Cellular/Molecular

Genetic Labeling Reveals Novel Cellular Targets of Schizophrenia Susceptibility Gene: Distribution of GABA and Non-GABA ErbB4-Positive Cells in Adult Mouse Brain

Jonathan C. Bean,[1] Thiri W. Lin,[1] Anupama Sathyamurthy,[1] Fang Liu,[1] Dong-Min Yin,[1,3] Wen-Cheng Xiong,[1,2] and Lin Mei[1,2]

[1]Department of Neuroscience and Regenerative Medicine, Medical College of Georgia, Georgia Regents University, Augusta, Georgia 30912, [2]Charlie Norwood Veterans Administration Medical Center, Augusta, Georgia 30904, and [3]Institute of Brain Functional Genomics, East China Normal University, Shanghai 200062, China

Neuregulin 1 (NRG1) **and its receptor *ErbB4* are schizophrenia risk genes. NRG1-ErbB4 signaling plays a critical role in neural development and regulates neurotransmission and synaptic plasticity. Nevertheless, its cellular targets remain controversial. ErbB4 was thought to express in excitatory neurons, although recent studies disputed this view. Using mice that express a fluorescent protein under the promoter of the *ErbB4* gene, we determined in what cells ErbB4 is expressed and their identity. ErbB4 was widely expressed in the mouse brain, being highest in amygdala and cortex. Almost all ErbB4-positive cells were GABAergic in cortex, hippocampus, basal ganglia, and most of amygdala in neonatal and adult mice, suggesting GABAergic transmission as a major target of NRG1-ErbB4 signaling in these regions. Non-GABAergic, ErbB4-positive cells were present in thalamus, hypothalamus, midbrain, and hindbrain. In particular, ErbB4 is expressed in serotoninergic neurons of raphe nuclei but not in norepinephrinergic neurons of the locus ceruleus. In hypothalamus, ErbB4 is present in neurons that express oxytocin. Finally, ErbB4 is expressed in a group of cells in the subcortical areas that are positive for S100 calcium binding protein β. These results identify novel cellular targets of NRG1-ErbB4 signaling.**

Key words: **ErbB4; Neuregulin; NRG1; oxytocin; S100β; serotonin**

Introduction

Neuregulin 1 (NRG1) is a trophic factor belonging to a family of proteins containing an EGF domain (Mei and Xiong, 2008; Mei and Nave, 2014). Its function is mediated by ErbB proteins, with ErbB4 being the only autonomous receptor (Guy et al., 1994; Sliwkowski et al., 1994; Garrett et al., 2003). Both *NRG1* and *ErbB4* are among well-characterized schizophrenia risk genes (Stefansson et al., 2002; Yang et al., 2003; Mei and Xiong, 2008; Mei and Nave, 2014). NRG1-ErbB4 signaling has been implicated in neural development (Flames et al., 2004; Li et al., 2007; Barros et al., 2009; Fazzari et al., 2010; Ting et al., 2011; Li et al., 2012a; Del Pino et al., 2013; Yin et al., 2013b). Both NRG1 and ErbB4 have been shown to regulate synaptic transmission and plasticity. Acute treatment with NRG1 increases GABA release (Woo et al., 2007) and thus inhibits pyramidal neuron firing and long-term potentiation (Huang et al., 2000; Chen et al., 2010; Wen et al., 2010; Li et al., 2012b; Tan et al., 2012).

However, cellular targets of the *ErbB4* gene remain controversial. ErbB4 was thought to express in excitatory neurons (Garcia et al., 2000; Huang et al., 2000; Ma et al., 2003; Kwon et al., 2005; Li et al., 2007; Iyengar and Mott, 2008; Barros et al., 2009; Pitcher et al., 2011) and regulate spines and synaptic plasticity via cell-autonomous mechanisms (Gu et al., 2005; Kwon et al., 2005; Li et al., 2007; Pitcher et al., 2011). However, ErbB4 transcripts are enriched in areas where interneurons are concentrated (Lai and Lemke, 1991; Woo et al., 2007), and its protein is expressed in GAD-positive hippocampal neurons (Huang et al., 2000; Woo et al., 2007). ErbB4 is present in newborn and migrating GABAergic interneurons (Yau et al., 2003) and, in adult, in parvalbumin (PV) and somatostatin interneurons (Vullhorst et al., 2009; Chen et al., 2010; Fazzari et al., 2010; Wen et al., 2010; Abe et al., 2011; Neddens et al., 2011; Ting et al., 2011). Recently, ErbB4 was shown to exclusively express in GABAergic interneurons in cortex and hippocampus (Vullhorst et al., 2009; Fazzari et al., 2010). ErbB4 mRNA was also detected in subcortical regions (Ozaki et al., 1997; Ma et al., 1999; Steiner et al., 1999; Bruce et al., 2002; Anton et al., 2004); however, cellular targets remain unclear due to low resolution of *in situ* hybridization.

To identify neurons or cells where ErbB4 protein is expressed in the brain, we generated *ErbB4-reporter* mice where tandem-

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Correspondence should be addressed to Dr. Lin Mei, Department of Neuroscience and Regenerative Medicine, Medical College of Georgia, Georgia Regents University, 1120 15th Street, CA4006, Augusta GA 30912. E-mail: lmei@gru.edu.

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Table 1. Antibodies used in the study

Species	Antigen	Clone	Source	Catalog #	Dilution
Mouse	ErbB4	H4.77.16	Lab Vision	MA5-12888	1:300
Rabbit	ErbB4	Poly	Gift (Zhu et al., 1995)	0618	1:500
Rabbit	PV	Poly	Swant	PV 25	1:1000
Rabbit	TH	Poly	Millipore	AB152	1:1000
Rabbit	5-HT	Poly	Protos Biotech	NT-102 5HTrab	1:1000
Rabbit	Oxytocin	Poly	Millipore	AB911	1:1000
Rabbit	Vasopressin	Poly	Millipore	PC234L	1:1000
Rabbit	S100β	Poly	Dako	Z031129-2	1:500
Mouse	GFAP	GA5	Millipore	MAB360	1:500
Mouse	MBP	1	Millipore	MAB382	1:500
Rabbit	Olig2	Poly	Millipore	AB9610	1:500

5-HT, Serotinin; GFAP, glial fibrillary acidic protein; Olig 2, oligodendrocyte transcription factor 2; PV, parvalbumin; MBP, myelin basic protein; TH, tyrosine hydroxylase.

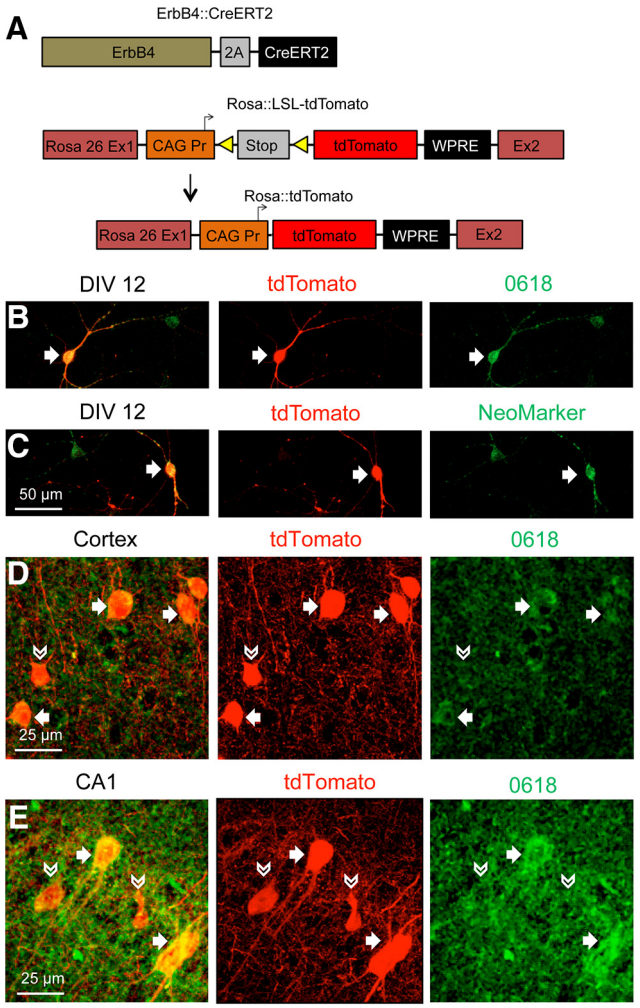

Figure 1. tdTomato labels ErbB4-expressing neurons. ***A***, Schematic of transgenes *ErbB4::CreERT2* and *Rosa::LSL-tdTomato*. A *ribosomal 2A skip* followed by an inducible *Cre recombinase (Cre) estrogen receptor T2 (ERT2)* was inserted after endogenous *ErbB4* gene stop codon. A *CMV-IE enhancer/chicken β-actin/rabbit β-globin hybrid (CAG) promoter, a loxP* (yellow triangles) flanked *stop* cassette, *tdTomato* red florescent protein, and a *woodchuck hepatitis virus post-translational regulatory element (WPRE)* were inserted between exon 1 (Ex1) and exon 2 (Ex2) of *Rosa26* gene. After CreERT2 is activated by tamoxifen, the stop cassette is removed from *Rosa::LSL-tdTomato*, resulting in *Rosa::tdTomato*, and tdTomato will be expressed in all cells that were expressing ErbB4 at time of induction. tdTomato-positive neurons in culture (***B***, ***C***) and in brain slices (***D***, ***E***) of *ErbB4-reporter* mice reacted to polyclonal, 0618 ErbB4 antibody (***B***, ***D***, ***E***) and monoclonal NeoMarker-H4.77.16 ErbB4 antibody (***C***). Arrows indicate neurons labeled by tdTomato and antibody. Arrowheads indicate neurons labeled by tdTomato alone.

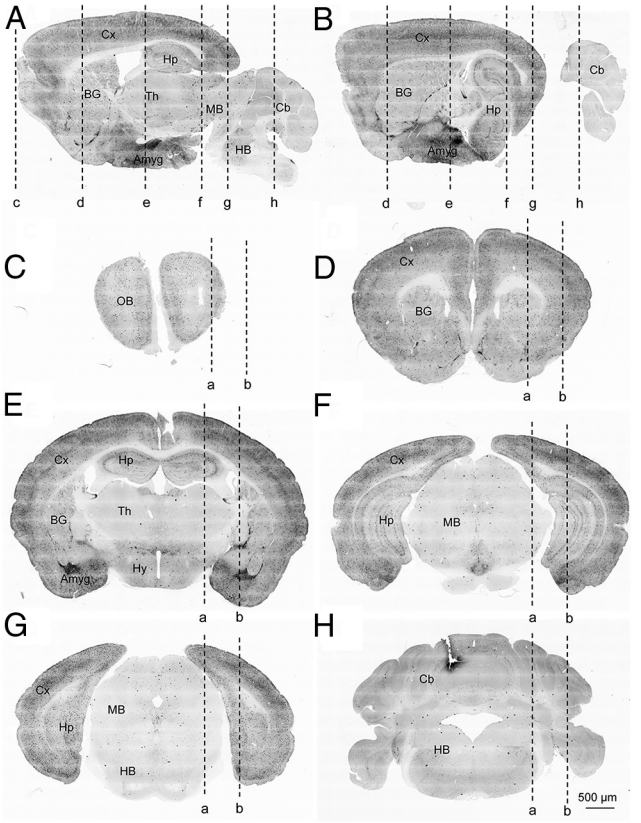

Figure 2. Unique distribution of ErbB4-positive cells in brain regions. Images (photographic negatives) of tdTomato florescence in *ErbB4-reporter* mouse brain slices illustrating overall ErbB4-positive cell expression. Composite images (***A***) from a sagittal section 1.80 mm lateral from midline; (***B***) from a sagittal section 2.52 mm lateral from midline; (***C***) from a coronal section 4.28 mm relative to bregma; (***D***) from a coronal section 1.54 mm relative to bregma; (***E***) from a coronal section −1.06 mm relative to bregma; (***F***) from a coronal section −3.80 mm relative to bregma; (***G***) from a coronal section −4.36 mm relative to bregma; and (***H***) from a coronal section −6.24 mm relative to bregma. ***A***, ***B***, Positions of the sagittal sections were indicated by lines a and b in ***C–H***. ***C–H***, Positions of the coronal sections were indicated by lines c–h in ***A***, ***B***. Amyg, Amygdala; BG, basal ganglia; Cb, cerebellum; Cx, cortex; HB, hindbrain; Hp, hippocampus; Hy, hypothalamus; MB, midbrain; OB, olfactory bulb.

Table 2. Density of ErbB4-positive cells in different brain regions[a]

Brain regions	ErbB4 cells/mm^3
Olfactory bulb	4600 ± 739
Cortex	7081 ± 780
Basal ganglia	3555 ± 607
Hippocampus	4667 ± 338
Amygdala	10,149 ± 795***
Thalamus	1245 ± 397*
Hypothalamus	5306 ± 1178
Midbrain	2271 ± 634
Hindbrain	1034 ± 270*
Cerebellum	1497 ± 469*
White matter	925 ± 199**
Total	4379 ± 599

[a]Data are mean ± SEM: $F_{(10,22)} = 20.53$, $p < 0.001$.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Bonferroni post hoc comparisons versus total.

dimer of DsRed (tdTomato) is expressed under the control of *ErbB4*'s promoter. ErbB4-positive cells were characterized with different markers and in hybrid with *GAD67::GFP* mice. Our results reveal that ErbB4 expression in cortex is restricted to GABAergic interneurons. In subcortical regions, ErbB4 is expressed in neurons or cells that express serotonin (5-HT) or oxy-

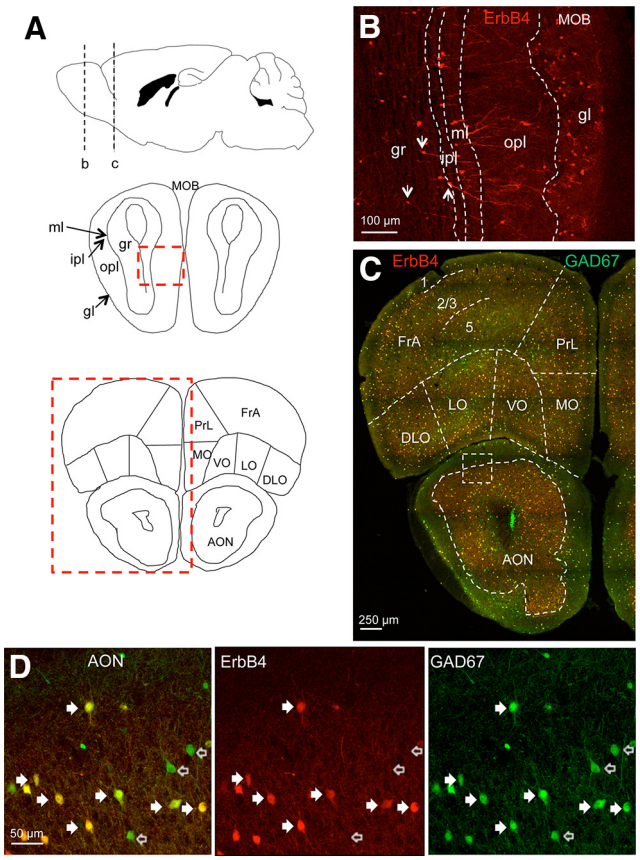

Figure 3. ErbB4-positive cells are enriched in the glomerulus and mitral cell layers of olfactory bulb. ***A***, Diagram of mouse brain sagittal section (top) and coronal sections (middle, bottom). Dashed lines b and c of the sagittal section diagram indicate the position of coronal sections. ***B***, Coronal section of *ErbB4-reporter* mouse brain, 4.28 mm relative to bregma. Area shown is indicated by the rectangle in the middle panel of ***A***. ***C***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, 2.68 mm relative to bregma. Area shown is indicated by the rectangle in the bottom panel of ***A***. ***D***, Enlarged image from the boxed area in ***C***. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. 1, Layer 1; 2/3, layers 2 and 3; 5, layer 5; AON, anterior olfactory nucleus; DLO, dorsal lateral orbital cortex; FrA, frontal association cortex; gl, glomerulus layer; gr, granular layer; ipl, inner plexiform layer; LO, lateral orbital cortex; MO, medial orbital cortex; MOB, main olfactory bulb; ml, mitral layer; opl, outer plexiform layer; PrL, prelimbic cortex; VO, ventral orbital cortex.

Table 3. ErbB4-positive cells are enriched in glomerular and mitral layers in the main olfactory bulb[a]

Main olfactory bulb layers	ErbB4 cells/mm^3
Glomerular	16,824 ± 3277*
Outer plexiform	1698 ± 409
Mitral	9304 ± 2358
Inner plexiform	5925 ± 1334
Granule cell	6232 ± 1472
Total	7467 ± 1583

[a]Data are mean ± SEM: $F_{(4,25)} = 7.76, p < 0.001$.

*$p < 0.05$, Bonferroni post hoc comparisons versus total.

tocin, or s100 calcium binding protein β (S100β). Our study identifies novel cellular targets of ErbB4 and suggests a role of NRG1-ErbB4 signaling in metabolism.

Materials and Methods

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Georgia Regents University. Mice were housed at 23°C with a 12 h light/dark cycle and food and water available *ad libitum*.

Table 4. Even distribution of ErbB4-postive cells in olfactory system[a]

Olfactory areas	ErbB4 cells/mm^3
Main olfactory bulb	5640 ± 1650
Accessory olfactory bulb	7221 ± 2146
Anterior olfactory nucleus	4546 ± 666
Olfactory tubercle	2713 ± 540
Piriform cortex	4428 ± 392
Taenia tecta	4830 ± 888
Dorsal peduncular	7069 ± 319
Total	4600 ± 739

[a]Data are mean ± SEM: $F_{(6,14)} = 1.94, p = 0.145$.

Table 5. Slight variation in density of ErbB4-positive cells in cortex[a]

Cortical regions	ErbB4 cells/mm^3
Frontal association	5840 ± 237
Prelimbic	6341 ± 662
Infralimbic area	8695 ± 858
Medial orbital	6243 ± 520
Ventral orbital	6219 ± 764
Lateral orbital	5907 ± 1143
Dorsal lateral orbital	6001 ± 583
Cingulate	7518 ± 1297
Retrosplenial	7410 ± 1309
Parietal association	9429 ± 778
Temporal association	8392 ± 514
Primary motor	6462 ± 899
Secondary motor	6798 ± 932
Primary somatosensory	6747 ± 1105
Secondary somatosensory	6082 ± 621
Gustatory/digestive	6422 ± 831
Primary visual	9180 ± 1173
Secondary visual	9256 ± 879
Primary auditory	8595 ± 788
Ventral auditory	7875 ± 438
Dorsal auditory	8249 ± 450
Insular	5636 ± 436
Ectorhinal	7527 ± 403
Entorhinal	7051 ± 548
Perirhinal	6776 ± 674
Total	7081 ± 780

[a]Data are mean ± SEM: $F_{(24,50)} = 2.15, p = 0.011$.

Generation of ErbB4-reporter and ErbB4-reporter; GAD67::GFP mice. Mice with targeted knock-in transgenes *ErbB4::CreERT2* and *Rosa::LSL-tdTomato* (Madisen et al., 2010) were purchased from the The Jackson Laboratory (stock #012360 and #007905, respectively). *GAD67::GFP* mice were a gift from Dr. Yuchio Yanagawa (National Defense Medical College Hospital, Saitama, Japan) (Tamamaki et al., 2003). In *ErbB4::CreERT2* mice, *CreERT2* was inserted after the stop codon of the *ErbB4* gene, following a ribosomal 2A skip (2A). CreERT2 expression is thus controlled by the promoter of the *ErbB4* gene and had no effect of ErbB4 expression or function because CreERT2 is expressed as a separate protein (Madisen et al., 2010). *Rosa::LSL-tdTomato* has a cassette inserted between exon 1 and exon 2 of the *Rosa26* gene, which contains the CMV-IE enhancer/chicken β-actin/rabbit β-globin hybrid promoter, a loxP-stop-loxP (LSL), tdTomato red florescent protein and a woodchuck hepatitis virus post-translational regulatory element (Madisen et al., 2010). *GAD67::GFP* has an enhanced green florescent protein inserted after exon 1 of *GAD67* (Tamamaki et al., 2003).

Primers for genotyping PCR were described as follows: *ErbB4::CreERT2*, 5′-GGGAG GATTG GGAAG ACAAT-3′, 5′-CCTGC AGGAA TACAG CACAA-3′, and 5′-AAAGA TGGGG CTCTT TGACA-3′; *Rosa::LSL-tdTomato*, 5′-AAGGG AGCTG CAGTG GAGTA-3′, 5′-CCGAA AATCT GTGGG AAGTC-3′, 5′-GGCAT TAAAG CAGCG TATCC-3′, and 5′-CTGTT CCTGT ACGGC ATGG-3′; *GAD67::GFP*, 5′-GGCAC AGCTC TCCCT TCTGT TTGC-3′, 5′-GCTCT CCTTT

CGCGT TCCGA CAG-3′, and 5′-CTGCT TGTCG GCCAT GATAT AGACG-3′. PCR of *ErbB4::CreERT2* resulted in a 465 bp product for mice lacking the transgene, a 190 bp product for mice with the transgene and both products for mice with one allele of each. PCR of *Rosa::LSL-tdTomato* resulted in a 350 bp product for mice lacking the transgene, a 200 bp product for mice with the transgene, and both products for mice with one allele of each. PCR of *GAD67::GFP* resulted in a 654 bp product for mice lacking the transgene, and both a 265 bp and 654 bp product for mice heterozygotic for the transgene. Mice with two copies of *GAD67::GFP* are not viable and are thus were not used.

Breeding *ErbB4::CreERT2* and *Rosa::LSL-td Tomato* mice generated double-transgenic *ErbB4::CreERT2; Rosa::LSL-tdTomato* mice (hereafter referred as *ErbB4-reporter* mice). On postnatal day 1 (P1) and postnatal day 60 (P60) mice were administered with 180 mg/kg tamoxifen, once orally and intraperitoneally, every other day over 5 d, respectively, and euthanized at P7 and P70, respectively. Tamoxifen activates CreERT2 in ErbB4-expressing cells and subsequent Cre-mediated removal of the stop signal in the *LSL-tdTomato* transgene. Ensuing tdTomato expression enabled detection of ErbB4-positive neurons. Five days after the final tamoxifen injection, mice were euthanized and brains were harvested. All experiments were performed on male mice.

Neuron culture. Neurons were prepared from embryonic day 18 (E18) *ErbB4::CreERT2; Rosa::LSL-tdTomato* mouse embryos and cultured in neurobasal medium (catalog #21103-049; Invitrogen) supplemented with B27 (catalog #17504-044; Invitrogen), 600 μM L-glutamine (catalog #25-005-CI; Cellgro), and penicillin-streptomycin (catalog #30-003-CI; Cellgro). For low-density culture, 2.5×10^4 cells were seeded on a glass coverslip (1.8 cm in diameter; catalog #12-545-84; Fisher Scientific) coated for 12 h with 1 μg/ml poly-L-lysine (catalog #P2636; Sigma) in 12 well plates (catalog #CLS3513-50EA, Sigma). At day *in vitro* (DIV) 7, neurons were treated with 2 μM tamoxifen for 1–3 h and fixed for at DIV 12.

Neurons were fixed in 4% PFA at room temperature for 20 min. Neurons were washed 3 times in PBS (10 min each) and incubated (10 min) in 0.3% Triton/PBS. Next, neurons were incubated in 10% goat serum for 1 h and with

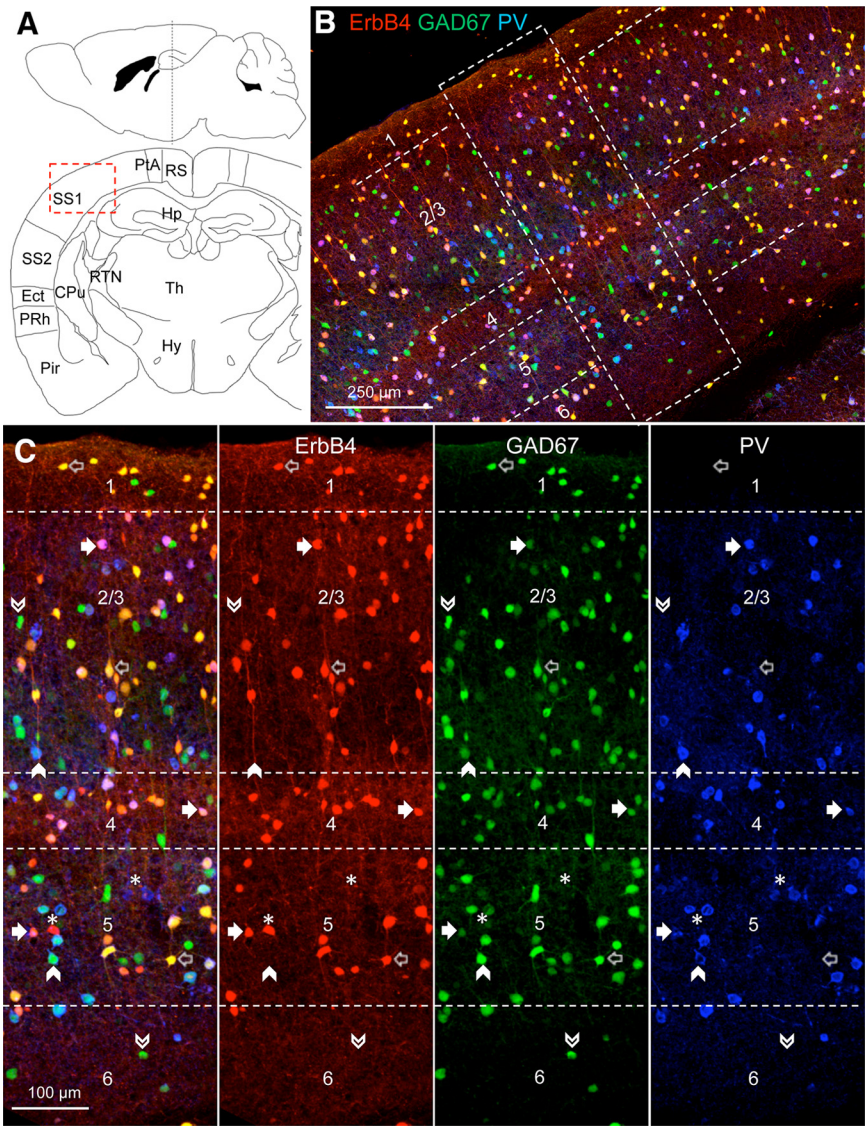

Figure 4. ErbB4-positive cells in cortex are enriched in layer 2/3, are GABAergic interneurons and positive for PV. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter* mouse brain counterstained with anti-PV, −1.58 mm relative to bregma. Area shown is indicated in the rectangle in ***A***. ***C***, Coronal section of the primary somatosensory cortex (SS1) spanning layers 1–6. Area shown is indicated in the rectangle in ***B***. Arrows indicate neurons positive for ErbB4, GAD67, and PV. Empty arrows indicate neurons positive for ErbB4 and GAD67 but not PV. Arrowhead indicates neurons positive for GAD67 and PV but not ErbB4. Empty arrowhead indicates neurons positive for GAD67 alone. *Cells positive for either ErbB4 or PV but not GAD67. CPu, Caudate–putamen; GI/DI, gustatory and digestive cortex; Hp, hippocampus; Hy, hypothalamus; Ins, insular cortex; LV, lateral ventricle; M1, primary motor cortex; M2, secondary motor cortex; Pir, piriform cortex; RS, retrosplenial cortex; SS1, primary somatosensory cortex; SS2, secondary somatosensory cortex; Th, thalamus.

Table 6. ErbB4-positive cells in cortex are GABAergic interneurons, many of which are PV-positive[a]

	Layer	GAD67/ErbB4	PV/ErbB4	ErbB4/GAD67	PV/GAD67	ErbB4/PV	GAD67/PV
SS1	1	100.0 ± 0.0	0.0 ± 0.0	87.7 ± 3.5	0.0 ± 0.0	NA	NA
	2/3	98.7 ± 0.7	38.3 ± 1.3	62.5 ± 2.4	35.6 ± 0.7	62.3 ± 3.0	91.5 ± 1.3
	4	97.9 ± 1.3	66.6 ± 2.9	62.4 ± 5.9	53.4 ± 1.5	72.9 ± 5.6	92.4 ± 2.6
	5	96.7 ± 1.0	55.9 ± 4.3	61.9 ± 1.9	56.4 ± 5.2	52.4 ± 3.5	82.2 ± 3.8
	6	95.6 ± 1.5	35.0 ± 6.7	65.5 ± 2.9	34.3 ± 5.4	65.7 ± 2.7	95.2 ± 3.0
	Total	97.9 ± 0.7	42.8 ± 0.7	64.1 ± 1.8	40.6 ± 1.0	61.1 ± 2.2	88.3 ± 1.6
FrA	1	100.0 ± 0.0	0.0 ± 0.0	79.6 ± 6.4	0.0 ± 0.0	NA	NA
	2/3	99.0 ± 0.5	31.7 ± 9.4	75.1 ± 3.6	24.9 ± 10.8	92.2 ± 4.9	99.0 ± 0.7
	5	98.3 ± 0.3	55.9 ± 6.8	70.5 ± 4.3	52.8 ± 12.2	78.3 ± 8.7	97.1 ± 1.0
	Total	98.8 ± 0.2	42.2 ± 6.0	72.4 ± 2.4	39.3 ± 9.3	81.1 ± 6.9	97.8 ± 0.4

[a]Data are mean percentage ± SEM. SS1, Primary somatosensory cortex; FrA, frontal association cortex; NA, not applicable.

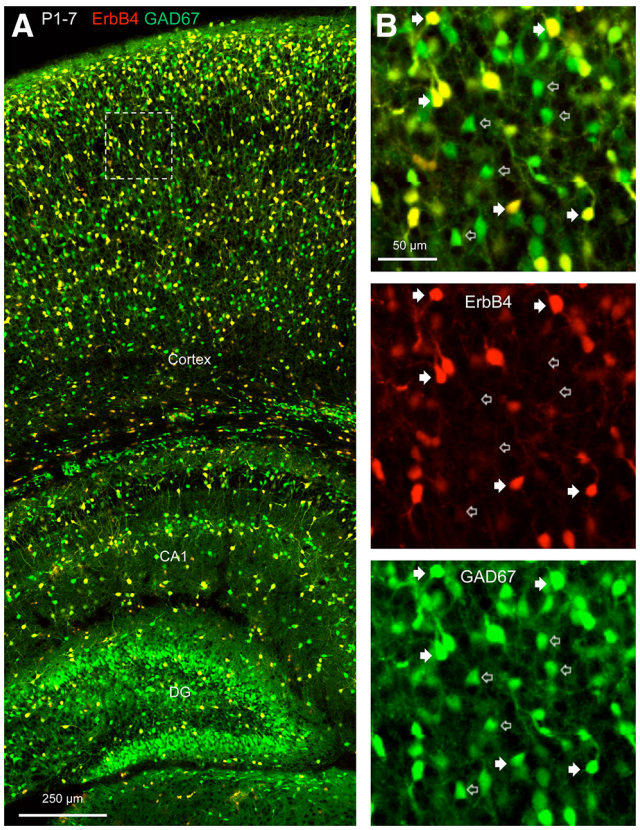

Figure 5. ErbB4-positive cells in cortex and hippocampus of neonatal mice are GABAergic interneurons. ***A***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain. Expression of tdTomato was induced on P1 and examined on P7. ***B***, Enlarged area of the squared region in ***A***. Arrows indicate neurons positive for ErbB4 and GAD67. Open arrows indicate neurons positive for GAD67 alone. CA1, CA1 area of hippocampus; DG, dentate gyrus of hippocampus; P1–7, postnatal days 1–7.

primary antibody for 24 h at 4°C (Table 1). After washing 3 times in PBS (10 min each), neurons were incubated with AlexaFluor-488 anti-mouse or rabbit antibody (Invitogen) for 1 h at room temperature. Neurons were washed 3 times in PBS before being mounted on Superfrost Plus Microscope Slides (Fisher Scientific) and sealed with Vectasheild Mounting Media (Vector). Images were taken using a Zeiss LSM 710 scanning confocal microscope under a 40 × oil-immersion objective.

Analysis of ErbB4-positive cells. After being anesthetized with a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture, mice were transcardially perfused with PBS (2 ml/g of body weight), followed by 4% PFA in PBS. Brains were harvested, incubated in 4% PFA overnight, and dehydrated at 4°C in two steps with 15% and 30% sucrose in PBS. Brains were frozen in OCT (catalog #14-373-65; Fisher) and sectioned into 25 μm slices on a cryostat microtome (Bosch Microm HM550) at −20°C. Slices were collected serially into 24 well plates in a cryoprotective solution (30% propylene glycol, 30% glycerin, and 40% PBS) and stored at −20°C until microscopy studies. Coronal brain slices from ~3.08 to 2.58 mm relative to bregma were used to access frontal association cortex, 0.74 to −1.94 mm relative to bregma to access primary somatosensory cortex, and −1.34 to −2.18 mm relative to bregma to access dorsal hippocampus.

Every 24th slice (one from each 24 well plate), 575 μm apart, was selected for analysis of ErbB4-positive cell density. Slices were first washed 3 times in PBS for 10 min each, mounted on Superfrost Plus Microscope Slides (Fisher Scientific), and sealed with Vectasheild Mounting Media (Vector) and Premium Cover Glass (Fisher Scientific). Images were taken on a Zeiss LSM 710 scanning confocal microscope with automated stage using a 20 × air objective and stitched together to make complete images of a brain region or section. Brain regions were identified by using anatomical atlases (Paxinos and Franklin, 2001; Lein et al., 2007). For cortical and hippocampal slices, layers were discerned by differences in neuropil and with the aid of anatomical atlas (Lein et al., 2007).

Table 7. Cells expressing ErbB4 at P1-P7 are GABAergic interneurons in cortex and hippocampus[a]

P1-P7	GAD67/ErbB4	ErbB4/GAD67
Cortex	99.4 ± 0.1	65.9 ± 0.4
CA1–3	99.2 ± 0.2	60.4 ± 0.3
DG	99.6 ± 0.2	39.2 ± 2.4

[a]Data are mean percentage ± SEM. CA1–3, CA1–3 areas of hippocampus; DG, dentate gyrus of hippocampus.

Table 8. ErbB4-positive cell densities vary in hippocampus[a]

Hippocampal regions	ErbB4 cells/mm^3
CA1	3689 ± 129
CA2	5000 ± 25
CA3	3594 ± 630
Dentate gyrus	3279 ± 54
Dorsal subiculum	4918 ± 1108
Ventral subiculum	7907 ± 620*
Presubiculum	7407 ± 1175
Parasubiculum	9329 ± 569**
Total	4667 ± 338

[a]Data are mean ± SEM: $F_{(7,16)} = 11.13$, $p < 0.001$.
*$p < 0.05$; **$p < 0.001$; Bonferroni post hoc comparisons versus total.

To quantify the density of tdTomato-positive cells, slices (25 μm in thickness) of every 575 μm in a given region or nucleus were examined for positive cells. Positive cells were marked using ImageJ software (National Institutes of Health) multipoint tool. The cell density (cell number per mm^3) was calculated by the following equation: cell density = number of positive cells of *n* slices/sum volume of *n* slices. Brains were divided into 11 major brain regions, which were subdivided to 173 unique regions.

Immunofluorescent labeling. Coronal sections from −0.58 to −1.22 mm relative to bregma were used to access if ErbB4 was expressed in oxytocin or vasopressin neurons. Coronal sections from −3.08 mm to −3.80 mm relative to bregma were used to determine whether ErbB4 was expressed in dopamine neurons of the ventral tegmental area (VTA) or substantia nigra compacta. Coronal sections from −3.80 mm to −4.72 mm relative to bregma were used to reveal whether ErbB4 was expressed in serotonin neurons of the raphe nuclei. Coronal sections from −5.34 to −5.52 mm relative to bregma were used to determine whether ErbB4 was expressed in norepinephrine neurons of the locus ceruleus. Coronal sections from −0.46 to −6.64 mm relative to bregma were used to access the identity of the non-neuronal cell that expressed ErbB4.

Brain slices were washed 3 times in PBS for 10 min each, blocked in a solution (10% goat serum, 1% BSA, 0.3% Triton) for 1 h at room temperature, and incubated with primary antibodies (Table 1) for 24 h at 4°C. After washing 3 times in PBS for 10 min each, slices were incubated with AlexaFluor-488 or -405 anti-rabbit or mouse antibodies (Invitrogen) for 1 h at room temperature. Slices were washed 3 times in PBS for 10 min each, mounted on Superfrost Plus Microscope Slides (Fisher Scientific) with Vectasheild Mounting Media (Vector), and covered with Premium Cover Glass (Fisher Scientific). Images were taken on Zeiss LSM 710 scanning confocal microscope with automated stage using a 40× oil-immersion objective. They were stitched together for complete view of regions of interest.

Statistical analysis. ANOVA was used to access variation of ErbB4-positive cell density across and within major brain regions. Regions were compared against an overall score *post hoc* using Bonferroni correction for multiple comparisons. Data were expressed as mean ± SEM.

Results

Generation and validation of *ErbB4-reporter* mice

We generated mice that expressed tdTomato (a red florescent protein) specifically in ErbB4-expressing cells by crossing *ErbB4::*

CreERT2 mice, where CreERT2 expression is under the control of endogenous *ErbB4* promoter, with *Rosa::LSL-tdTomato* mice (hereafter referred to as *ErbB4-reporter* mice) (Madisen et al., 2010). CreERT2 was inactive in the absence of tamoxifen; thus, no tdTomato expression was detected (data not shown). In mice that were treated with tamoxifen, the CreERT2 became active and thus removed the LSL cassette to enable tdTomato expression (Fig. 1*A*). To determine whether tdTomato is faithfully expressed in cells that express ErbB4, we cultured cortical neurons from *ErbB4-reporter* mice at E18. Neurons were stained with 0618 antibody, a rabbit polyclonal antibody raised against the intracellular domain of ErbB4. This antibody has been used widely to label ErbB4 in cultured cortical neurons and in brain slices (Zhu et al., 1995; García-Rivello et al., 2005; Ghashghaei et al., 2006; Woo et al., 2007; Chen et al., 2010; Wen et al., 2010; Del Pino et al., 2013). As shown in Figure 1*B* (solid arrow), cortical neurons expressing tdTomato were stained positive. To further validate ErbB4-positive cells, cortical neurons were stained with another antibody NeoMarker-H4.77.16, a mouse monoclonal antibody that was raised against the extracellular domain of ErbB4 and reacts with ErbB4-expressing neurons (Vullhorst et al., 2009; Neddens and Buonanno, 2011; Neddens et al., 2011). The monoclonal antibody labeled more strongly tdTomato-expressing neurons than tdTomato-negative neurons (Fig. 1*C*). These observations demonstrate that tdTomato is expressed in neurons that express endogenous ErbB4 and can be used as a faithful indicator of ErbB4-positive neurons. This notion was supported by staining of cortical and hippocampal slices with the 0618 antibody (Fig. 1*D*,*E*).

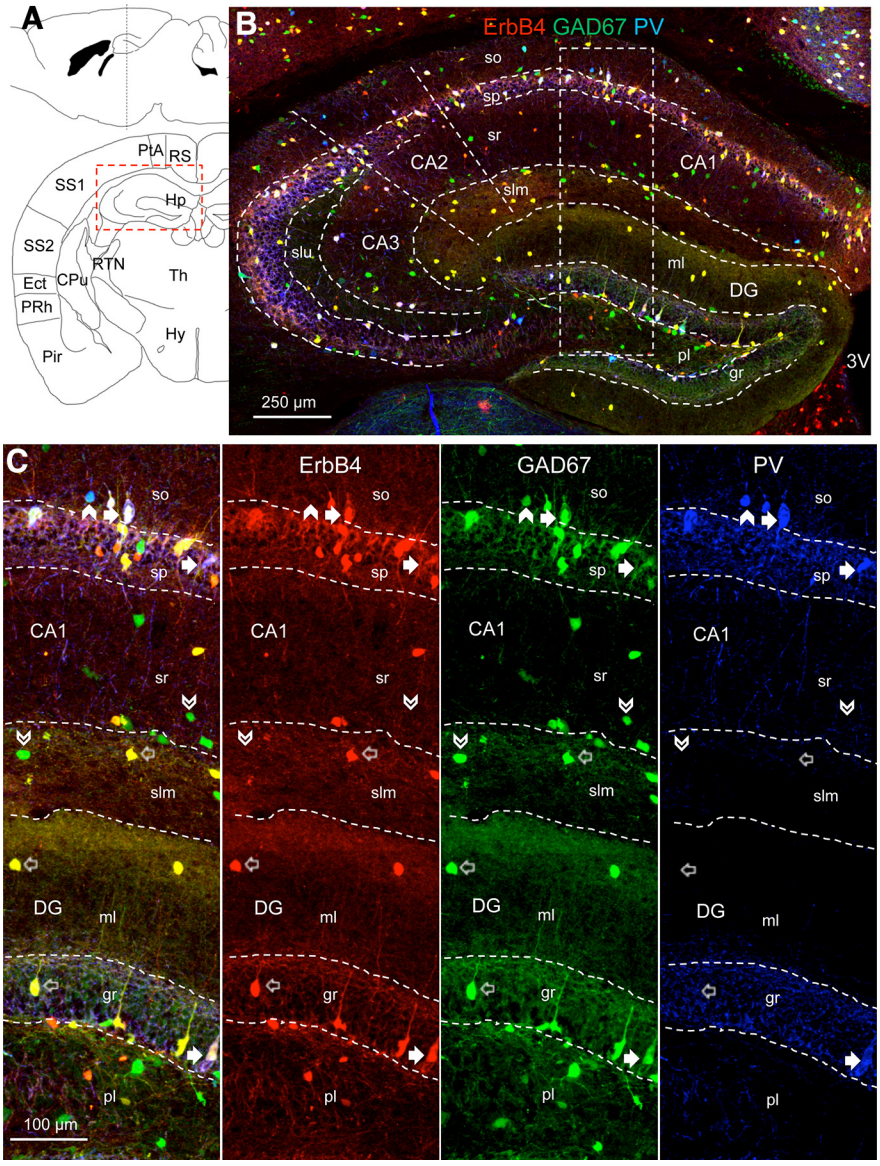

Figure 6. ErbB4-positive cells in hippocampus are concentrated in stratum pyramidal and are GABAergic interneurons and positive for PV. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain counterstained with anti-PV, −1.58 mm relative to bregma. Area shown is indicated in the rectangle in ***A***. ***C***, Layers of CA1 and DG areas indicated by rectangle in ***B***. Arrows indicate neurons positive for ErbB4, GAD67, and PV. Empty arrows indicate neurons positive for ErbB4 and GAD67 but not PV. Arrowhead indicates neurons positive for GAD67 and PV but not ErbB4. Empty arrowhead indicates neurons positive for GAD67 alone. 3V, Third ventricle; CPu, caudate–putamen; Ect, ectorhinal cortex; gr, granular layer; Hp, hippocampus; Hy, hypothalamus; ml, molecular layer; Pir, piriform cortex; pl, polymorph layer; PRh, perirhinal cortex; PtA, parietal association cortex; slu, stratum lucidum; slm, stratum lacunosum-moleculare; so, stratum oriens; sp, stratum pyramidal; sr, stratum radiatum; RS, retrosplenial cortex; RTN, reticular thalamic nucleus; SS1, primary somatosensory cortex; SS2, secondary somatosensory cortex; Th, thalamus.

Density of ErbB4-positive cells varies across brain regions

We first assessed the global distribution of ErbB4-positive cells in the brain. ErbB4-positive cells were widely expressed but showed obvious regional differences. Numerically, cortex had a higher concentration of positive cells than the overall average (Fig. 2; Table 2). Amygdala had a particular enrichment of ErbB4-positive cells with a significant difference from the overall total (Fig. 2*A*,*B*,*E*). Thalamus, hindbrain, and cerebellum had significantly lower concentrations than that of overall brain (Fig. 2*E*,*G*,*H*). Midbrain had a numerical lower number of ErbB4-positive cells than whole brain (Fig. 2*F*,*G*). The density of ErbB4-positive cells in olfactory bulb, basal ganglia, and hypothalamus was similar to that of entire brain (Fig. 2*C*–*E*). It is known that LTP is difficult to induce in amygdala presumably because of high GABAergic activity (Tang et al., 1999; Rammes et al., 2000; Sigurdsson et al., 2010). Our result may provide an underlying mechanism, considering NRG1-ErbB4 promotion of GABA release (Woo et al., 2007).

Since recent immunolabeling reports demonstrating that ErbB4 is exclusively expressed in GABAergic interneurons in cortex and hippocampus (Vullhorst et al., 2009; Neddens and Buonanno, 2011; Neddens et al., 2011), we began our studies with experiments to test this hypothesis. *ErbB4-reporter* mice were crossed with mice that expressed GFP under the endogenous promoter of *GAD67*, which effectively labels all GABAergic cells (Tamamaki et al., 2003). Unexpectedly, we identified many ErbB4-positive cells to be non-GABAergic in subcortical areas. We characterized these cells by

Table 9. ErbB4-positive cells in hippocampus are GABAergic interneurons, many of which are PV-positive[a]

	Layer	GAD67/ErbB4	PV/ErbB4	ErbB4/GAD67	PV/GAD67	ErbB4/PV	GAD67/PV
CA1–3	so	95.6 ± 1.7	41.1 ± 3.2	52.7 ± 0.8	47.9 ± 5.6	47.2 ± 2.8	98.2 ± 1.1
	sp	98.9 ± 0.7	48.3 ± 5.3	84.6 ± 1.8	49.6 ± 5.8	82.2 ± 2.4	98.4 ± 1.6
	slu	96.9 ± 3.1	41.8 ± 7.5	71.8 ± 8.1	33.7 ± 6.8	93.3 ± 3.9	100.0 ± 0.0
	sr	98.7 ± 1.3	15.4 ± 3.8	56.6 ± 2.6	11.2 ± 3.2	67.9 ± 7.6	82.7 ± 11.2
	slm	99.6 ± 0.4	1.8 ± 1.8	87.0 ± 2.5	1.5 ± 1.5	NA	NA
	Total	98.4 ± 0.2	29.8 ± 0.7	71.4 ± 1.3	29.7 ± 1.2	71.0 ± 1.42	97.5 ± 0.1
DG							
	ml	94.7 ± 3.7	4.3 ± 1.5	98.2 ± 1.8	4.6 ± 1.6	100.0 ± 0.0	100.0 ± 0.0
	gr	99.0 ± 1.0	16.3 ± 2.3	81.5 ± 5.5	16.3 ± 3.9	86.7 ± 8.2	100.0 ± 0.0
	pl	96.9 ± 3.1	5.0 ± 5.0	43.0 ± 4.5	4.2 ± 4.2	66.7 ± 0.0	100.0 ± 0.0
	Total	96.9 ± 1.2	10.4 ± 1.2	75.1 ± 1.7	9.7 ± 1.2	83.9 ± 6.8	100.0 ± 0.0

[a]Data are mean percentage ± SEM. so, Stratum oriens; sp, stratum pyramidal; slu, stratum lucidum; sr, stratum radiatum; slm, stratum lacunosum-moleculare; ml, molecular layer; gr, granule layer; pl, polymorph layer.

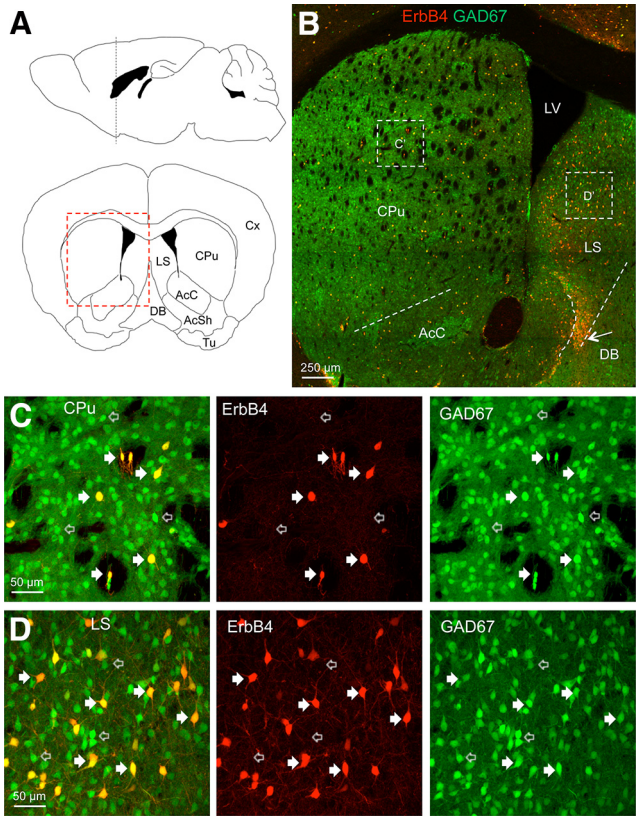

Figure 7. ErbB4-positive cells in the basal ganglia show unique distribution and are GABAergic interneurons. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, 0.98 mm relative to bregma. Area shown is indicated by the rectangle in ***A***. ***C***, Caudate–putamen area indicated by square ***C′***. ***D***, Lateral septum area indicated by square ***D′***. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. AcC, Nucleus accumbens core; AcSh, nucleus accumbens shell; CPu, caudate–putamen; Cx, cortex; DB, diagonal band nucleus; LS, lateral septum; LV, lateral ventricle; Tu, olfactory tubercle.

Table 10. ErbB4-positive cell densities vary in basal ganglia[a]

Basal ganglia regions	ErbB4 cells/mm^3
Caudate putamen	2743 ± 407
Nucleus accumbens core	4194 ± 433
Nucleus accumbens shell	5372 ± 618
Claustrum	4562 ± 403
Globus pallidus	707 ± 285
Ventral pallidum	2017 ± 672
Lateral septum	4904 ± 908
Medial septum	2593 ± 903
Diagonal bands nucleus	5582 ± 1808
Substantia innominata	4533 ± 1432
Fundus of striatum	9615 ± 2207*
Bed nucleus of the stria terminalis	6019 ± 1476
Total	3555 ± 607

[a]Data are mean ± SEM: $F_{(11,24)} = 4.09, p = 0.002$.

*$p < 0.01$; Bonferroni post hoc comparisons versus total.

immunohistochemical analysis using different antibody markers to elucidate their cell type. In the following, we describe the distribution of ErbB4-positive cells, cellular identity, and functional implications.

Olfactory system

The olfactory system, responsible for the sense of smell, can be divided into several sections: main olfactory bulb, accessary olfactory bulb, and olfactory cortical regions. The main olfactory bulb has five characteristic layers (Fig. 3*A*,*B*): the glomerular layer, the most superficial where axons of olfactory neurons form synapses onto dendrites of mitral cells in glomeruli; the outer plexiform layer containing axons and dendrites; the mitral layer where the soma of mitral cells are located; the internal plexiform layer with dendrites and axons; and the granule cell layer composed of granule interneurons (Scott, 2008; Nolte, 2009a). ErbB4-positive cells were localized in all five layers; however, the density was highest in the glomerular layer, followed by the mitral cell layer (Table 3). Moreover, ErbB4-positive cells in the granule cell layer appeared to project into the mitral cell layer as well as the outer plexiform layer (Fig. 3*B*, arrows). In agreement, ErbB4 is present in migrating neuroblasts in the rostral migratory stream of the developing brain, which go on to populate the olfactory bulb (Anton et al., 2004; Ghashghaei et al., 2006; Long et al., 2007).

The olfactory bulb relays information to olfactory cortical regions, such as anterior olfactory nucleus, piriform cortex, olfactory tubercle, and dorsal tenia tectum. ErbB4-positive cells were present throughout the olfactory system (Table 4). Most, if not all, ErbB4-positive cells colocalized with GAD67-GFP, indicating they were GABAergic. However, many GAD67-positive cells did not show evidence of being ErbB4-positive, indicating that not all GABAergic neurons in the olfactory system express ErbB4 (Fig. 3*C*,*D*).

Cortex

The cerebral cortex is the outermost structure of the vertebrate brain, where high-level processing occurs for varied processes, including motor control and sensory perception. There appeared

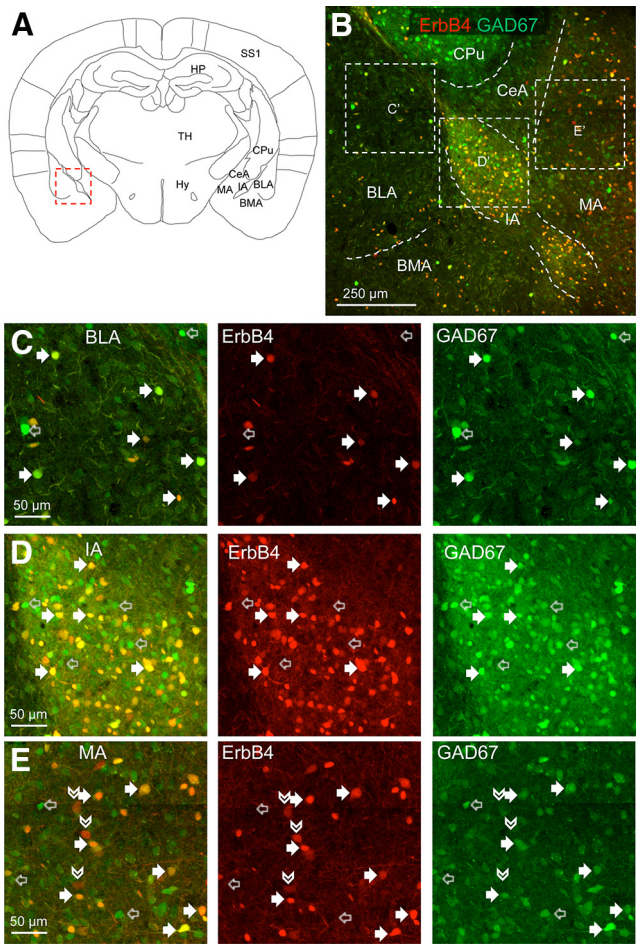

Figure 8. ErbB4-positive cells in the amygdala are concentrated in the intercalated nucleus. ***A***, Diagram of mouse coronal section. The position is indicated by the dashed line in Figure 6*A*. ***B***, Amygdala of *ErbB4-reporter; GAD67::GFP* mouse brain, −1.58 mm relative to bregma. Dashed lines indicate nuclei of the amygdala. Areas in squares ***C′***, ***D′***, and ***E′*** are shown in panels ***C–E***. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. Arrowheads indicate neurons positive for ErbB4 alone. BLA, Basal lateral amygdala; BMA, basal medial amygdala; CeA, central amygdala; Hp, hippocampus; Hy, hypothalamus; IA, intercalated nucleus of the amygdala; MA, medial amygdala; SS1, primary somatosensory cortex; Th, thalamus.

Table 11. ErbB4-positive cells are enriched in the intercalated nucleus and medial amygdala, within the amygdala[a]

Amygdala regions	ErbB4 cells/mm^3
Basal lateral amygdala	8609 ± 1058
Basomedial amygdala	8254 ± 1675
Medial amygdala	13,600 ± 2120
Central amygdala	2834 ± 552
Amygdala cortex	10,190 ± 694
Anterior amygdala area	6874 ± 1665
Posterior amygdala area	13,590 ± 1626
Intercalated nucleus	30,800 ± 5365*
Total	10,150 ± 795

[a]Data are mean ± SEM: $F_{(7,16)} = 13.10$, $p < 0.001$.

*$p < 0.001$; Bonferroni post hoc comparisons versus total.

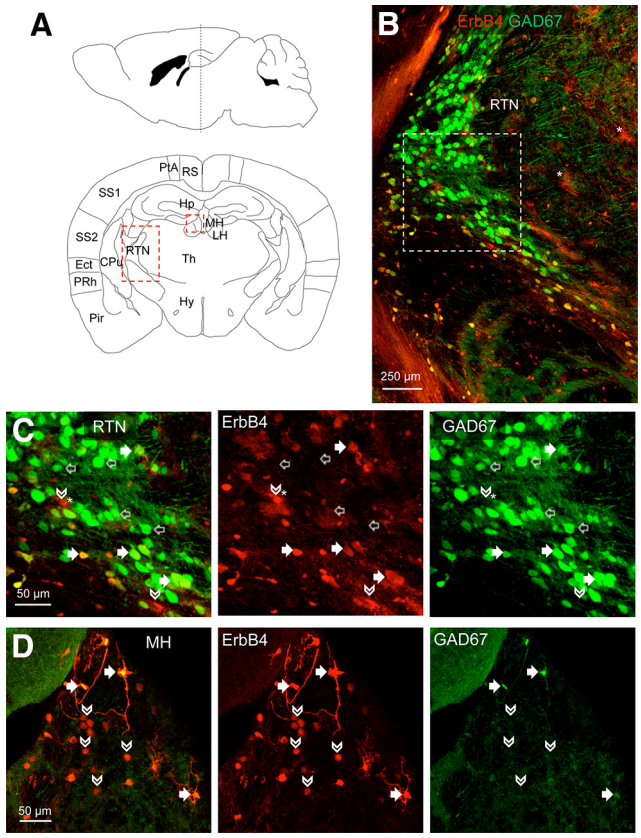

Figure 9. ErbB4-positive cells are enriched in medial habenula. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Dashed line indicates coronal section position. ***B***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, −1.58 mm relative to bregma. Position of the section is indicated by rectangle in ***A***. *ErbB4-positive cells with non-neuronal morphology. ***C***, Reticular thalamic nucleus area indicated by square in ***B***. ***D***, Coronal section of medial habenula, −1.58 mm relative to bregma. Position of the section is indicated by smaller box in ***A***. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. Arrowheads indicate neurons positive for ErbB4 alone. CPu, Caudate–putamen; Ect, ectorhinal cortex; Hp, hippocampus; Hy, hypothalamus; LH, lateral habenula; MH, medial habenula; Th, thalamus; Pir, piriform cortex; PRh, perirhinal cortex; PtA, parietal association cortex; SS1, primary somatosensory cortex; SS2, secondary somatosensory cortex; RS, retrosplenial cortex; RTN, reticular thalamic nucleus.

to be more ErbB4-positive cells in the cortex than other brain regions except the amygdala. However, the density of ErbB4-positive cells varied in different cortical regions: with highest in parietal association, primary visual and secondary visual cortices and lowest in frontal association, lateral orbital, and insular cortices (Table 5).

The cortex has a well-defined layered structure. We analyzed ErbB4-positive cell distribution in cortical layers of the primary somatosensory cortex (Fig. 4*A*,*B*). Among the six layers, ErbB4-positive cells were densest in the external granular/external pyramidal layers (or layer 2/3) that is populated by pyramidal neurons projecting to other cortical areas. Following that were the molecular layer (or layer 1) where apical dendrites of pyramidal neurons are localized; the internal granular layer (or layer 4) where spiny stellate neurons are located; the internal pyramidal layer (or layer 5) that is populated with pyramidal neurons that project to the spinal cord and subcortical areas; and the polymorphic layer (or layer 6) that is composed of morphologically variable excitatory neurons that project to thalamus (Hendry et al., 2008; Nolte, 2009b). Similar distribution was observed in the frontal association cortex (Fig. 3*C*).

In both neonatal and adult mice, nearly all ErbB4-positive cells were GAD67-positive in various layers of both cortices, suggesting that ErbB4-positive cells are GABAergic interneurons (Figs. 4 and 5; Tables 6 and 7). In adult mice, >40% of ErbB4-positive cells were also positive for PV, indicating that they were basket and chandelier subtypes of interneurons (Fig. 4C; Table 6). However, only a fraction of GAD67-positive cells were positive for ErbB4. In layer 5, for example, 38.1% of GAD67-positive

Table 12. ErbB4-positive cells are enriched in the medial habenula of the thalamus[a]

Thalamus regions	ErbB4 cells/mm^3
Paraventricular thalamic nucleus	2860 ± 936
Central medial thalamic nucleus	342 ± 150
Reuniens thalamic nucleus	1798 ± 470
Reticular thalamic nucleus	1267 ± 429
Rhomboid thalamic nucleus	535 ± 70
Anterior thalamic area	414 ± 155
Posterior thalamic nucleus	949 ± 603
Submedius	869 ± 482
Stria medullaris	5451 ± 4024
Mediodorsal thalamic nucleus	573 ± 231
Laterodorsal thalamic nucleus	834 ± 377
Ventromedial thalamic nucleus	415 ± 54
Ventrolateral thalamic nucleus	276 ± 171
Ventroposterior thalamic nucleus	403 ± 51
Medial geniculate	952 ± 407
Lateral geniculate	2598 ± 814
Posterior intralaminar thalamic nucleus	2664 ± 1283
Medial habenula	8832 ± 2655*
Lateral habenula	2538 ± 862
Intermediodorsal nucleus	629 ± 317
Parafascicular nucleus	2277 ± 857
Total	1245 ± 397

[a]Data are mean ± SEM: $F_{(20,42)} = 3.01$, $p = 0.001$; $F_{(19,40)} = 1.51$, $p = 0.134$; excluding medial habenula.
*$p < 0.001$; Bonferroni post hoc comparisons versus total.

cells were negative for ErbB4 (Fig. 4*C*; Table 6). These results suggest that a subgroup of GABAergic interneurons in the cortex is a direct target of the NRG1-ErbB4 pathway. Interestingly, higher colocalization rates were observed in layer 1 (≥80%) for both cortices, suggesting a larger role of NRG1-ErbB4's regulatory role in layer 1.

Hippocampus

Hippocampus, a seahorse-shaped structure deep to the cortex, is responsible for memory consolidation, retrieval, and spatial navigation (Morris et al., 1982; Zola-Morgan and Squire, 1986). The hippocampus can be divided into cornu ammonis (CA) 1, 2, and 3 areas and the dentate gyrus (DG) (Buzsaki, 2011). Axons and neurons in the hippocampus form a trisynaptic loop. In the perforant pathway, axons from the entorhinal cortex synapse onto granule cells in the granule cell layer of the DG, which relay the information, in the mossy fiber pathway, to pyramidal neurons in CA3. CA3 pyramidal neurons project to CA1 pyramidal neurons in the Schaffer collateral pathway, which project via subiculum back to the entorhinal cortex (Buzsaki, 2011). ErbB4-positive cells are detectable in various regions of the hippocampus at comparable densities, except the subiculum areas (Table 8). In the DG, ErbB4-positive neurons are enriched in the granule cell layer and polymorphic layer, but lower in the molecular layer. ErbB4-positive cell densities varied across the layers of CA1–3 regions (areas of hippocampus). They were localized primarily in stratum pyramidal where pyramidal neurons are concentrated and stratum lacunosum-moleculare where perforant path axons synapse onto distal dendrites of pyramidal neurons. The density was low in stratum oriens where many basket interneurons are found as well as basal dendrites from pyramidal neurons, stratum lucidum where mossy fibers from DG granule cells pass, or stratum radiatum where Schaffer collaterals are contained (Fig. 6*B*,*C*). As observed in the cortex, most ErbB4-positive cells in the CA1–3 regions were GAD67-positive, regardless of layers (Fig. 5*B*,*C*). A total of 30% of ErbB4-positive cells were positive for PV, whereas

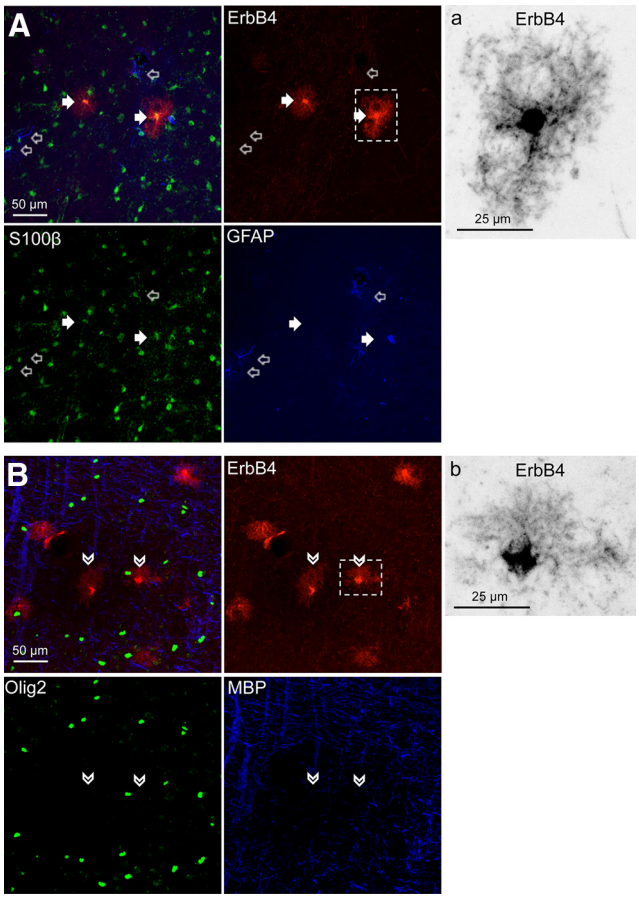

Figure 10. S100β was detected in ErbB4-positive cells in thalamus, hypothalamus, midbrain, and hindbrain. ***A***, ***B***, ErbB4-positive cells in thalamus, with small soma and radiating processes. ***a***, ***b***, Enlarged images of ErbB4-postive cells in rectangles in ***A*** and ***B***, respectively. These cells were detected in low density in areas, including hypothalamus (Fig. 11), midbrain (Fig. 14), and hindbrain (Fig. 16) as well as thalamus (Figs. 2*E* and 9*B*). Arrows indicate cells positive for ErbB4 and S100β. Empty arrows indicate cells positive for S100β and GFAP but negative for ErbB4. Arrowheads indicate cells positive for ErbB4 alone. Olig2, Oligodendrocyte transcription factor 2.

70%–80% of PV-positive cells were positive for ErbB4. In agreement, NRG1 has been shown to suppress LTP via activating ErbB4 in GABAergic neurons (Table 9) (Huang et al., 2000; Woo et al., 2007; Pitcher et al., 2008; Chen et al., 2010; Wen et al., 2010).

Basal ganglia

Basal ganglia are responsible for modulation of movement and intimately related to reward and motivation. Unique to the rest of the brain, >90% of neurons in the basal ganglia are medium spiny GABAergic neurons. Its nuclei including the caudate–putamen, the nucleus accumbens, and lateral septum receive input from dopaminergic neurons in the substantia nigra and VTA in the midbrain. In addition, nuclei of the basal ganglia receive glutamatergic inputs from motor cortices (to caudate–putamen), from the subthalamic nucleus in the hypothalamus (to globus pallidus), and from the amygdala (to nucleus accumbens) (Mink, 2008; Nolte, 2009d). The motor circuit culminates in inhibitory projections from the internal segment of the globus pallidus to the thalamus where the signal is relayed back to the motor cortices. ErbB4-positive cells were throughout in the basal ganglia but enriched in fundus of striatum, bed nucleus of the stria terminalis, diagonal bands nucleus, and ventral tip of the lateral septum

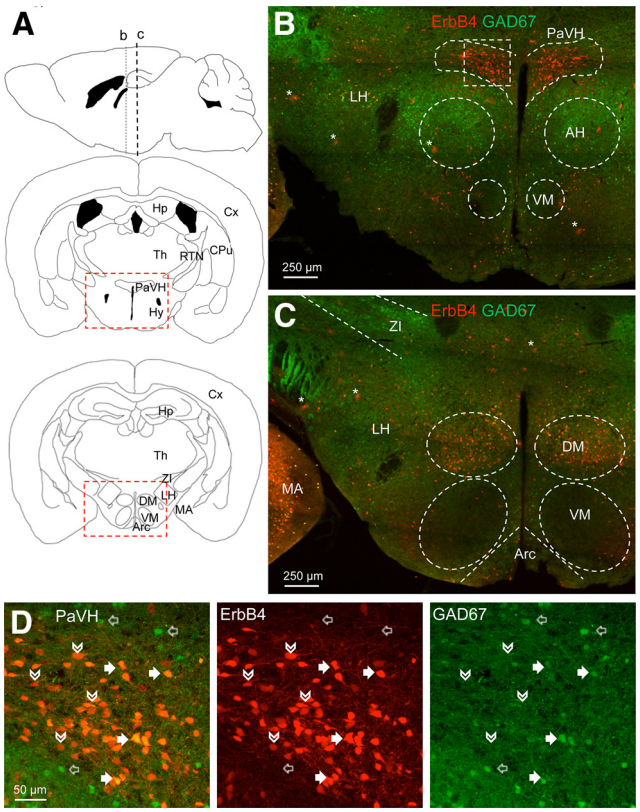

Figure 11. ErbB4-positive cells enriched in the paraventricular and dorsomedial hypothalamic nuclei. ***A***, Diagram of mouse brain sagittal (top) and coronal (middle and bottom) sections. Coronal section positions were indicated by dashed lines b and c. ***B***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, −1.06 mm relative to bregma. Area shown is indicated by the rectangle in ***A*** middle. Dashed lines indicate hypothalamus nuclei. ***C***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, −1.58 mm relative to bregma. *ErbB4-positive cells with non-neuronal morphology. Dashed lines indicate hypothalamus nuclei. ***D***, PaVH, enlarged from the area in the square. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. Arrowheads indicate neurons positive for ErbB4 alone. AH, Anterior hypothalamic nucleus; Arc, arcuate nucleus; CPu, caudate–putamen; Cx, cortex; DM, dorsomedial hypothalamic nucleus; Hp, hippocampus; Hy, hypothalamus; LH, lateral hypothalamus; MA, medial amygdala; RTN, reticular thalamic nucleus; Th, thalamus; VM, ventral medial hypothalamic nucleus; ZI, zona incerta.

(Fig. 7*A*,*B*, arrow; Table 10), suggesting a role of ErbB4 in reward circuitry. Expression of ErbB4 was low in ventral pallidum, caudate–putamen, and globus pallidus. Most ErbB4-positive cells are GABAergic, although many that were positive for GAD67 were not ErbB4-positive (Fig. 7*C*,*D*). This suggests that ErbB4-expressing cells were always GABAergic in the basal ganglia but that most GABAergic neurons do not express ErbB4. This disparity was largest in the caudate–putamen (Fig. 7*C*). The sparsity and randomness of ErbB4-expressing cells suggest that these are most likely interneurons that play a modulatory role and not the principle cells of this area.

Amygdala

Amygdala is implicated in modulation of fear and emotional memory. It has two input pathways. First, information from sensory cortices goes to the basal lateral amygdala, which relays the signal to the central amygdala via the intercalated nucleus (Fig. 8*A*,*B*). Neurons in the central amygdala project to monoaminergic cells of the midbrain and hindbrain, and to cells in hypothalamus. Second, the signal from sensory cortices passes from the lateral amygdala to the ventral portion of the basal lateral

Table 13. ErbB4-positive cells are enriched in paraventricular hypothalamic nucleus[a]

Hypothalamus regions	ErbB4 cells/mm^3
Medial preoptic nucleus	5200 ± 595
Lateral preoptic nucleus	4746 ± 1483
Median preoptic nucleus	4067 ± 586
Ventromedial hypothalamic nucleus	3308 ± 788
Dorsomedial hypothalamic nucleus	13,640 ± 1441
Posterior hypothalamic nucleus	13,780 ± 3773
Anterior hypothalamic nucleus	6614 ± 1969
Lateral hypothalamic nucleus	3825 ± 1388
Premammillary nucleus	8008 ± 1671
Medial mammillary nucleus	703 ± 189
Supramammillary nucleus	8165 ± 2965
Arcuate nucleus	7824 ± 1744
Periventricular nucleus	8645 ± 1431
Suprachiasmatic nucleus	7616 ± 2183
Paraventricular hypothalamic nucleus	15,820 ± 4529*
Tuber cinereum area	2163 ± 369
Zona incerta	1819 ± 473
Median eminence	7323 ± 1855
Subthalamic nucleus	8865 ± 1877
Total	5306 ± 1178

[a]Data are mean ± SEM: $F_{(18,38)} = 4.34, p < 0.001$.

*$p < 0.01$; Bonferroni post hoc comparisons versus total.

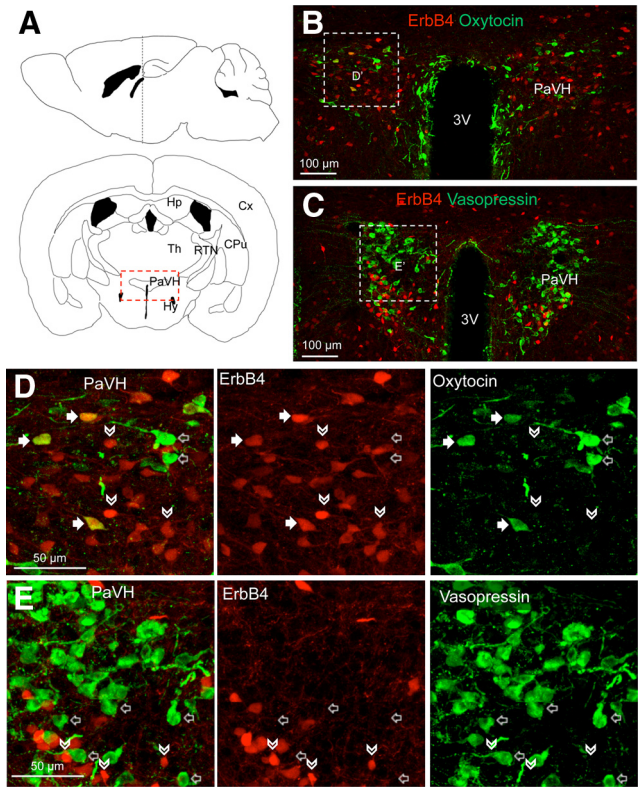

Figure 12. ErbB4-positive cells colocalize with oxytocin but not vasopressin in the paraventricular hypothalamic nucleus. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, ***C***, Coronal section of *ErbB4-reporter* mouse brain, −1.06 mm relative to bregma. Shown was the PaVH area, indicated by the rectangle in ***A***. Sections were stained with antibodies against oxytocin (***B***) or vasopressin (***C***). ***D***, ***E***, PaVH areas stained with anti-oxytocin (***D***) or anti-vasopressin (***E***). Areas shown were indicated by squares in ***B*** and ***C***, respectively. Arrows indicate neurons positive for ErbB4 and oxytocin. Empty arrows indicate neurons positive for oxytocin or vasopressin alone. Arrowheads indicate neurons positive for ErbB4 alone. 3V, Third ventricle; CPu, caudate–putamen; Cx, cortex; Hp, hippocampus; Hy, hypothalamus; RTN, reticular thalamic nucleus; Th, thalamus.

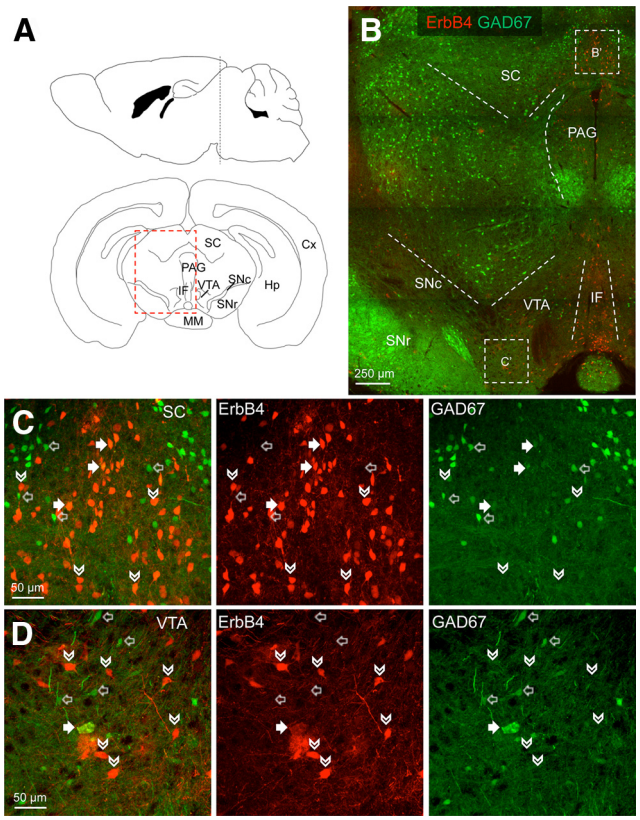

Figure 13. ErbB4-positive cells in the midbrain. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, −3.08 mm relative to bregma. Dashed lines indicate midbrain nuclei. ***C***, ***D***, Superior colliculus and VTA, indicated by square ***C′*** and ***D′*** in ***B***, respectively. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. Arrowheads indicate neurons positive for ErbB4 alone. Cx, Cortex; Hp, hippocampus; IF, interfascicular nucleus; MM, medial mammillary nucleus; PAG, periaqueductal gray; SC, superior colliculus; SNr, substantia nigra reticular part; SNc, substantia nigra compact part; VTA, ventral tegmental area.

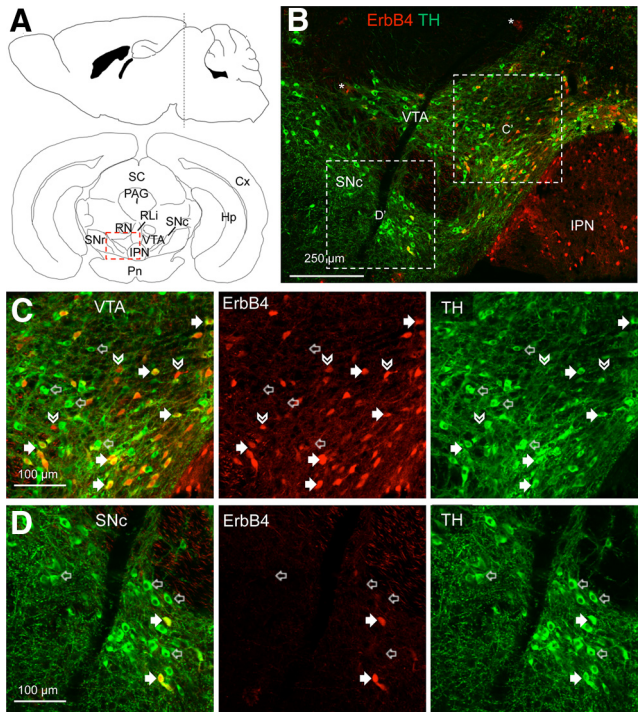

Figure 14. ErbB4-positive cells colocalize with TH in midbrain. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter* mouse brain, −3.80 mm relative to bregma. *Non-neuronal ErbB4-positive cells. ***C***, ***D***, VTA and SNc, indicated by squares ***C′*** and ***D′***, respectively. Arrows indicate neurons positive for ErbB4 and TH. Empty arrows indicate neurons positive for TH alone. Arrowheads indicate neurons positive for ErbB4 alone. Cx, Cortex; Hp, hippocampus; IPN, interpeduncular nucleus; PAG, periaqueductal gray; PN, pontine nucleus; RLi, rostral linear nucleus; RN, red nucleus; SC, superior colliculus; SNr, substantia nigra reticular part; SNc, substantia nigra compact part; VTA, ventral tegmental area.

amygdala or basal amygdala. From here the signal is relayed to the nucleus accumbens. The effect is to modulate arousal and emotional state and encode them into memory associated with certain stimuli (LeDoux, 2008). Among all brain areas, amygdala was the region that had the highest ErbB4-positive cell density (Table 2), in agreement with *in situ* hybridization analysis (Lai and Lemke, 1991; Woo et al., 2007). In amygdala, ErbB4-positive cell density varied significantly in different subregions: with the highest concentration in the intercalated nucleus (Table 11; Fig. 8*B*,*D*). Almost all ErbB4-positive cells in amygdala were GAD67-positive (Fig. 8), except a few cells in the medial amygdala (Fig. 8*E*). Some GAD67-positive cells were not positive for ErbB4, as observed in other brain regions (Fig. 8*C*–*E*). These results suggest that ErbB4-expressing cells in the amygdala are mostly GABAergic interneurons, although not all GABAergic interneurons express ErbB4 and a small population of ErbB4-expressing cells, in particular those in the medial amygdala, are non-GABAergic.

Thalamus

The dorsal thalamus acts as a relay center for sensory information traveling to and from sensory cortices other than olfaction (Fig. 9*A*,*B*). Information from the cerebellum, basal ganglia, and limbic structures is also relayed at the ventral thalamus. ErbB4-positive cell density was low in the thalamus (Figs. 2*E* and 9*B*; Table 12). They are mainly distributed in the medial habenula, in

Table 14. ErbB4-positive cells are enriched in raphe nuclei in the midbrain[a]

Midbrain regions	ErbB4 cells/mm^3
Ventral tegmental area	3459 ± 1115
Substantia nigra pars compacta	2457 ± 694
Substantia nigra pars reticulata	1263 ± 354
Interpeduncular nucleus	9893 ± 3456*
Interfascicular raphe nucleus	14,430 ± 4055***
Rostral linear raphe nucleus	11,700 ± 3145**
Central linear raphe nucleus	3977 ± 2945
Dorsal raphe nucleus	10,500 ± 2167*
Red nucleus	1146 ± 307
Periaqueductal gray	4412 ± 1535
Pretectal area	2611 ± 610
Zonal layer of superior colliculus	3408 ± 1066
Superficial gray layer of superior colliculus	2093 ± 644
Optic nerve layer of superior colliculus	3255 ± 812
Intermediate gray layer of superior colliculus	3609 ± 1349
Intermediate white layer of superior colliculus	1590 ± 604
Deep gray layer of superior colliculus	2547 ± 932
External cortex of the inferior colliculus	645 ± 255
Brachium of the inferior colliculus	1438 ± 406
Central nucleus of the inferior colliculus	465 ± 244
Dorsal cortex of the inferior colliculus	1227 ± 471
Midbrain reticular nucleus	939 ± 307
Total	2271 ± 634

[a]Data are mean ± SEM: $F_{(21,44)} = 5.48$, $p < 0.001$.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Bonferroni post hoc comparisons versus total.

agreement with ErbB4 mRNA localization (Lai and Lemke, 1991; Steiner et al., 1999; Bruce et al., 2002; Woo et al., 2007). Only a small fraction of ErbB4-positive cells were GAD67-positive; more appeared to be negative for GAD67. However, almost all GAD67-positive cells in the medial habenula are ErbB4-positive (Fig. 9*D*). We observed ErbB4-positive cells that are scattered in the central thalamus. Their soma size was small but had radiating processes that look more like glial cells (Fig. 9*B*,*C*, asterisks). These cells were positive for S100β but not with GFAP, oligodendrocyte transcription factor, or MBP (Fig. 10). The identity and function of these cells remain unclear. They may be GFAP-negative astrocytes or unidentified oligodendrocytes. They appeared prominent in the thalamus because of low background ErbB4 signal. Careful examination indicated that they were detectable in the hypothalamus, midbrain, and hindbrain (Fig. 9, asterisks, and figures hereafter). The majority of GABAergic neurons in the thalamus are concentrated in the reticular thalamic nucleus that project to inhibit glutamatergic neurons within the thalamus (Hendry et al., 2008; Nolte, 2009f). In agreement, most GAD67-GFP cells were strictly localized in the reticular thalamic nucleus. Previous studies indicated that ErbB4 mRNA was enriched in the nucleus (Lai and Lemke, 1991; Woo et al., 2007). Intriguingly, most GAD67-positive cells were not positive for ErbB4 in the region (Fig. 9*C*).

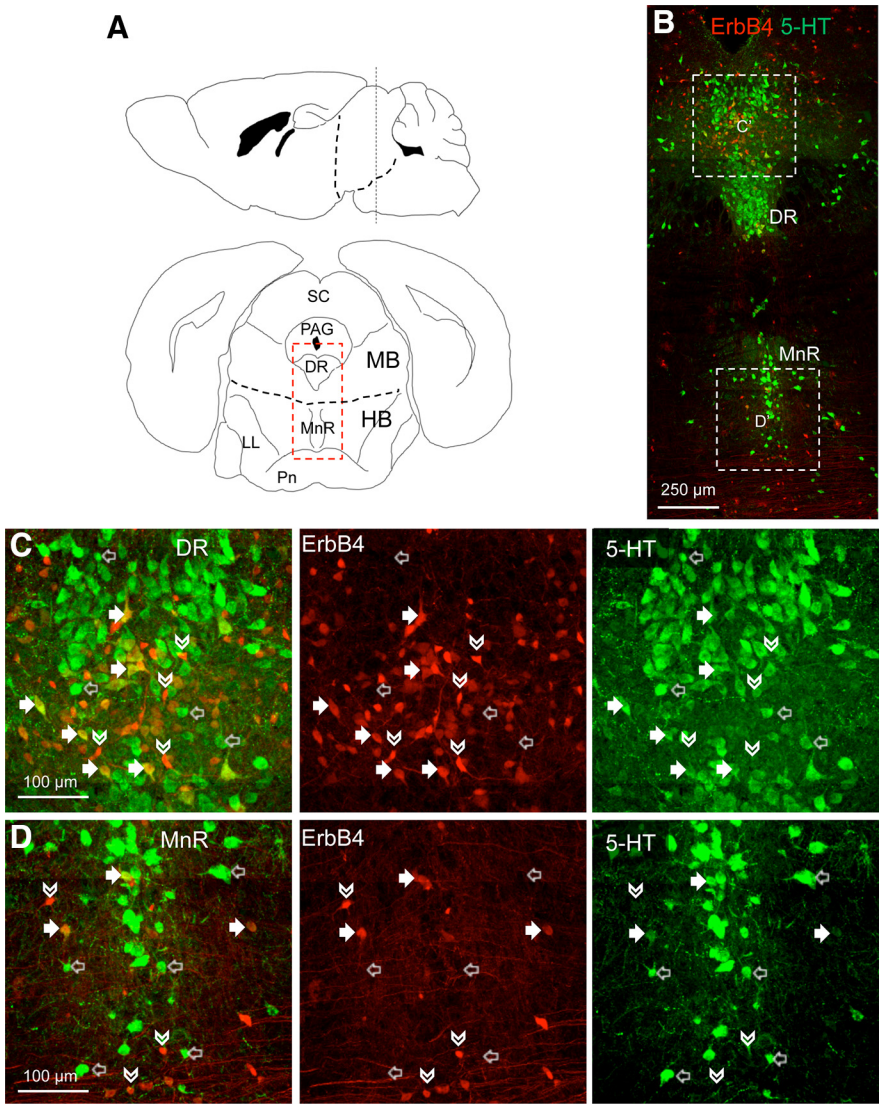

Figure 15. ErbB4-positive cells colocalize with serotonin neurons in raphe nuclei. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter* mouse brain, −4.36 mm relative to bregma. Sections were stained with anti-5-HT antibody (green). ***C***, ***D***, Dorsal raphe of the midbrain and median raphe of the hindbrain, indicated by squares ***C′*** and ***D′***, respectively. Arrows indicate neurons positive for ErbB4 and 5-HT. Empty arrows indicate neurons positive for 5-HT alone. Arrowheads indicate neurons positive for ErbB4 alone. DR, Dorsal raphe; LL, lateral lemniscus; MnR, median raphe; PAG, periaqueductal gray; PN, pontine nucleus; SC, superior colliculus.

Hypothalamus

The hypothalamus receives input from nearly all sensory modalities and from hippocampus, amygdala, and cingulate cortex. It also contains sensors for blood temperature, sugar, mineral, and hormone levels (Nolte, 2009c). It plays integral roles in regulating metabolism, thermoregulation, sleep, stress response, and sexual behavior. ErbB4-positive cell densities varied in the hypothalamus: apparently high in the paraventricular hypothalamic nucleus (PaVH), dorsal medial hypothalamic nucleus, and posterior hypothalamic nucleus (Figs. 2*E* and 11*A–C*; Table 13). The expression profile generally agrees with that of previous *in situ* reports (Ma et al., 1999; Woo et al., 2007; Lai and Lemke, 1991) As observed in other areas, some, but not all, ErbB4-positive cells in the PaVH and dorsal medial hypothalamic nucleus were positive for GAD67 (Fig 11*D*) (data not shown). Likewise, some GAD67-positive cells did not express ErbB4. These results suggest that ErbB4-positive cells in the hypothalamus were a mixture of GABAergic interneurons and non-GABAergic cells (Fig 11*D*). Neurons in the PaVH are known to release oxytocin, a factor implicated in social bonding, sexual response, maternal care, and lactation (Lee et al., 2009). Costaining analysis indicated that ~50% of oxytocin-expressing cells in the hypothalamus are positive for ErbB4, suggesting that NRG1-ErbB4 signaling may regulate sociality or maternal instincts (Fig. 12*A*,*B*,*D*). In contrast, none of the cells expressing vasopressin, a factor related to oxytocin sometimes opposing its effects, was positive for ErbB4 (Fig. 12*C*,*E*) (Keverne and Curley, 2004). Together, these results suggest that the NRG1-ErbB4 signaling may regulate metabolism or other hypothalamus-associated functions.

Table 15. ErbB4-positive cells colocalize with monoamines[a]

	5-HT/ErbB4	TH/ErbB4	GAD67/ErbB4	ErbB4/5-HT	ErbB4/TH	ErbB4/GAD67
VTA	NA	53.7 ± 3.5	10.3 ± 4.2	NA	22.4 ± 8.2	15.0 ± 6.4
SN	NA	44.9 ± 2.2	2.4 ± 2.4	NA	6.9 ± 2.8	3.0 ± 3.0
RLi	29.7 ± 14.9	NA	0.0 ± 0.0	30.8 ± 11.1	NA	0.0 ± 0.0
MnR	18.8 ± 7.1	NA	10.6 ± 5.3	9.4 ± 5.1	NA	5.2 ± 2.7
DR	42.2 ± 3.0	NA	16.4 ± 5.3	20.4 ± 5.0	NA	22.2 ± 4.0
LC	NA	0.0 ± 0.0	45.8 ± 20.8	NA	0.0 ± 0.0	6.9 ± 2.2

[a]Data are mean percentage ± SEM. 5-HT, Serotonin; DR, dorsal raphe; LC, locus coeruleus; MnR, median raphe; RLi, rostral linear raphe nucleus; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

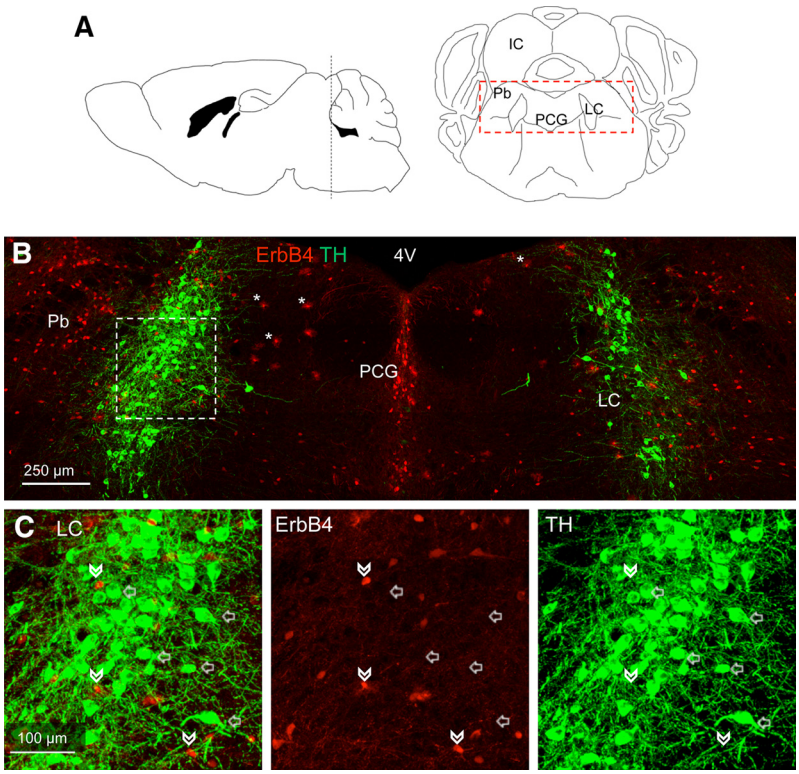

Figure 16. ErbB4-positive cells do not colocalize with TH in the locus ceruleus. ***A***, Diagram of mouse brain sagittal (left) and coronal (right) sections. Coronal section position is indicated by the dashed line at left. ***B***, Coronal section of *ErbB4-reporter* mouse brain, −5.34 mm relative to bregma. Sections were stained with anti-TH antibody (green). *ErbB4-positive non-neuronal cells. ***C***, Locus ceruleus, indicated by the square in ***B***. Arrows indicate neurons positive for TH alone. Arrowheads indicate neurons positive for ErbB4 alone. 4V, Fourth ventricle; IC, inferior colliculus; LC, locus ceruleus; Pb, parabrachial nucleus; PCG, pontine central gray.

Table 16. ErbB4-positive cell density varies in hindbrain despite low levels[a]

Hindbrain regions	ErbB4 cells/mm^3
Pontine central gray	1077 ± 140
Reticulotegmental nucleus of pons	1148 ± 444
Pontine reticular nucleus	731 ± 176
Median raphe nucleus	2987 ± 918
Paramedian raphe nucleus	1585 ± 515
Periolivary region	335 ± 113
Nucleus of raphe magnus	1301 ± 290
Principal sensory trigeminal nucleus 5n	2125 ± 652
Motor trigeminal nucleus 5n	769 ± 261
Supratrigeminal nucleus 5n	3882 ± 1974*
Spinal trigeminal nucleus 5n	635 ± 85
Parvocellular reticular nucleus	1414 ± 838
Intermediate reticular nucleus	513 ± 115
Dorsomedial tegmental area	758 ± 162
Posterodorsal tegmental area	2995 ± 848
Medial vestibular 8n	808 ± 231
Lateral vestibular 8n	1147 ± 393
Superior vestibular 8n	691 ± 322
Facial nucleus 7n	492 ± 118
Gigantocellular reticular nucleus	468 ± 71
Medial longitudinal fasciculus	1010 ± 327
Lateral lemniscus	500 ± 217
Parabrachial nucleus	2992 ± 497
Abducens nucleus 6n	319 ± 142
Cochlear 8n	830 ± 182
Locus ceruleus	2700 ± 456
Nucleus prepositus	1333 ± 501
Total	1034 ± 270

[a]Data are mean ± SEM: $F_{(26,54)} = 3.02$, $p < 0.001$.

*$p < 0.05$; Bonferroni post hoc comparisons versus total.

Midbrain

The midbrain is located below and behind the cerebral cortex and in front of the hindbrain. In the midbrain, ErbB4-positive cells are clustered in superior colliculus, periaqueductal gray, raphe nuclei, and interpeduncular nucleus (Figs. 13, 14, and 15; Table 14). Overall, most GAD67-positive cells were negative for ErbB4 in midbrain (Fig. 13*A*,*B*). Among the cells that were positive for ErbB4, some were GAD67-positive but many were not (Fig. 13*C*).

Interestingly, ErbB4-positive cells are localized in regions that are enriched in monoaminergic neurons, including substantia nigra and VTA where dopamine neurons are concentrated (Figs. 13*B*,*D* and 14). This is consistent with previous *in situ* reports that dopamine neurons express ErbB4 (Steiner et al., 1999; Abe et al., 2009; Zheng et al., 2009). The dopaminergic neurons in the substantia nigra pars compacta (SNc) project to the caudate–putamen in the basal ganglia and are implicated in motor control. On the other hand, dopamine neurons in the VTA project to nucleus accumbens for reward and motivation. They also project to cortical and limbic structures for cognition. The serotoninergic neurons in raphe nuclei have been implicated in alertness and mood regulation (Nolte, 2009e). In both VTA and SNc, ~50% of ErbB4-positive cells were positive for tyrosine hydroxylase (TH), a marker of dopaminergic neurons (Nagatsu, 1995) (Table 15; Fig. 14). Less than 10% of ErbB4-positive cells are GAD67-positive in both regions. However, ErbB4-positive cells accounted for only a fraction (7%–22%) of dopaminergic neurons (Table 15). This is inconsistent with the notion that nearly all dopamine neurons express ErbB4 (Abe et al., 2009; Zheng et al., 2009). These data suggest that ErbB4-expressing cells in the VTA and SNc are often dopaminergic and sometimes GABAergic.

Having found high densities of ErbB4-positive cells in many raphe nuclei, we examined whether they were serotoninergic neurons by staining with anti-5-HT antibody. We focused on the rostral linear and dorsal raphe nuclei of the midbrain and the median raphe nucleus of the hindbrain, where large amounts of 5-HT neurons are located. ErbB4-positive cells were indeed positive for 5-HT, indicating that ErbB4 is expressed in serotoninergic neurons (Fig. 15). Quantitatively, 20%–40% of ErbB4-positive cells were positive for 5-HT; conversely, 10%–30% of 5-HT cells expressed ErbB4 (Table 15). In the raphe nuclei, most GAD67-positive cells were not positive for ErbB4. These data indicate that ErbB4 is expressed in serotoninergic neurons in raphe nuclei, identifying a novel cellular target of NRG1-ErbB4 signaling.

Hindbrain and cerebellum

The density of ErbB4-positive cells was low in the hindbrain, compared with other brain areas (Table 2). In hindbrain, ErbB4-positive cells were concentrated in the median raphe (Fig. 15), parabrachial nucleus, locus ceruleus (Fig. 16), supratrigeminal

nucleus, and posterodorsal tegmental area (Table 16).

Most of GAD67-positive cells were negative for ErbB4 in hindbrain (Fig. 17*A–C*). Among the cells that were positive for ErbB4, ~45% were GAD67-positive, for example, in locus ceruleus (Table 15). Although ErbB4-positive cells in the midbrain could be monoaminergic, those in the hindbrain were negative for TH that marks norepinephrine neurons (Nagatsu, 1995) (Fig. 16; Table 15). These results suggest that ErbB4 expression in monoaminergic neurons is regional specific.

Previous studies using *in situ* hybridization suggested that ErbB4 is expressed in the cerebellum (Ozaki et al., 1997, 1998; Rio et al., 1997; Rieff et al., 1999). Compared with other brain areas, ErbB4-positive cells were fewer in the cerebellum (Table 2; Table 17). As shown in Figure 17, GAD67 expression marked Purkinje cells and some smaller interneurons in the granule layer. Occasionally, ErbB4-positive cells were observed in the granule layer. GAD67 signal was not observed in these ErbB4-positive cells (Fig. 17*D*). These data suggest that GABAergic neurons of the cerebellum may not express ErbB4.

White matter areas and choroid plexus

ErbB4-positive cells were observed in the white matter, including the corpus callosum (Fig. 18*A*,*B*). The positive cell density did not vary across white matter areas (Table 18). Interestingly, most ErbB4-positive cells in the corpus callosum were GAD67-positive. However, there were also cells that were positive for GAD67 but not ErbB4 (Fig. 18*B*). Finally, large ErbB4-positive cells were observed in the choroid plexus, a tissue that generates the CSF. These mostly colocalized with GAD67 (Fig. 18*C*).

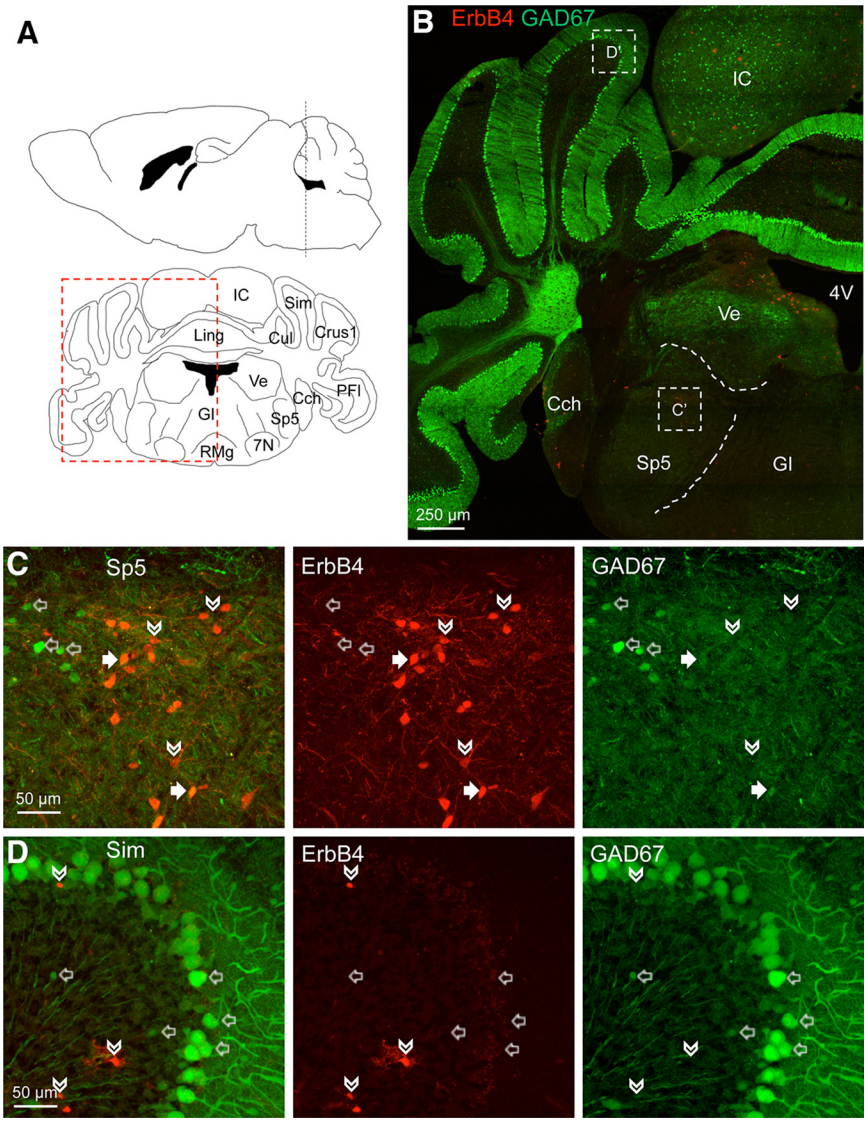

Figure 17. ErbB4-positive cells in the cerebellum and hindbrain. ***A***, Diagram of mouse brain sagittal (top) and coronal (bottom) sections. Coronal section position is indicated by the dashed line in top. ***B***, Coronal section of *ErbB4-reporter; GAD67::GFP* mouse brain, −5.52 mm relative to bregma. ***C***, ***D***, Spinal trigeminal nucleus and simple lobule of cerebellum. Areas shown were indicated by squares ***C′*** and ***D′*** in ***B***. Arrows indicate neurons positive for ErbB4 and GAD67. Empty arrows indicate neurons positive for GAD67 alone. Arrowheads indicate cells positive for ErbB4 alone. 4V, Fourth ventricle; 7N, facial nucleus; Cch, cochlear nucleus; Cul, culmen lobule; Crus 1, Crus 1 of the ansiform lobule; Gl, gigantocellular reticular nucleus; IC, inferior colliculus; Ling, lingual lobule; PFl, paraflocculus lobule; RMg, raphe magnus nucleus; Sim, simple lobule; SP5, spinal trigeminal nucleus; Ve, vestibular nucleus.

Discussion

The major findings of this study are as follows. First, ErbB4 is widely expressed in the adult mouse brain. Notably ErbB4-positive cell density was highest in the amygdala, followed by the cortex, and by hypothalamus. Expression of ErbB4 in thalamus, hindbrain, and cerebellum was low. Second, ErbB4-positive cells (>98%) were almost always GABAergic in cortex, hippocampus, basal ganglia, and most of amygdala in both neonatal and adult stages, suggesting GABAergic transmission as a major target of NRG1-ErbB4 signaling in these regions. Third, non-GABAergic, ErbB4-positive cells were present in thalamus, hypothalamus, midbrain, and hindbrain. In particular, ErbB4 is expressed in serotoninergic neurons of raphe nuclei but not in norepinephrinergic neurons of the locus ceruleus. In hypothalamus, ErbB4 is present in neurons that express oxytocin. Finally, ErbB4 is expressed in a group of

Table 17. Slight variation of ErbB4-positive cell densities in cerebellum[a]

Cerebellum regions	ErbB4 cells/mm^3
Lingula (II) lobule	3744 ± 1304
Central (III) lobule	3460 ± 1838
Culmen (IV/V) lobule	2219 ± 645
Central simple (VI) lobule	1375 ± 259
Lateral simple (VI) lobule	1033 ± 555
Crus 1 of the ansiform (VII) lobule	933 ± 245
Crus 2 of the ansiform (VII) lobule	595 ± 269
Paraflocculus (VIII/IX) lobule	719 ± 158
Central flocculus (X) lobule	1091 ± 399
Lateral flocculus (X) lobule	547 ± 45
Fastigial nucleus	708 ± 204
Interposed nucleus	461 ± 293
Dentate nucleus	1010 ± 495
Total	1497 ± 469

[a]Data are mean ± SEM: $F_{(12,26)} = 2.35$, $p = 0.034$.

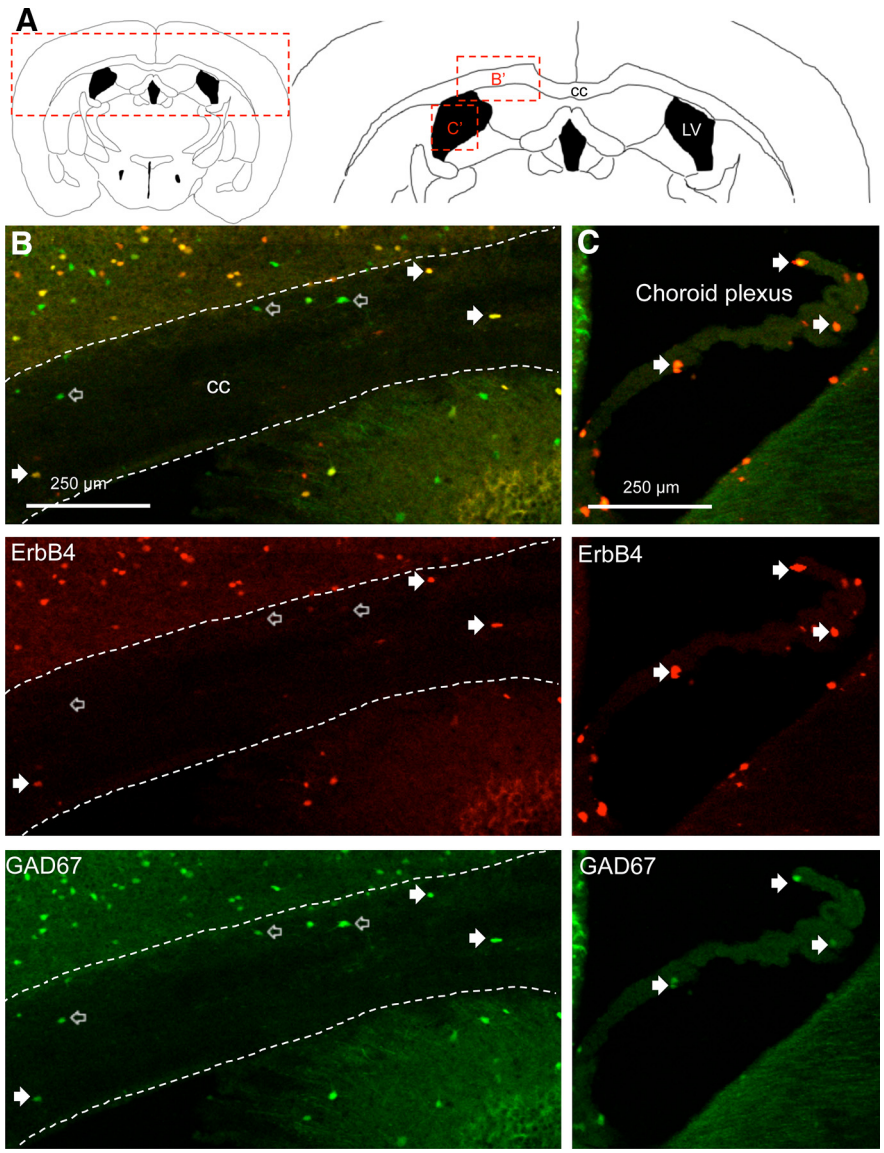

Figure 18. ErbB4-positive cells in the corpus callosum and choroid plexus. ***A***, Diagram of mouse brain coronal sections, −0.94 mm relative to bregma. Coronal section position is indicated by the dashed line in Figure 12*A*. ***B***, ***C***, Corpus callosum and choroid plexus in the lateral ventricle. Areas shown were indicated by squares ***B′*** and ***C′***, respectively. Arrows indicate cells positive for ErbB4 and GAD67. Empty arrows indicate cells positive for GAD67 alone.

Table 18. ErbB4-positive cells are evenly distributed in white matter areas[a]

White matter areas	ErbB4 cells/mm^3
Corpus callosum	864 ± 181
Anterior commissure	1373 ± 152
Internal capsule	1001 ± 285
External capsule	981 ± 201
Fimbria	1142 ± 295
Medial lemniscus	415 ± 184
Cerebral peduncle	969 ± 470
Cerebellar peduncle	486 ± 177
Arbor vitae	533 ± 111
Cerebellar commissure	672 ± 88
Total	925 ± 199

[a]Data are mean ± SEM: $F_{(9,20)} = 1.69, p = 0.432$.

cells that sparsely populate the subcortical areas. Morphologically, they have small soma and short, dense processes and are positive for S100β. These results identify novel cellular targets of NRG1-ErbB4 signaling.

ErbB4 was previously reported to be expressed in excitatory neurons (Garcia et al., 2000; Huang et al., 2000; Ma et al., 2003; Kwon et al., 2005; Li et al., 2007; Iyengar and Mott, 2008; Barros et al., 2009; Pitcher et al., 2011). ErbB4 in excitatory neurons has been implicated in synapse formation and synaptic plasticity through cell-autonomous mechanisms (Gu et al., 2005; Kwon et al., 2005; Li et al., 2007; Pitcher et al., 2011). Contrary to this idea, ErbB4 transcripts were found to be present in areas enriched with interneurons (Lai and Lemke, 1991; Woo et al., 2007). ErbB4 protein was detectable in GAD-positive neurons in the hippocampus (Huang et al., 2000; Woo et al., 2007) and was shown to be exclusively expressed in interneurons (Vullhorst et al., 2009; Fazzari et al., 2010; Neddens and Buonanno, 2011; Neddens et al., 2011). Nevertheless, spine morphology and behavior are impaired by *ErbB4* gene mutation via *GFAP::Cre*, *Nestin::Cre*, and *Emx1::Cre*, which are expressed in cells, including pyramidal neurons (Barros et al., 2009). Therefore, it becomes critically important to determine exactly in what cells ErbB4 is expressed. Unfortunately, this question cannot be addressed by current antibodies against ErbB4 because of poor specificity and availability. We attempted to address this question by taking advantage of the endogenous promoter of ErbB4 (Fig. 1). The cells that expressed the tdTomato protein were also labeled by anti-ErbB4 antibodies 0618 and NeoMarker-H4.77.16, validating the feasibility of the approach. Both antibodies showed nonspecific labeling that would make it difficult to interpret data from staining with these antibodies.

ErbB4 protein is expressed in the forebrain areas that are increasingly implicated in schizophrenia, including the amygdala, which is associated with emotional memory (LeDoux, 2008). Schizophrenics often have reduced emotionality or flattened affect (Tsuang et al., 2000) and alteration in amygdala activity (Brunet-Gouet and Decety, 2006; Pinkham et al., 2007; Rasetti et al., 2009). High levels of ErbB4 in amygdala may provide a pathophysiological mechanism of abnormal NRG1-ErbB4 signaling. Finding ErbB4 is primarily expressed in GABA neurons in cortex, hippocampus, and striatum provides further evidence for a role of NRG1 and ErbB4 in the development and function of the GABAergic circuitry. In agreement, ErbB4 is critical for GABAergic neuron migration and differentiation (Flames et al., 2004). NRG1 was shown to promote GABA release and to suppress LTP in a manner that requires ErbB4 (Woo et al., 2007; Chen et al., 2010; Wen et al., 2010). GABAergic dysfunction is well documented in schizophrenia (Gonzalez-Burgos et al., 2011; Lewis et al., 2011). Intriguingly, *ErbB4* gene mutation causes schizophrenia-relevant behavior deficits, including hyperactivity, impaired prepulse inhibition, abnormal working memory, and contextual fear conditioning

(Stefansson et al., 2002; Chen et al., 2010; Wen et al., 2010; Shamir et al., 2012; Del Pino et al., 2013; Yin et al., 2013a).

Besides interneurons, we found ErbB4-positive cells enriched in the raphe nuclei. They were serotoninergic neurons because they costained with the neurotransmitter (Fig. 15). Serotonin has long been implicated in psychiatric disorders, including schizophrenia (Laruelle et al., 1993; Ngan et al., 2000). This identifies a novel cellular target for NRG1-ErbB4 signaling and adds a layer of complexity to how NRG1 and ErbB4 may be related to the disorder. Moreover, our study reveals that ErbB4-positive cells were enriched in hypothalamic nuclei, including posterior hypothalamic, dorsal medial hypothalamic, PaVH nuclei. In PaVH, ErbB4 was detectable in ~50% of oxytocin-positive neurons (Fig. 12). Oxytocin is involved in social bonding, sexual arousal, orgasm, maternal care, and lactation (Lee et al., 2009). In light of impaired social interaction in schizophrenia (Tsuang et al., 2000), the finding of ErbB4 in oxytocin-expressing neurons suggests a previously unappreciated pathophysiological mechanism of abnormal NRG1-ErbB4 signaling.

Previous studies suggested that ErbB4 mRNA is enriched in the reticular thalamic nucleus (Lai and Lemke, 1991; Steiner et al., 1999; Bruce et al., 2002; Woo et al., 2007). However, tdTomato signal was not detectable in the reticular thalamic nucleus, except a few cells in the ventral region (Fig. 9). Moreover, ErbB4 mRNA was shown to be in most (80%–99%) dopamine neurons in midbrains of rodents, monkey, and human (Abe et al., 2009; Zheng et al., 2009). In the current study, however, only 20% of midbrain DA neurons expressed tdTomato in *ErbB4-reporter* mice (Fig. 14). Our result is more consistent with the report of low levels of ErbB4 in dopamine neurons (Neddens and Buonanno, 2011). It is worth pointing out that, in our study, the readout depends on activation of the CreERT2 by tamoxifen that is controlled by tamoxifen bioavailability and expression and stability of the tdTomato protein.

In conclusion, nearly all ErbB4-positive cells were GABAergic in cortex, hippocampus, basal ganglia, and most of amygdala in neonatal and adult mice. This suggests that GABAergic transmission may be a major target of NRG1-ErbB4 signaling in these regions. However, there were non-GABAergic ErbB4-positive cells in subcortical areas, including thalamus, hypothalamus, midbrain, and hindbrain. Intriguingly, ErbB4 was expressed in serotoninergic neurons of raphe nuclei, oxytocin-positive cells in hypothalamus, and glia-like cells that are positive for S100β. These results identify novel cellular targets of NRG1-ErbB4 signaling.

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