a Cre-inducible GFP-expressing AAV (AAV-GFP.DIO) was used as a negative control. T-maze and Y-maze behavioral tasks and *in vivo* microdialysis were performed 8 and 10 weeks post-injection, respectively.

RESULTS

Midbrain DAergic neurons express ErbB4 on cell bodies and axonal processes

Previous studies reported expression of ErbB4 transcripts in the rodent mesencephalic area, which contains GABAergic and DAergic neurons.[29–32] Using a sensitive double-fluorescence *in situ* hybridization approach (RNAscope) on adult mouse sections (Figure 1a), we observed that the vast majority of ventral tegmental area/substantia nigra compacta neurons expressing TH co-express ErbB4 (1015 out of 1025 TH+ cells, $n=4$ sections) but not ErbB3, another NRG-binding receptor (Supplementary Figure S1a). The negative control probe DapB did not generate any hybridization signals (Supplementary Figure S1b), illustrating the low background of this approach.

Consistent with the above results, ErbB4 receptor protein was found to be expressed in cultured DAT+ primary midbrain neurons (Figure 1b). Interestingly, in contrast to the somatodendritic pattern of ErbB4 immunoreactivity previously reported for GABAergic interneurons (see Discussion and[20]), ErbB4 in DAergic neurons was found not only on somata and dendrites but also on axonal projections co-labeled with Ankyrin G and Tau antibodies. Importantly, immunoreactive puncta for ErbB4 were absent from cultured DAT+ neurons from ErbB4-KO mice (Figure 1c).

ErbB4 in DAergic neurons directly regulates extracellular DA levels in hippocampus, mPFC and striatum

The expression of ErbB4 in DAergic axonal projections, together with our earlier work demonstrating that local infusion of NRG1 by microdialysis increases extracellular DA levels in the rat hippocampus,[33] prompted us to explore a possible role of direct ErbB4 signaling in DAergic projections to the hippocampus, mPFC and striatum. Using reverse microdialysis, we measured the effects of local NRG1 infusion on extracellular DA levels in TH-Cre;ErbB4$^{f/f}$ KO mice lacking ErbB4 in DAergic neurons and their ErbB4$^{f/f}$ littermate controls. As shown in Figure 2a–c (left), local infusion of NRG1 (1 nM, 15 min) completely failed to increase extracellular DA levels in the hippocampus, mPFC and striatum of TH-Cre;ErbB4$^{f/f}$ KO mice relative to baseline levels. In stark contrast, in ErbB4$^{f/f}$ littermate controls NRG1 produced a robust augmentation of extracellular DA levels in all three areas; of note, NRG1 responses in ErbB4$^{f/f}$ mice were indistinguishable from those in C57BL/6J mice (not shown). Importantly, consistent with a prior study,[53] a KCl pulse (50 mM) elicited a similar accumulation of extracellular DA in both TH-Cre;ErbB4$^{f/f}$ KOs and ErbB4$^{f/f}$ controls

(Figures 2a–c), ruling out a general impairment in the development of DAergic axonal projections and terminals in TH-Cre; ErbB4$^{f/f}$ KO mice.

As shown in Figure 2a–c (right), in a parallel set of experiments we found that NRG1 robustly increased DA levels in both PV-Cre; ErbB4$^{f/f}$ KOs and their littermate ErbB4$^{f/f}$ controls, indicating that the local effects of NRG1 on extracellular DA levels in these structures are not indirectly mediated via ErbB4-expressing GABAergic interneurons.

As norepinephrinergic (NE) neurons express TH and can co-release DA,[54] we considered their potential contribution to the NRG1-dependent increase in extracellular DA levels. However, we found that 1nM of NRG1 did not increase extracellular NE levels relative to baseline in the hippocampus of wild-type C57BL/6J mice (Supplementary Figure S2), despite a robust augmentation of extracellular NE levels following a 50 mM KCl pulse. The absence of a NRG1 response is consistent with the lack of ErbB4 expression in TH+ NE neurons in the locus coeruleus.[55] Taken together, these results not only extend our previous findings in the hippocampus[33] to the mPFC and striatum, but furthermore suggest that NRG1/ErbB4 signaling augments extracellular DA by acting directly on DAergic axonal projections.

NRG1/ErbB4 signaling reduces DAT uptake efficiency in LUHMES cells

Elevations of extracellular DA concentration in response to local NRG1 infusion could result from increased DA release, decreased DA clearance, or both. To differentiate between these possibilities, we initially used fast-scan cyclic voltammetry and found that NRG1 has no effect on electrically evoked DA release from terminals in mouse dorso-striatal slices (Supplementary Figure S3). We next used DAergic LUHMES cells to investigate if NRG/ErbB signaling directly modulates DAT activity. Upon differentiation, LUHMES cells express ErbB4 (Supplementary Figure S4) as well as numerous DAergic markers, including DAT.[50] Moreover, they accumulate measurable amounts of DA in the medium (0.289 ± 0.027 pg µl^{-1}, $n = 27$). As shown in Figure 3a, NRG1 (1 nM) increased DA levels in conditioned media relative to baseline, and this increase was blocked by the pan-specific ErbB receptor kinase inhibitor PD158780. Consistent with a potential effect of NRG1 on DAT function, we found that treatment with the DAT blocker GBR12935 increases DA in the media (Figure 3b) and occluded any further NRG1-mediated increases of DA ($P > 0.05$). Next, we directly measured transporter activity in LUHMES cells by assaying DAT-mediated uptake of tritiated DA ([^{3}H]-DA) or MPP$^+$ ([^{3}H]-MPP$^+$), a stable DAT substrate analog whose uptake is regulated in a dose-dependent manner by NRG1 (Figure 3c). As shown in Figure 3d, relative to vehicle, NRG1 was equally effective in reducing the uptake of [^{3}H]-DA or [^{3}H]-MPP$^+$ and reduced by PD158780. As cell surface protein biotinylation experiments failed to show an effect of NRG1/ErbB4 signaling on DAT surface levels (data not shown), it is conceivable that other previously identified regulatory mechanisms are involved.[56–58] Taken together, our *in vivo* and *in vitro* findings indicate that acute NRG/ErbB4 signaling directly on DAergic axons increases extracellular DA levels, at least in part, by reducing DAT-mediated uptake.

Mice lacking ErbB4 in TH+ neurons show steady-state DA imbalances and spatial/working memory behavioral deficits

We evaluated the effects of chronic deletion of ErbB4 on basal extracellular DA levels in TH-Cre;ErbB4$^{f/f}$ and PV-Cre;ErbB4$^{f/f}$ KO mice. Interestingly, we found regional variability of both basal extracellular DA levels as well as its metabolites DOPAC and HVA in TH-Cre;ErbB4$^{f/f}$ KO mice, relative to their littermate ErbB4$^{f/f}$

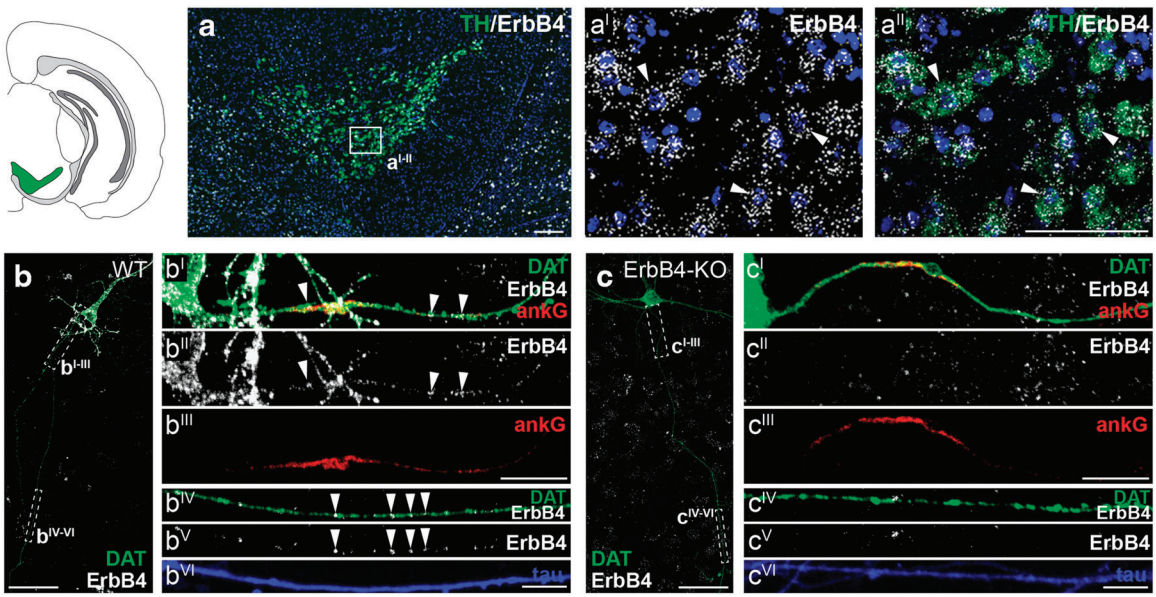

Figure 1. ErbB4 mRNA and protein is expressed in soma and axons of midbrain DAergic neurons. (**a**) Double-fluorescence *in situ* hybridization (RNAscope) for ErbB4 (white) and TH (green) transcripts in midbrain coronal sections from wild-type C57BL/6J mice; anatomical region corresponds to area highlighted in green in the adjacent scheme. The boxed area in (**a**) is enlarged in the two panels on the right (**a$^{I–II}$**) to visualize the numerous DA neurons abundantly expressing ErbB4 transcripts (arrowheads); nuclei were labeled with DAPI (blue). (**b** and **c**) Representative immunofluorescence images of dissociated primary midbrain neurons isolated from (**b**) wild-type C57BL/6J or (**c**) ErbB4-KO mice. (**b$^{I–III}$**) Higher magnification of the area demarked in **b** shows that ErbB4 receptor puncta (*arrowheads*) distribute on the cell soma and along DAT-positive axonal processes that are positive for the axon hillock marker Ankyrin G (ankG). (**b$^{IV–VI}$**) A second magnified area from the same neuron shows ErbB4 immunoreactive puncta along a more distal DAergic Tau-positive axonal process. ErbB4 immunoreactivity is specific, because somatic (**c**) and axonal (**c$^{I–III}$** and **c$^{IV–VI}$**) puncta are absent from DAT-positive mesencephalic neurons isolated from ErbB4-KO mice. For panels (**b**) and (**c**), 9 DAT-positive neurons from C57BL/6J WT and 10 DAT-positive neurons derived from ErbB4-KO mice were analyzed in two independent cell culture preparations, respectively. Scale bars = 200 µm (**A** and **a$^{I–II}$**), 100 µm (**B** and **C**), 10 µm (**b$^{I–VI}$** and **c$^{I–VI}$**).

controls (Figure 4a, red bars; and Supplementary Table S1). Whereas basal DA levels were elevated in the hippocampus and mPFC, they were reduced in the striatum. Importantly, DA, DOPAC and HVA levels in PV-Cre;ErbB4$^{f/f}$ KO mice (Figure 4a, green bars; and Supplementary Table S1) were not significantly different from their control littermates in these brain areas. Hence, NRG/ErbB4

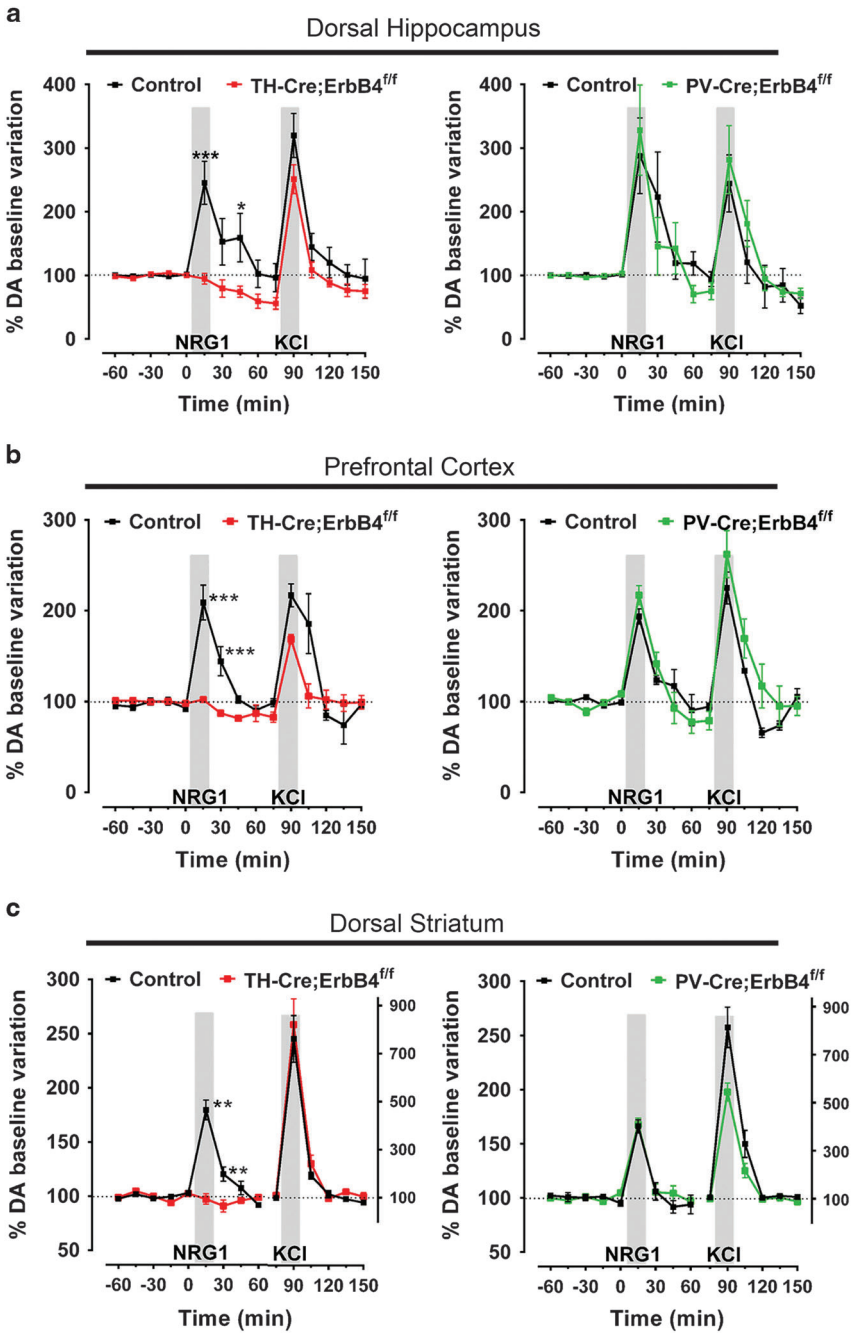

Figure 2. NRG1-mediated increases in extracellular DA requires direct ErbB4 signaling in DAergic neurons *in vivo*. Local delivery of NRG1 (left shaded area) and measurements of extracellular DA were performed using reverse microdialysis in the (**a**) dorsal hippocampus, (**b**) mPFC and (**c**) dorsal striatum of freely moving adult TH-Cre;ErbB4$^{f/f}$ (left, red lines) or PV-Cre;ErbB4$^{f/f}$ KO mice (right, green lines), and their corresponding ErbB4$^{f/f}$ littermate controls (black lines). Samples were collected for 15 min to account for low DA levels in the hippocampus and mPFC. Local ErbB4 activation with NRG1 (1 nM, 15 min) elicits a robust increase of extracellular DA in control ErbB4$^{f/f}$ mice (controls for TH-Cre;ErbB4$^{f/f}$, hippocampus: 245.6 ± 33.8%, $n = 6$, mPFC: 208.9 ± 19.2%, $n = 6$, striatum: 179.7 ± 9.1, $n = 6$) (controls for PV-Cre;ErbB4$^{f/f}$, hippocampus: 288.0 ± 59.4%, $n = 6$; mPFC: 193.6 ± 8.5%, $n = 6$, striatum: 166.1 ± 6.0, $n = 6$) and in PV-Cre;ErbB4$^{f/f}$ mutant mice (hippocampus: 328.2 ± 71.0%, $n = 6$, mPFC: 217.1 ± 10.6%, $n = 6$, striatum: 167.8 ± 6.0%. $n = 6$), but not in TH-Cre;ErbB4$^{f/f}$ mice (hippocampus: 94.8 ± 8.7%, $n = 7$, $F(1,11) = 11.73$, $P = 0.0057$; mPFC: 102.2 ± 1.6%, $n = 6$, $F(1,10) = 20.02$, $P = 0.0012$; striatum: 97.3 ± 4.8%, $n = 6$, $F(1,10) = 30.83$, $P = 0.0002$). The functionality of DA processes in each anatomical area was assayed 60 min after the NRG1 application when DA levels had returned to baseline by delivering a depolarizing KCl pulse (50 mM, 15 min). Extracellular DA levels increased in all genotypes, indicating that dopaminergic processes retained normal capacity for depolarization-dependent release. Data represent the mean ± s.e.m. of the percentage of baseline variation. In **c**, DA increases in striatum during the KCl pulse were plotted on a second y axis shown on the right side of the graph. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$.

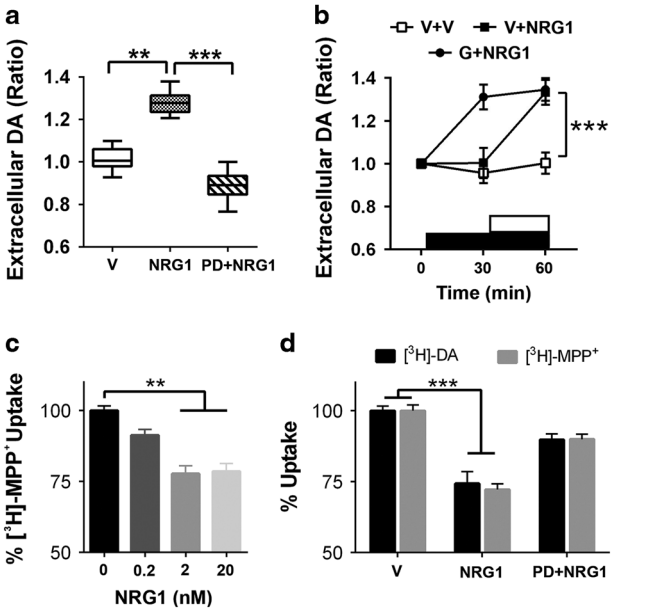

Figure 3. NRG/ErbB4 signaling increases extracellular DA levels by reducing DAT uptake efficiency. Experiments were performed in differentiated LUHMES cells, which exhibit numerous properties of DAergic neurons,[44] to determine if NRG/ErbB signaling cell-autonomously regulates extracellular DA levels. (**a**) Cells were treated for 30 min with either vehicle (V), 1 nM NRG1, or 10 μM PD158780+1 nM NRG1 (PD+NRG1); DA levels in conditioned media are plotted as ratios of post- over pre-treatment values. ($n = 8$/treatment). NRG1 increased DA levels relative to baseline (vehicle vs NRG1: 1.010 ± 0.019 vs 1.280 ± 0.019, $n = 8$/treatment; $F(2,21) = 88.77$, $P < 0.0001$), and this increase was blocked by PD158780 (PD+NRG1: 0.889 ± 0.025). (**b**) The DAT blocker GBR12935 increased DA in the media ($F(2,15) = 31.09$, $P < 0.0001$, $n = 6$/treatment) and occluded any further NRG1-mediated increases of DA ($P > 0.05$). Extracellular DA levels in LUHMES cell media were measured 30 min following the vehicle (V) control, and again 30 min later after addition of either more vehicle (V+V, open squares) or NRG1 (V+NRG1, solid squares). In parallel samples, cultures were initially treated for 30 min with 100 nM GBR12935 (G) and followed by 30 min treatment with GBR12935 and NRG1 (G+NRG1; solid circles). The solid bar represents either vehicle or GBR12935 treatment during 1 h of treatment, and the open bar represents addition of NRG1 (1 nM) or vehicle to the media ($n = 6$/treatment). (**c**) NRG1 treatment (20 min) dose-dependently reduced [^{3}H]-MPP+ uptake in LUHMES cells (0 nM: $100 \pm 1.7\%$, $n = 16$; 0.2 nM: $91.3 \pm 2.0\%$, $n = 13$; 2 nM: $77.8 \pm 2.8\%$, $n = 20$; and 20 nM: $78.6 \pm 2.7\%$, $n = 20$). One-way analysis of variance (ANOVA) $F(3,65) = 18.10$, $P < 0.0001$. (**d**) [^{3}H]-MPP$^+$ and [^{3}H]-DA uptake in LUHMES treated for 20 min with vehicle (V), 2 nM NRG1 (N) or 10 μM PD158780+2 nM NRG1 (PD+N) during 20 min. NRG1 treatment reduced [^{3}H]-MPP$^+$ ($72.2 \pm 2.0\%$, $n = 25$) and [^{3}H]-DA uptake ($74.4 \pm 4.2\%$, $n = 18$) similarly and uptake was blocked by co-application of PD158780 ([^{3}H]-DA: $89.8 \pm 2.0\%$, $n = 17$; [^{3}H]-MPP$^+$: $90.0 \pm 1.8\%$, $n = 23$), consistent with an effect of NRG1 on transporter function. Two-way ANOVA revealed a primary effect of NRG1 treatment on DAT-mediated uptake ($F(2,119) = 63.95$, $P < 0.0001$). Data in (**c** and **d**) are plotted as percentages, with 100% defined as uptake under vehicle-treatment conditions, and represent mean ± s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$.

signaling in DAergic afferents, but not in PV+ cells, is required to maintain DA homeostasis in the hippocampus, mPFC and striatum.

The observed DA imbalances in TH-Cre;ErbB4$^{f/f}$ KO mice prompted us to investigate their performance in a variety of tasks previously reported to be sensitive to DA function, such as spatial, working and emotional memory, locomotor activity, amphetamine sensitivity, sensorimotor gating and anxiety-related behaviors. In tests designed to evaluate the function of mPFC and hippocampus in spatial and working memory,[59] TH-Cre;ErbB4$^{f/f}$ KO mice performed poorly relative to their littermate ErbB4$^{f/f}$ controls in both the T-maze (Figure 4b) and Y-maze (Figure 4c). Performance of TH-Cre;ErbB4$^{f/f}$ KO mice in the Barnes maze, which additionally requires learning and spatial reference memory,[60] was also lower relative to littermate controls. KO mice showed longer latency times (Figure 4d; $F(1,22) = 19.25$, $P = 0.0002$), required more training ($F(3,66) = 26.70$, $P < 0.0001$) and made more errors during the first 3 days of training (Figure 4d; right panel; genotype: $F(1,22) = 18.02$, $P = 0.0003$; days of training: $F(3,66) = 25.46$, $P < 0.0001$); no differences were observed by the fourth day. Importantly, TH-Cre;ErbB4$^{f/f}$ KO mice exhibited reduced memory retrieval during testing on day 5, manifesting as an increased number of errors (Figure 4e), reduced time spent in the correct zone and reduced number of target pokes. Interestingly, and in contrast to full ErbB4-KO and PV-Cre;ErbB4$^{f/f}$ KO mice[11,14,15] (Table 1), TH-Cre;ErbB4$^{f/f}$ KO mice performed similarly to their controls in a battery of tests designed to assess: (1) novelty-induced hyperactivity in the open field (Figure 4f), (2) hypersensitivity to amphetamine-induced locomotor activity (Figure 4g), (3) sensorimotor gating using PPI (Figure 4h), (4) basal anxiety in the elevated plus maze (Figure 4i) and (5) cued and contextual fear memory (Figure 4j); all data and statistical analyses are summarized in Supplementary Table S2. These findings indicate that modulation of NRG/ErbB4 signaling in DAergic neurons vs PV+ GABAergic interneurons regulates distinct, non-overlapping behavioral domains, and that NRG/ErbB4-dependent maintenance of DA homeostasis is required for normal performance in spatial/working memory (cognitive-related) behaviors.

Selective expression of ErbB4 in midbrain DAergic neurons of TH-Cre;ErbB4f/f KO mice restores DA homeostasis and behaviors

To test whether DA imbalance, acute responses to NRG1 and behavioral deficits in TH-Cre;ErbB4$^{f/f}$ KO mice are the result of developmental compensatory adaptations of affected neural circuits, we rescued ErbB4 function selectively in midbrain DAergic neurons by stereotaxic microinjection of an AAV expressing Cre-inducible ErbB4 (ErbB4.DIO) bilaterally into the mesencephalic area of adult TH-Cre;ErbB4$^{f/f}$ KO mice (Figure 5a). Mice microinjected with AAV-GFP.DIO were used as negative controls (see Supplementary Methods for details). We began by testing mice in the T-maze and Y-maze to assess spatial learning memory 8 weeks post-injection (Figure 5b). Relative to the AAV-GFP.DIO controls, injection of AAV-ErbB4.DIO resulted in significant improvements in T-maze and Y-maze performance. To determine if spatial/working memory improvements correlated with changes in DA levels, we measured extracellular DA and its metabolites in the mPFC and striatum (dual cannulation/mouse); hippocampal measurements were not performed due to technical limitations (exceedingly low DA levels requiring large numbers of mice; quadruple cannulation was not feasible). Interestingly, we found that the elevated basal DA levels observed in the mPFC of TH-Cre;ErbB4$^{f/f}$ KO mice (Figure 4a) were selectively normalized in mice microinjected with AAV-ErbB4.DIO, but they remained high in mice injected with AAV-GFP.DIO (Figure 5c). Conversely, reduced striatal DA was augmented after AAV-ErbB4.DIO but not after AAV-GFP.DIO injection (Figure 5c). Consistent with these findings, a comparison of the basal concentrations of DA and its metabolites in the mPFC and striatum of control (ErbB4$^{f/f}$), KO (TH-Cre;ErbB4$^{f/f}$) and 'rescued' mice (AAV-ErbB4.DIO-injected TH-Cre;ErbB4$^{f/f}$) showed that re-expression of ErbB4 in mesencephalic neurons normalized extracellular DA levels (Figure 5c and Supplementary Table S1). Finally, we analyzed if re-expression of ErbB4 in DAergic neurons restored responsiveness to acute NRG1 application. As shown in Figure 5d, local infusion of NRG1 in ErbB4-rescued mutants, but not in GFP.DIO controls, acutely increased extracellular DA levels

in both the mPFC and striatum, as observed earlier in control mice (see Figure 2). In conclusion, our data show that NRG1/ErbB4 signaling in midbrain DAergic neurons directly regulates DA function in mesocortical and nigrostriatal systems, which are important for cognitive behaviors such as spontaneous alternation and spatial/working memory during T- and Y-maze.

DISCUSSION

Using a combination of *in vivo* microdialysis, biochemical, genetic engineering and behavioral approaches, we demonstrate for we believe the first time that NRG1 acutely augments extracellular DA levels by acting directly on DAergic axonal projections expressing ErbB4 to modulate DAT activity, and that ErbB4

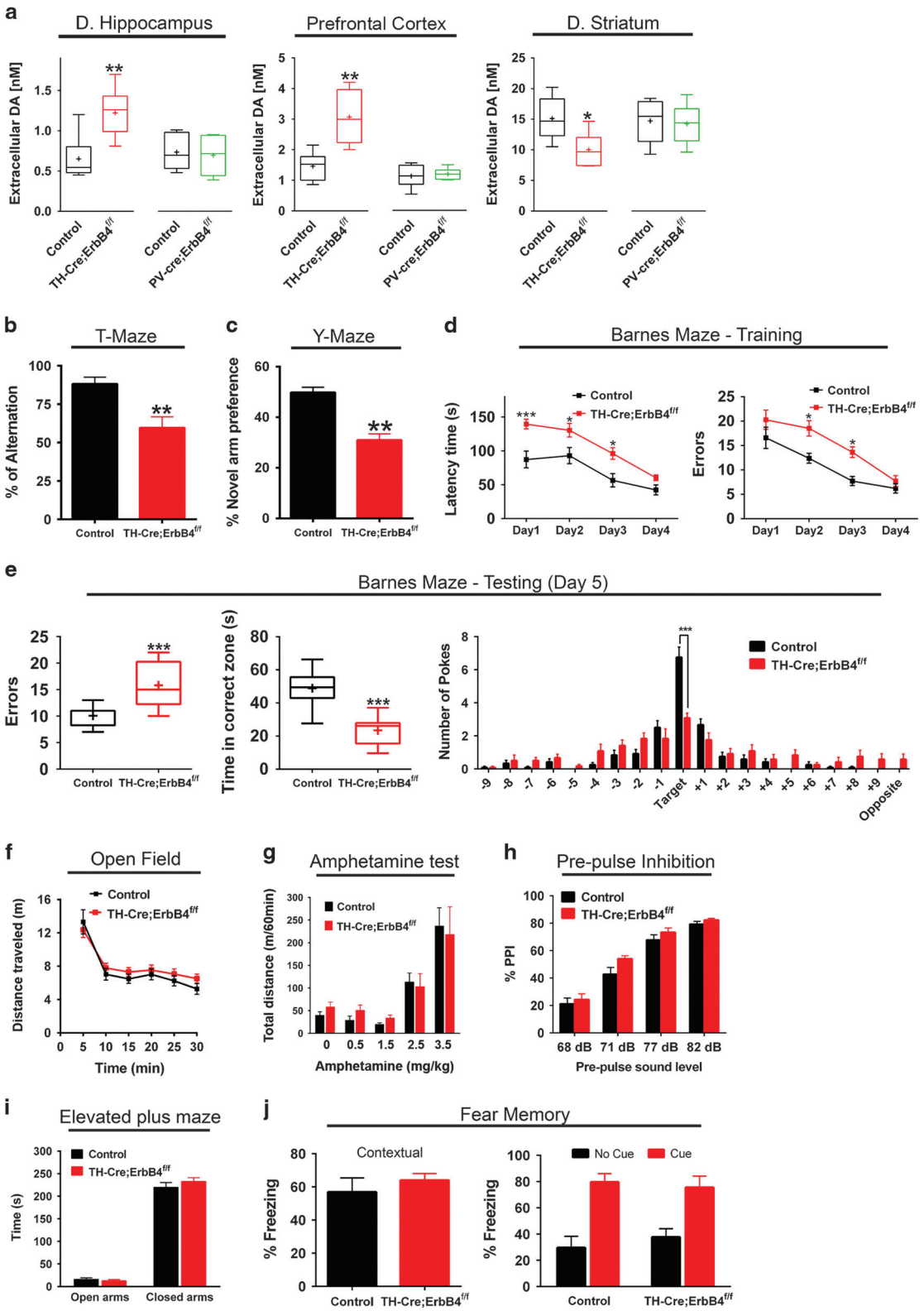

Figure 4. TH-Cre;ErbB4f/f mice exhibit altered DA homeostasis and display spatial learning memory deficits. (**a**) TH-Cre;ErbB4$^{f/f}$ mice (red) show elevated basal extracellular dopamine (DA) levels in the dorsal hippocampus (KO vs control: 1.22 ± 0.11 nM vs 0.65 ± 0.12 nM; $U = 3$, $P = 0.0076$; Mann-Whitman *U*-test) and mPFC (3.07 ± 0.36 nM vs 1.46 ± 0.19 nM; $U = 1$, $P = 0.0043$), but reduced levels in dorsal striatum (10.0 ± 1.13 nM vs 15.11 ± 1.42 nM; $U = 5$, $P = 0.0411$), compared to their corresponding control littermates (black). By contrast, PV-Cre;ErbB4$^{f/f}$ mice (green) did not show altered DA levels (KO vs control: 0.70 ± 0.10 nM vs 0.73 ± 0.09 nM (hippocampus); 1.19 ± 0.07 nM vs 1.12 ± 0.16 nM (mPFC); 14.23 ± 1.32 nM vs 14.72 ± 1.48 nM (striatum); all $P > 0.05$). (**b–e**) Assessment of behaviors that differ between TH-Cre;ErbB4$^{f/f}$ (red) and their littermate controls (ErbB4$^{f/f}$, black). (**b**) Spontaneous alternation (in %) during the T-maze test ($n = 14$/genotype). (**c**) Memory for familiar/unfamiliar environments in the Y-maze test, plotted as preference (in %) for the novel arm ($n = 12$/genotype). (**d**) Spatial learning and memory in the Barnes maze during training days 1–4, including latency time to reach the escape target (left panel) and number of errors (right panel). (**e**) Barnes maze test at day 5 (test day), showing number of incorrect nose pokes (errors, left), time spent in the correct zone (middle) and number of nose pokes plotted as a function of target location (right) ($n = 12$/genotype). (**f–j**) Performances were indistinguishable between TH-Cre;ErbB4$^{f/f}$ (red) and their littermate controls (ErbB4$^{f/f}$) in a variety of tasks, including: (**f**) Novelty-induced horizontal locomotor activity in the open field (cumulative distance traveled during 5-min intervals); (**g**) Amphetamine-induced horizontal locomotor activity (total distance traveled in 1 h following vehicle, 0.5, 1.5, 2.5 and 3.5 mg/kg amphetamine i.p. injection); (**h**) Pre-pulse inhibition of the acoustic startle response (40 ms sound, 120 dB intensity); (**i**) Basal anxiety assessed as time spent in open vs closed arms in the elevated plus maze; and (**j**) Contextual and cued fear memory (unconditioned stimulus (US): 0.5 mA foot shock, 2 ms). Data are represented as means ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$.

Table 1. Comparison of DAergic function and behavioral deficits observed in TH-Cre;ErbB4$^{f/f}$ and PV-Cre;ErbB4$^{f/f}$ mutant mice

Experimental test	*TH-Cre; ErbB4$^{f/f}$*	*PV-Cre; ErbB4$^{f/f}$*
Acute NRG1-dependent DA response	Not observed	Observed
Basal extracellular DA levels	↑Hippocampus ↑mPFC ↓Striatum	↔ All brain areas
Basal extracellular DOPAC levels	↓Hippocampus ↓mPFC ↑Striatum	↔ All Brain areas
Basal extracellular HVA levels	↔ All brain areas	↔ All brain areas
T-maze, Y-maze, Barnes maze (spatial learning/working memory)	Impaired	Not tested
Radial arm maze (spatial learning and memory)	Not tested	Impaired[16]
Open field test (novelty-induced locomotor activity)	Normal	Increased[15,16]
Amphetamine challenge test (amphetamine-induced locomotion)	Normal	Not tested
Pre-pulse inhibition (Sensorimotor gating to the startle)	Normal	Impaired[15,16]
Elevated plus maze (anxiety)	Normal	Normal[15]
Contextual/cued freezing behavior (fear memory)	Normal	Normal[15] Impaired,[11,14]

Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; mPFC, medial prefrontal cortex; NRG1, neuregulin 1; PV, parvalbumin; TH, tyrosine hydroxylase. ↔Unchanged, ↑increased or ↓decreased extracellular concentrations of DA and its metabolites DOPAC and HVA levels in tested brain areas of conditional TH-Cre;ErbB4$^{f/f}$ and PV-Cre;ErbB4$^{f/f}$ mice, as compared to ErbB4$^{f/f}$ control littermates.

receptors expressed on GABAergic PV+ interneurons are not required for this increase. Moreover, we show that chronic loss of ErbB4 from DAergic neurons, but not from PV+ interneurons, alters DA homeostasis in the hippocampus, mPFC and striatum, and results in spatial/working memory deficits. As discussed below, the present findings, when taken together with prior studies on the functional role of ErbB4 in PV+ neurons, begin to reveal how NRG/ErbB4 signaling in DAergic and GABAergic neurons directly affect their function and suggest that their functional interactions can synergistically modulate E/I balance, local network activity and synaptic plasticity-processes thought to be affected in psychiatric disorders.

ErbB4 signaling in DAergic neurons underlies the acute effects of NRG1 on extracellular DA levels: implications for the modulation of local networks

We previously reported that infusion of recombinant NRG1 into the rat hippocampus rapidly increases extracellular DA levels *in vivo*.[33] Moreover, in hippocampal slices in which DAergic afferents are severed from their midbrain cell bodies, NRG1 reverses LTP and increases the power of gamma oscillations via DA D4 receptor signaling.[33,61] Although these studies established a requirement for DA to mediate the effects of NRG1/ErbB4 signaling on local circuit functions, they did not identify the cellular and subcellular loci of the pertinent population of ErbB4 receptors mediating NRG1-dependent increases in extracellular DA. Our conclusion that NRG1-elicited increases in extracellular DA are mediated directly by ErbB4 receptors located on DAergic terminals, and not indirectly through networks requiring ErbB4+ GABAergic interneurons (the only neuron type in the neocortex and hippocampus expressing ErbB4[19,20]), rests on several lines of evidence that include: (1) selective genetic ablation of ErbB4 in TH + neurons, but not in PV+ interneurons, modulates NRG1-evoked and basal extracellular DA levels, (2) re-expression of ErbB4 selectively in midbrain DAergic neurons restores normal basal levels of extracellular DA and responsiveness to NRG1, (3) ErbB4 is expressed on axons of DAergic neurons as previously suggested[30] (contrasting the lack of detectable ErbB4 immunoreactivity on axons of GABAergic neurons[19,20]), (4) NRG1 increases extracellular DA levels in LUHMES cells that are grown in isolation, in the absence of other neuronal inputs and (5) this effect is regulated by DAT, expressed specifically on DAergic neurons including their axonal processes.

Numerous studies have investigated how NRG/ErbB4 signaling in PV+ interneurons regulates cortical E/I balance and resultant neuronal network activity.[11,18,22,62] Despite the fact that DA signaling via D1- and D2-type receptors has been shown to

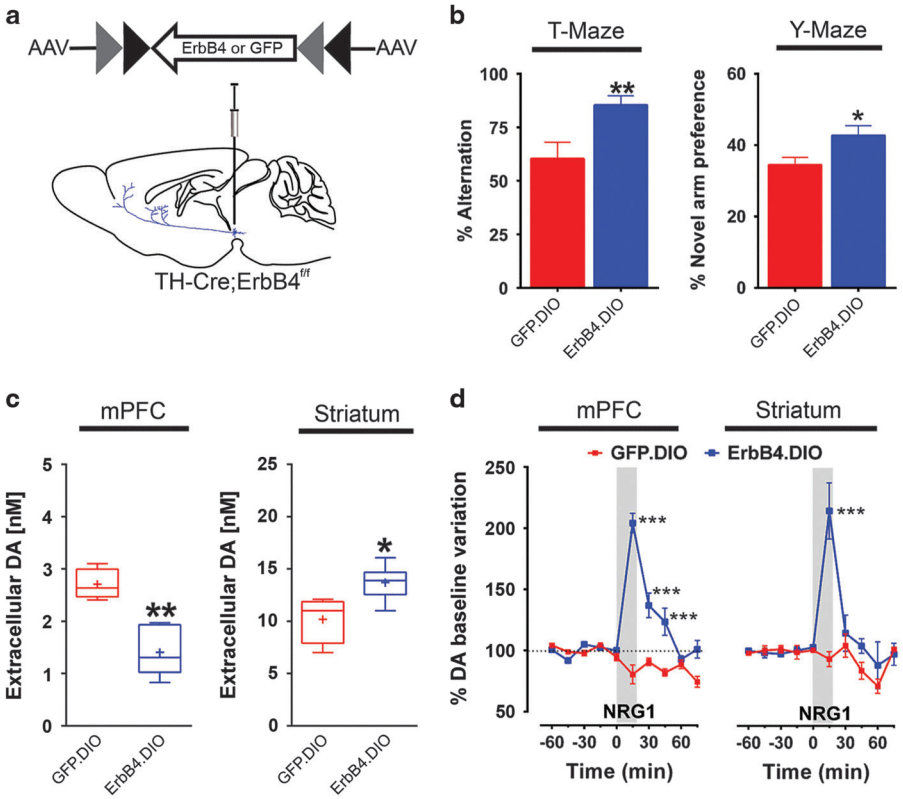

Figure 5. Rescuing ErbB4 expression in midbrain DAergic neurons of TH-Cre;ErbB4$^{f/f}$ mice restores behavioral deficits and dopamine (DA) balance. (**a**) Schematic depiction of AAV-ErbB4.DIO (ErbB4.DIO) or AAV-GFP.DIO (GFP.DIO) stereotaxic bilateral microinjections (0.5 μl/hemisphere) into the midbrain of 2-month-old TH-Cre;ErbB4$^{f/f}$ mice to cre-dependently express either ErbB4 or GFP in DAergic neurons. (**b**) Spontaneous alternation in the T-maze (left) was restored after injections of AAV-ErbB4.DIO (ErbB4.DIO vs GFP.DIO: 85.4 ± 4.3% vs 60.4 ± 7.6%, $n = 16$/group; $U = 67$, $P = 0.0158$) as was time spent in unfamiliar arms during the Y-maze test (right; ErbB4.DIO vs GFP.DIO: 42.7 ± 2.7% vs 34.5 ± 2.1%; $n = 12$/ErbB4.DIO; $n = 11$/GFP.DIO; $t(21) = 2.380$, $P = 0.0269$). (**c**) Basal extracellular DA levels in the mPFC (ErbB4.DIO vs GFP.DIO: 1.4 ± 0.2 nM vs 2.7 ± 0.1 nM, $n = 6$/group; $U = 0$, $P = 0.0022$) and striatum (ErbB4.DIO vs GFP.DIO: 13.7 ± 0.7 nM vs 10.2 ± 0.8 nM; $n = 6$/group; $U = 3$, $P = 0.0152$) were normalized after AAV-ErbB4.DIO injections. (**d**) Relative extracellular DA levels in TH-Cre;ErbB4$^{f/f}$ mice [% of baseline] increased after local delivery of 1 nM NRG1 (shaded area) in the mPFC (ErbB4.DIO vs GFP.DIO: 204.1 ± 8.0% vs 80.5 ± 7.7%, $n = 6$/group; two-way ANOVA treatment $F(1,10) = 38.14$, $P = 0.0001$) and striatum (ErbB4.DIO vs GFP.DIO: 214.5 ± 22.9% vs 93.4 ± 6.2%, $n = 6$/group; $F(1,10) = 8.8$, $P = 0.0141$) 10 weeks following AAV microinjection. Data are represented as means ± S.E.M. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

regulate the properties and trafficking of numerous voltage-gated ion channels and NMDA receptors to modulate synaptic plasticity and stabilization of cortical E/I balance,[63–68] relatively less attention has been devoted to the functional neuromodulatory role of NRG/ErbB4 signaling in DAergic neurons. Therefore, based on our findings, henceforth it will be important that electrophysiology studies on the indirect effects of NRGs on intrinsic properties and synaptic plasticity of neurons lacking ErbB4 (that is, hippocampal and cortical glutamatergic neurons) do not limit their interpretations to effects originating exclusively from ErbB4-expressing GABAergic neurons, but also consider the neuromodulatory effects of DAergic signaling.

DA homeostasis and its importance in endophenotypes relevant to psychiatric disorders

Transgenic mice and acute pharmacological models affecting central nervous system DA homeostasis have been valuable tools to understand their relevance for endophenotypes associated with psychiatric disorders. A major confound in these types of studies is that developmental compensatory mechanisms often complicate the interpretation of how acute effects of factors regulating DA levels and behaviors are related to effects stemming from chronic manipulations.[34,37] In this work and in a prior study,[33] we observed that acute ErbB4 stimulation increases extracellular DA levels in the hippocampus, mPFC and striatum. Based on these observations, chronic loss of NRG/ErbB4 signaling would be expected to result in a global hypo-DAergic state due to elevated DAT activity. Although basal extracellular DA levels were indeed reduced in the striatum of TH-Cre;ErbB4$^{f/f}$ KO mice, they were increased in the mPFC and hippocampus (Table 1). Similar discrepancies between acute and chronic treatments were observed when acute systemic administration of a NRG1 EGF-like peptide in neonatal mice, which results in a hyper-DAergic state in adults,[37] was compared to mice that chronically over-express NRG1 and that exhibit a hypo-DAergic state.[34] As is the case with other rodent and human studies (see below), we presently do not understand the mechanisms that account for the differential hypo- and hyper-DAergic states in the striatum and mPFC/hippocampus observed in TH-Cre;ErbB4$^{f/f}$ KO mice. These regional differences could potentially be due to the underlying circuitry regulating the mesocortical/nigrostriatal systems[69] or to the crosstalk between the NMDA receptor and DAergic signaling pathways.[42,43] Alternatively, impaired functional crosstalk between NRG/ErbB4 signaling in cortical PV+ interneurons and DAergic neurons, as observed in mice lacking the schizophrenia risk gene 'disrupted in schizophrenia 1' (DISC1),[70–73] could affect the

functional relationship between GABAergic and DAergic neurons and regionally alter DA homeostasis.

We and others have reported that ErbB4-KO mice exhibit many behavioral deficits relevant to endophenotypes in psychiatric disorders, particularly schizophrenia.[15,45] Interestingly, in contrast to PV-Cre;ErbB4$^{f/f}$ KO mice that predominantly manifest alterations reminiscent of positive symptoms in schizophrenia such as novelty-induced hyperlocomotor activity, reduced PPI and impaired fear memory,[11,14–16] TH-Cre;ErbB4$^{f/f}$ KO mice are indistinguishable from their WT littermates for those behaviors and instead exhibit cognitive-related impairments (Figure 4 and Table 1). DA regulates the maturation of cortical microcircuits during adolescence by modulating neuronal excitability,[43] NMDA receptor trafficking[42] and the activity of PV+ fast-spiking basket cells,[66,67,74,75] which are thought to underlie adult behaviors.[43] Importantly, optimal performance in tasks requiring working memory exhibits a non-linear (inverted-U) relationship with DA levels,[76–78] and alterations in E/I balance and reduced power of evoked gamma oscillations in DLPFC are associated with reduced cognitive performance in schizophrenia patients.[21,22] Because TH-Cre;ErbB4$^{f/f}$ KO mice have augmented mPFC and hippocampal DA levels and perform poorly in tasks requiring spatial/working memory, and because both parameters are restored by re-expression of ErbB4 in adult midbrain DAergic neurons, it will be interesting in future studies to determine if ErbB4 in DA neurons modulates neuronal network synchrony, particularly in the gamma range.

Studies in humans have shown that PPI is sensitive to systemic administration of drugs such as amphetamine that induce a generalized hyper-DAergic state,[79] and that individuals with schizophrenia frequently manifest an enhanced vulnerability to stimulant-induced psychosis.[80] Moreover, reduced PPI and amphetamine hypersensitivity in rodents have also been associated with increased striatal DA.[13,81,82] In contrast, TH-Cre;ErbB4$^{f/f}$ KO mice are normal in novelty- and amphetamine-induced locomotor activity and PPI tests, despite their observed DA imbalance. We speculate that a reason these behaviors are unperturbed is because TH-Cre;ErbB4$^{f/f}$ KO mice are hypo-DAergic, rather than hyper-DAergic, in the striatum; moreover, these mice display a more complex DA imbalance that includes hyper-DAergic states in the mPFC and hippocampus. Consistent with the former, previous DA depletion studies in humans have shown no effect on PPI.[83] Conversely, although PV-Cre;ErbB4$^{f/f}$ KO mice have normal DA function, they exhibit increased novelty-induced locomotor activity and PPI deficits[11,15,16] as well as altered anxiety and fear memory behaviors (Table 1), findings that are consistent with the general idea that behaviors are modulated by more than one neurotransmitter system but that also highlight the particular importance of GABAergic neurotransmission in the regulation of behaviors sensitive to generalized hyper-DAergia.[84] Taken together, this and prior studies show that NRG/ErbB4 signaling modulates complex interactions between the DAergic and GABAergic neurotransmitter systems, which could explain why the phenotypes of PV- and TH neuron-targeted ErbB4 mutant mice[11,14,15] overlap with those of other pharmacological and genetic rodent models that either directly or indirectly affect DA homeostasis.[34–37,39,41]

As optimal DLPFC DA levels are strongly associated with performance in working memory tasks,[76–78] it will be important to identify the mechanisms that regulate DA levels and how they might be altered in schizophrenia and other conditions associated with alterations in DA homeostasis like Parkinson's disease, Tourette's syndrome, depression and addiction.[85] Functional imaging studies measuring amphetamine-induced DA accumulation in control and schizophrenia subjects have reported opposite effects of amphetamine in the sensorimotor striatum and DLPFC.[80,86] Although methodological differences (neurochemical measurements of baseline DA vs functional imaging of DA receptor occupancy) preclude a direct comparison between our findings and these human studies, they suggest that DA imbalances do not result from an overall loss of DA content or DAergic terminals. Instead, in aggregate these studies point to alterations in the functional coupling of DA release mechanisms in schizophrenia patients.[80] Our observation that local KCl-mediated depolarization of DAergic terminals in the striatum and hippocampus/mPFC of TH-Cre;ErbB4$^{f/f}$ KO mice results in a normal accumulation of extracellular DA, and that ErbB4 deficiency does not result in a loss of DA terminals (consistent with a prior report[53]), suggest that TH-Cre;ErbB4$^{f/f}$ KO mice may serve as an important tool to dissect mechanisms relevant to schizophrenia and other diseases that affect DA homeostasis. Because re-expression of ErbB4 in DAergic midbrain neurons of adult TH-Cre;ErbB4$^{f/f}$ KO mice restores DA homeostasis and performance in behavioral tasks requiring spatial/working memory, it is tempting to speculate that ongoing NRG/ErbB4 signaling in the adult brain could constitute a potential target for therapeutic intervention to improve working memory and other cognitive parameters.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp).