Developmental, neurochemical, and behavioral analyses of ErbB4 Cyt-1 knockout mice

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Abstract
Neuregulins (NRGs) and their cognate neuronal receptor ERBB4, which is expressed in GABAergic and dopaminergic neurons, regulate numerous behaviors in rodents and have been identified as schizophrenia at-risk genes. ErbB4 transcripts are alternatively spliced to generate isoforms that either include (Cyt-1) or exclude (Cyt-2) exon 26, which encodes a cytoplasmic domain that imparts ErbB4 receptors the ability to signal via the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway. Although ErbB4 Cyt-1/2 isoforms have been studied in transfected cultured cells, their functions in vivo remain unknown. Here, we generated ErbB4-floxed (ErbB4-Cyt1fl/fl) mice to investigate the effects of germline (constitutive) and conditional (acute) deletions of the Cyt-1 exon. Overall receptor mRNA levels remain unchanged in germline ErbB4 Cyt-1 knockouts (Cyt-1 KOs), with all transcripts encoding Cyt-2 variants. In contrast to mice lacking all ErbB4 receptor function, GABAergic interneuron migration and number are unaltered in Cyt-1 KOs. However, basal extracellular dopamine (DA) levels in the medial prefrontal cortex are increased in Cyt-1 heterozygotes. Despite these neurochemical changes, Cyt-1 heterozygous and homozygous mice do not manifest behavioral abnormalities previously reported to be altered in ErbB4 null mice. To address the possibility that Cyt-2 variants compensate for the lack of Cyt-1 during development, we microinjected an adeno-associated virus expressing Cre-recombinase (AAV-Cre) into the DA-rich ventral tegmental area of adult ErbB4-Cyt1fl/fl mice to acutely target exon 26. These conditional Cyt-1 KOs were found to exhibit behavioral abnormalities in the elevated plus maze and startle response, consistent with the idea that late exon 26 ablations may circumvent compensation by Cyt-2 variants. Taken together, our observations indicate that ErbB4 Cyt-1 function in vivo is important for DA balance and behaviors in adults.

Abbreviations: AAV, adeno-associated virus; aCSF, artificial cerebral spinal fluid; CNS, central nervous system; Cyt, cytoplasmic; Cyt-1 KOs, Cyt-1 knockout mice; DA, dopamine/dopaminergic; DAT, dopamine transporter; Dbh, dopamine beta-hydroxylase; DLPCF, dorsal lateral prefrontal cortex; ErbB4 KOs, heart-rescued ErbB4 knockout mice; ES, embryonic stem; GFP, green-fluorescent protein; HPLC, high performance liquid chromatography; Hpp, hippocampus; IHC, immunofluorescence histochemistry; ISH, in-situ hybridization; JM, juxtamembrane; KO, knockout; mPFC, medial prefrontal cortex; NPP, no pre-pulse; NRGs, neuregulins; P13K, phosphatidylinositol-3-kinase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PPI, pre-pulse inhibition; PPXY, WW domain motif; PV, parvalbumin; RRIIDs, Research Resource Identifier (see scicrunch.org); RT-qPCR, reverse-transcription polymerase chain reaction; Scz, Schizophrenia; SSCtx, somatosensory cortex; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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1 | INTRODUCTION

The Neuregulin (NRG) family of neurotrophic factors, as well as their cognate neuronal receptor tyrosine kinase ErbB4, are genetically associated with a risk for schizophrenia (Scz) and its endophenotypes (Buonanno, 2010; Greenwood et al., 2012; Mei & Nave, 2014; Mostaid et al., 2016; Silberberg, 2006; Stefansson et al., 2002). NRG/ErbB4 signaling is best characterized in the cortex and hippocampus, where ErbB4 expression is confined to GABAergic interneurons, (Fazzari et al., 2010; Vullhorst et al., 2009), as well as in the mesencephalon, where ErbB4 is highly expressed in dopaminergic (DAergic) neurons (Abé et al., 2009; Skirzewski et al., 2018; Steiner et al., 1999). Germline ablation of ErbB4 results in embryonic lethality because of failure of heart trabeculation (Gassmann et al., 1995), which can be circumvented by selective transgenic expression of ErbB4 in the heart (Tidcombe et al., 2003). Using heart-rescued ErbB4 null mice, NRG/ErbB4 signaling has been shown to regulate the migration, allocation, and the number of cortical GABAergic interneurons during embryonic and neonatal development (Bartolini et al., 2017; Flames et al., 2004; Neddens & Buonanno, 2010). Later in development, NRG/ErbB4 signaling regulates the maturation of glutamatergic synapses onto GABAergic interneurons (Del Pino et al., 2013; Fazzari et al., 2010; Kotzadimitriou et al., 2018; Vullhorst et al., 2015; Woo et al., 2007; Yang et al., 2013; Yang et al., 2018), particularly parvalbumin (PV)-positive fast-spiking interneurons, that modulate critical period plasticity (Gu et al., 2016; Sun et al., 2016) and neuronal network activity (Andersson et al., 2012; Fisahn et al., 2009; Nason Jr. et al., 2011). NRG/ErbB4 signaling in midbrain DAergic neurons also regulates DA homeostasis and cognitive function (Kwon et al., 2008; Namba et al., 2016; Skirzewski et al., 2018; Skirzewski et al., 2020; Yan et al., 2018). Interestingly, both NRG2 and ErbB4 KOs, as well as conditional ErbB4 Cyt-1/iCyt-2 mutant mice that lack the receptor in tyrosine hydroxylase (Th)-expressing neurons (Th-Cre; ErbB4 Cyt-1/iCyt-2), exhibit imbalances of basal extra- cellular DA levels in distinct projection areas (Kato et al., 2011; Mizuno et al., 2013; Skirzewski et al., 2018; Skirzewski et al., 2020; Yan et al., 2018) that are reminiscent of the imbalances in DA release reported in the dorsal striatum and dorsolateral prefrontal cortex (DLPFC) of Scz patients (Silfstein et al., 2015; Weinstein et al., 2017). Consistent with the association of the NRG/ErbB4 signaling pathway with a risk for Scz, mice with targeted mutations in either nrg1, nrg2, nrg3, or ErbB4 manifest many behavioral abnormalities that are associated with traits observed in Scz (Chen et al., 2010; Hayes et al., 2016; Lu et al., 2014; Muller et al., 2018; Shamir et al., 2012; Skirzewski et al., 2018; Tan et al., 2018; Wen et al., 2010; Yan et al., 2018), and in some studies shown to be ameliorated by antipsychotic treatment (Tan et al., 2018; Yan et al., 2018). In addition to having altered levels of extracellular dopamine (Skirzewski et al., 2018), ErbB4 null mice are hyperactive, exhibit anxiety-like behaviors on the elevated plus maze, have reduced pre-pulse inhibition (PPI) responses to startle, and perform poorly relative to wild-type littermates in numerous tasks (i.e., T-, Y- and Barnes-maze) that require hippocampal/prefrontal cortical functions (Shamir et al., 2012; Skirzewski et al., 2020; Wen et al., 2010).

Members of the ErbB receptor family (ErbB1-ErbB4) are type-I transmembrane proteins comprised of an extracellular NRG-binding domain and an intracellular tail encoding a tyrosine kinase and regulatory binding sites necessary for downstream signaling. NRG binding to ErbB3 or ErbB4 receptors promotes their heteromeric or homomeric dimerization to initiate MAPK or phosphatidylinositol-3-kinase (PI3K)/Akt downstream signaling. In the brain, ErbB4 is the major neuronal NRG receptor (reviewed by (Buonanno & Fischbach, 2001; Mei & Nave, 2014). Whereas several studies have investigated the consequences of ablating ErbB4 on the functions of cortical/hippocampal GABAergic interneurons and DAergic neurons, relatively little is known about the selective functions of different ErbB4 splice variants. Four ErbB4 isoforms are generated by alternative splicing of exons encoding sequences in either the extracellular juxtamembrane (JM) domain (exon 16) that encodes a cleavage site for metalloproteases, or at the cytoplasmic (Cyt) domain where inclusion or exclusion of exon 26 generates ErbB4 Cyt-1 and Cyt-2 isoforms, respectively (Elenius et al., 1997; Elenius et al., 1999). The additional 16 amino acids encoded by exon 26 of Cyt-1 receptor variants form a docking site for PI3K and a recognition motif for WW domain proteins (PPXY) (Elenius et al., 1999; Omorovic et al., 2004). As a result, Cyt-1-containing ErbB4 receptors can directly stimulate PI3K and Akt phosphorylation (Elenius et al., 1999; Kainulainen et al., 2000). By contrast, ErbB4 Cyt-2 subunits require heterodimerization with either ErbB3 (also activates PI3K) or ErbB4 Cyt-1 subunits to initiate downstream PI3K/Akt signaling (Gambarotta et al., 2004). Interestingly, in the postmortem DLPFC of Scz patients, the ratio of ErbB4 Cyt-1/Cyt-2 variants and the downstream target PI3KCD are increased compared to healthy controls (Chung et al., 2016; Joshi et al., 2014; Law et al., 2007; Law et al., 2012; Silberberg et al., 2006), suggesting a selective association of the ErbB4 Cyt-1 isoform with risk for Scz.

Both convergent and divergent biological functions have been reported for ErbB4 Cyt-1 and Cyt-2 isoforms based on experiments performed in non-neuronal cell lines (reviewed in [Veikkolainen et al., 2011]). In most regions of the CNS, Cyt-2 accounts for approximately 60% of total ErbB4 transcripts, whereas Cyt-1 variants predominate (~80%) in the heart (Chung et al., 2016; Erben et al., 2018; Veikkolainen et al., 2011). Several studies have suggested ErbB4 isoform-specific functions in the CNS (Fornasari et al., 2016; Fregnan et al., 2011; Gambarotta et al., 2004; Veikkolainen et al., 2011), including GABAergic interneuron migration during embryonic and postnatal development in vivo (Rakic et al., 2015). However, the relative contribution of Cyt-1 receptor isoforms to the numerous aforementioned functions attributed to ErbB4, based on studies using ErbB4 null mice, remains unknown. To investigate the specific functions of ErbB4 Cyt-1 on GABAergic and DAergic pathways in vivo, we generated ErbB4 Cyt-1 knockout (KO) mice using...
cre Cre/loxP site-directed homologous recombination to selectively target exon 26 either by germline deletion (Elia-Cre:ErbB4 Cyt-1fl/fl mice) or by acute ablation in the adult animals using adeno-associated virus expressing Cre-recombinase (AAV-Cre) microinjections in Cyt-1fl/fl mice.

2 | METHODS

Time-line of the study design, experimental procedures, and number of mice used: see Figure S1.

2.1 | Animals & generation of Cyt-1 KOs

Mice were kept on a 12–12 h light–dark schedule with access to food and water ad libitum. All studies were conducted in female and male mice during the light cycle. Animals were handled in accordance with the ARRIVE guidelines and the NIH Animal Welfare guidelines and all animal procedures were approved by the NIH Animal Care and Use Committee (ASP #18–074). The Cyt-1 targeting construct, harboring loxP sites flanking ErbB4 exon 26, was generated by "recombineering" using vector pL253 (Liu et al., 2003) (Figure 1a). Site-specific recombination of the erbb4 locus and generation of conditional Cyt-1 mutant mice was performed at the Transgenic & Genetically Engineered Models Core of the University of Alabama at Birmingham. Successfully targeted and PCR-validated embryonic stem (ES) cells derived from C57BL/6J mice (Li et al., 2016) were injected into blastocysts from albino C57BL/6J mice (JAX stock # 000058). Chimeric offspring were bred with albino C57BL/6J mice; only black F1 mice were used for further breeding. Mice were then crossed to FLP deleter strain B6;SJL-Tg(ACTFLPe)9205Dym/J (JAX stock # 003800) to remove the FRT- flanked neomycin cassette. Null (germline) Cyt-1 mutant mice, hereafter referred as Cyt-1 knockouts (KOs), were generated by crossing floxed Cyt-1 mice to mice expressing Cre recombinase under the control of ubiquitously active Elia promoter (also on C57BL/6 background; JAX stock # 003724). Heterozygous Cyt-1 mice were used for breeding and yielded normal litter sizes (6.4 pups ±0.11, N = 2 cohorts, n = 648 mice; compared to C57BL/6J 6.2 ±0.2 pups [Nagasawa et al., 1973]), balanced sexes (52.9 ±1.29% males, N = 2 cohorts, n = 648 mice), and Mendelian genotype frequencies (24.5 ±0.03% +/-, 50.3 ± 1.47%, 25.1 ± 1.44%, N = 2 cohorts, n = 648 mice). Routine genotyping was performed by PCR using forward primers 5′-AGTTTCTTTATCCAGGTTAAGG/GAGAGAGATGTAATGAGGTAAGG and reverse primer 5′-GATTGATCCAGAATGCTACACACATGGCC-3′. ErbB4 Cyt-1 and Cyt-2 probes were custom-made to respectively target mouse ErbB4 exon junctions 25/26 (5′-ACCTACACATCCAGAATGCTACACATGGCC-3′) and 25/27 (5′-CCATCTACACATCCAGAACAGAATGCTACACATGGCC-3′). Briefly, paraffin sections were deparaffinized with xylene and pretreated with hydrogen peroxide (10 min at RT), antigen-retrieved (15 min at 100°C), and incubated with proteases (protease III, 30 min at 40°C). Next, probes were incubated for 2 h at 40°C, the signal was chemically amplified with 7/8 amplification steps (BaseScope version 1/2) and subsequently detected using alkaline phosphatase and FastRED dye. For post hoc immunostaining, sections were immediately labeled with rat anti-DA transporter (DAT) antibody (clone 6-5G10; Santa Cruz; catalog # sc-32258; 1:200) followed by secondary donkey anti-rat antibody conjugated to Alexa 488 (1:1000, Thermo Fisher; catalog # A-21208) as described (Erben & Buonanno, 2019), using standard immunofluorescence histochemistry (IHC) protocol (see below).

2.2 | Isoform-specific in situ hybridization (ISH)

Exon-specific ISH (BaseScope version 1 & 2; Advanced Cell Diagnostics) was performed on coronal formalin-fixed paraffin-embedded serial 8 μm-sections of 8–11 week-old adult mice (C57BL/6J or Cyt-1 homozygote/ heterozygote mutants and control littersmates; both sexes) as previously described (Erben et al., 2018; Erben & Buonanno, 2019). ErbB4 Cyt-1 and Cyt-2 probes were custom-made to respectively target mouse ErbB4 exon junctions 25/26 (5′-CATCTACACATCCAGAATGCTACACATGGCC-3′ and 25/27 (5′-CCATCTACACATCCAGAACAGAATGCTACACATGGCC-3′). Cyt-1 KOs were crossed to GAD67- GFP and heterozygote/controls (n = 4, both sexes) were performed as previously described (Vullhorst et al., 2017). Briefly, mice were euthanized using an overdose of anesthetic Avertin and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline, pH 7.4. Dissected brains were post-fixed overnight in the same fixative at 4°C and 50 μm-thick serial sections cut on a vibratome. Sections were blocked in 10% normal donkey serum, 0.3% Triton X-100 in 0.1 M PBS for 1 h at RT and incubated with primary antibodies in blocking solution overnight at 4°C. Following three 10 min washes in 0.1 M PBS with 0.25% Triton X-100, sections were incubated with secondary antibodies in blocking buffer for 2 h at RT. Sections were extensively washed with 0.1 M PBS, counterstained with DAPI, and mounted with Mowiol-DABCO. Primary antibodies were rat monoclonal anti-GFP (clone GF090R, Nacalai Tesque, Japan; catalog # 04404–84; 1:2000), chicken anti-GFP (Life Technologies, catalog # A10262; 1:2000), rabbit anti-TH (Pel Freez; catalog # P40101; 1:3000) and rat monoclonal DAT (clone 6-5G10, Santa Cruz; catalog # sc-32258; 1:200), which were visualized with secondary
antibodies raised in donkey (Invitrogen, Thermo Fisher & Jackson Immuno Research). For GABAergic interneuron densities, bilateral cortical analyses were performed at three different bregma levels (~+0.26, -0.46, -1.22), and bilateral hippocampal analyses at four different bregma levels (~-1.22, -1.82, -2.46, -3.08). Damaged sections were excluded from analysis (n = 6 bregma locations excluded because of damage).

2.4 | Image analysis

Fluorescent FastRED ISH signals were analyzed at 20x magnification using Zeiss LSM710/800 confocal microscopes. Unbiased automated quantitative analysis of maximum intensity projections obtained from Z-stack images was performed using CellProfiler (Carpenter et al., 2006), as previously described (Erben et al., 2018).
For ISH-IHC analyses, >30% overlap of the cell with DAT staining was used to define a neuron as DAergic. Similarly, IHC in GAD-GFP mice was analyzed at 10x magnification using a Zeiss LSM800 confocal microscope. Density of GABAergic interneurons was analyzed from maximum intensity projections using a custom-made CellProfiler pipeline to identify GAD-GFP+ cells (size >25 μm² (SSCtx) or 50 μm² (Hpp); threshold = 1.5x median background intensity; eccentricity >0.94). Hippocampal subregions and cortical layers were defined based on morphology, DAPI stain, and interneuron density. Injection sites in floxed Cyt-1 mutant mice were verified at 10x magnification using a Zeiss LSM800 confocal microscope (see below). For all analyses, areas examined were not different between transcripts and/or genotype or group.

2.5 | Quantitative reverse-transcription PCR (RT-qPCR)

Real-time RT-qPCR using TaqMan probes was performed as previously described (Erben et al., 2018). Briefly, whole-brain (n = 3/ genotype) and hippocampal (n = 4/ genotype) total RNA was isolated from male and female ten-week-old Cyt-1 heterozygote, null, and WT littermates using TRI Reagent (Thermo Fisher; catalog # AM9738), cDNA was synthesized from 1 μg RNA using SuperScript IV Reverse Transcriptase (Thermo Fisher; catalog #18090050) and random hexamers for 20 min at 55°C. Gene expression was subsequently assessed using TaqMan universal PCR Master Mix (Thermo Fisher; catalog #4304437), 0.25 μM FAM/VIC-labeled TaqMan probes, and 0.9 μM primers from 2.5 to 5 ng cDNA in a total volume of 10 μl. Cycling was performed in 384-well plates using a QuantStudio 6 Thermocycler (Thermo Fisher) and the following parameters: 2 min at 50°C and 10 min at 92°C, followed by 40 cycles of 15 s at 95°C, 1 min at 65°C (for Cyt probes) or 60°C (for all other probes). Custom-made isoform-specific ErbB4 TaqMan probes (Thermo Fisher) were FAM-ATGACGCGGCAATCCATTTAACA-MGB for JM, FAM-TTCAAGCATTAGAGACTGACCGGCTGAC-MGB for JMb, FAM-TGAAATTGGACAGCCCTCGTCTCG-MGB for Cyt-1 and FAM-AA TTGACTTCAATAGGAATCAGTGTGTGTAAGAGT-MGB for Cyt-2. Flanking primers were 5′-CCACCTTTGCCATCCAAA-3′ and 5′-CCA TGACTCTGGGTGCAATCA-3′ for JM isoforms, and 5′-CAACATACCC TCTCTTACTTAC-3′ and 5′-GCATTTCTTGTGTGCAAA-3′ for Cyt isoforms. ErbB3 (Thermo Fisher, Mm01159999_m1) and ErbB4 (Thermo Fisher, Mm01256793_m1) TaqMan probes were commercial. A VIC-labeled TaqMan probe for GAPDH (Thermo Fisher, Mm9999915_g1) was used for normalization. Standard curves (10 ng to 1 ng) of cloned DNA for ErbB4 JMα/Cyt-1 and JMβ/Cyt-2 and of a synthetic DNA fragment for ErbB3, confirmed linearity and showed similar amplification efficiencies for isoform-specific assays. Assay specificity was tested using 100 pg DNA of non-matching ErbB4 isoforms or ErbB receptor.

2.6 | DA measurements

Basal extracellular DA levels in the medial prefrontal cortex (mPFC) of freely moving mice (n = 6–7/ genotype, both sexes, 2.5–4 months old) were measured using the non-net flux (NNF) microdialysis approach (Friend et al., 2017). Mice were anesthetized using aerosolized isoflurane first at a 3% flow rate for a 5-min induction period and then maintained throughout surgery at a 1.5–2% flow rate. Briefly, counterbalanced unilateral stainless-steel guide shafts (21-gauge, 6 mm-long) were chronically implanted into the mPFC (AP: 2.0 mm, L: 0.3 mm, V: 0.3 mm with respect to bregma). One week after surgery, NNF measurements were made after lowering a laboratory-made microdialysis probe through the implanted guide shaft as reported (Hernandez et al., 1986). The tip of the microdialysis probe consisted of a 2 mm-long cellulose hollow fiber (18 kDa MWCO, SpectrumLabs Inc) protruding from the tip of the guide shaft. Artificial cerebrospinal fluid (aCSF; 136 mM NaCl, 3.7 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, and 10 mM NaHCO₃ at pH 7.4) was continuously perfused through the microdialysis probe at a flow rate of 1 μl/min for 2 h before sample collection. Five different DA standard concentrations (0, 0.5, 1.0, 1.5, and 2.0 nM) were randomly perfused for 60 min through the microdialysis probe, and duplicate 30-min
samples per standard were collected in 10 μl of 100 mM HCl+ 1 mM EDTA to prevent catecholamine degradation. Samples were immediately frozen and stored at −80°C until subsequent analysis within a week. Probe placements were verified by Nissl staining in 50-μm-thick vibratome sections (Figure S5a); animals with misplaced probes were excluded from the analysis. DA standards and samples were injected using a HPLC autosampler (INSIGHT, Eicom) into an isocratic HPLC system coupled to amperometric detection according to the manufacturer’s instructions (HTEC-510, Eicom). Regression curves were generated by subtracting the area under the curve of the corresponding standard (IN) minus the area under the curve of the dialysate sample (OUT). Regression equations were used to estimate the NNF DA concentration (at y = 0) for each mouse.

2.7 | Stereotaxic injections

Adult male and female 3- to 4-month-old Cyt-1fl/fl mice were used for stereotaxic bilateral mesencephalic microinjections. To acutely target ErbB4 Cyt-1fl/fl recombination and visualize the transduced neurons, AAV-Cre (serotype 9; pENN.AAV.hSyn.Cre.hGH, Addgene; catalog #105555, Lot: v35950) was mixed with DIO-GFP (pAAV.synP.DIO.EGFP:WRPE.hGH, Addgene; catalog #100043, Lot: v25058) at 1.0 × 10^10 GC/mL (Cre) and 3.0 × 10^12 GC/ml (DIO-GFP) in sterile PBS. As controls, mice were microinjected with 3.0 × 10^12 GC/ml AAV-GFP (pAAV:hSyn.eGFP:WRPE.bGH, Addgene; catalog #105539, Lot: 38081) diluted in sterile PBS. Cyt-1fl/fl mice were anesthetized during surgery using isoflurane as described above. Equal numbers of Cyt-1fl/fl mice were microinjected with 200 nl of either AAV Cre/DIO-GFP or AAV-GFP at a flow rate of 5 nl/s using the following stereotaxic coordinates: AP -2.7 mm (with respect to bregma), L ± 0.75 mm (with respect to bregma), V -4.35/-4.3 mm (relative to brain surface: 100nl/each 2) to target the VTA. Microinjections using glass capillaries (Drummond Nanoinjector) were performed for 8 min and capillaries were left in place for an additional 2 min before withdrawing to avoid retrograde mass flow. Mice were allowed to recover and closely monitored for 2 weeks before they were used in behavioral tests and given i.p. ketoprofen injections (5 mg/kg) three consecutive days post-op to manage pain. Upon completion, injection sites were verified by immunostaining using anti-GFP, and markers for DAergic neurons anti-DAT and anti-TH antibodies; misinjected mice were excluded from analyses (n = 4 mice excluded from analyses for misinjection).

2.8 | Behavioral tests

Behavioral testing of conditional ErbB4 Cyt-1 KO mice (3–7 months old) was conducted in the following sequence: open field, elevated plus maze, PPI, Y-maze, and Barnes maze testing. Acute AAV-injected Cyt-1fl/fl mice (3 months old; both sexes) were tested as follows: in the open field, elevated plus maze, Y-maze, and PPI. Behavioral testing was performed on multiple cohorts comprised of male and female mice during the light cycle and all apparatuses were cleaned with 70% ethanol between trials. Tracking in the open field and the elevated, Y and Barnes mazes were conducted with ANY-maze software. The experimenter was blind to the mouse genotypes except for behaviors performed with acute AAV-injected Cyt-1fl/fl mice.

2.8.1 | Open field

Mice (n = 12–14/genotype) were habituated to the testing environment for 30 min prior to measuring locomotor activity in a 50 × 50 × 30 cm white chamber (the center was defined as 28 × 28 cm, 70–80 lux in the perimeter, 80–90 lux in the center). Mice were permitted to freely explore the maze for 30 min, and the traveled distance and time spent in the center were recorded.

2.8.2 | Elevated plus maze

The elevated plus maze test for anxiety-like behavior was performed in a plus-shaped white plastic apparatus (30 × 5 cm arms) consisting of two closed arms (18 cm-high black walls, 60–70 lux) and two open arms (130–140 lux) elevated 40 cm above the ground. ErbB4 Cyt-1 mutant and WT controls (n = 11–14/genotype) were habituated for 30 min prior to testing. Mice were permitted to explore the maze for 5 min and time spent in the open and closed arms was recorded.

2.8.3 | PPI

Startle reflex and PPI were measured in a SR-LAB-Startle Response System (San Diego Instruments). ErbB4 Cyt-1 mutant mice (n = 17–18/genotype) were habituated to a 65 dB background noise in the plexiglass tube of the testing chamber for 5 min on 3 consecutive days. Startle reflex was measured by pseudo-random presentations of tones ranging from 70 to 120 db (5 db increments, 5 tones each) and normalized to the average startle response to 120 db pulses of the entire cohort. During PPI testing on the following day, animals were presented with a pseudorandom sequence of 20ms pre-pulse tones (PP; 66, 70, 74, 78 dB; 12 times each) and 40ms 120 db pulse pairings with the variable inter-trial intervals between 10 and 45 s. A 120 db no pre-pulse (NPP) presented 28 times was used to calculate the percentage of PPI of startle response as [(average startle to NPP–average startle to PPI)/ average startle to NPP]*100.

2.8.4 | Y-maze

Working memory was tested in Cyt-1 mutant mice (n = 12–14/genotype) using a Y-maze apparatus (30 × 18 × 9.5 cm three-arm maze with opaque tan walls, 50 lux). Mice were habituated for 30 min to the testing environment and then permitted to explore the maze
for 5 min. Novel arm entries (spontaneous alternation) were identified as entries into the third arm different from the current and previously explored arm. Consecutive arm entry is considered as a single entry but accounted for total arm entries (Heredia-Lopez et al., 2016). Percentage of alternation was calculated as [(number of alternations)/(total arm entries - 2)] * 100.

2.8.5 | Barnes maze

Barnes maze spatial learning and memory test was conducted as previously described (Skirzewski et al., 2018). The edge of the round elevated apparatus (Stoelting, 90 cm diameter, 800 lux, 90 cm above ground) was marked by 20 open holes (~10 cm diameter) with a hidden escape chamber placed underneath one of the holes (target, 0) and the remaining holes labeled as +1 to +10, and −1 to −9. The edges of the maze were covered with a wall providing spatial cues and 85 db background white noise was played during all phases of the test. The Barnes Maze test consisted of three phases: training, probe test, and new escape test. During the training, mice (n = 8–11/ genotype) were allowed to explore the maze. The trial ended when the animal entered the escape chamber; at that point, the noise switched off automatically and the aversive light was blocked. The mouse was held in the chamber for 1 min. Mice that could not locate the chamber within 3 min were manually guided to the escape hole. The training was conducted over 4 days (4 trials per day). Mice that failed to escape the maze in more than one trial on the fourth day of training were excluded from the analysis. On the fifth day, the escape chamber was removed, and mice were tested for 90 s (probe test). Latency to escape and time in the correct zone were recorded using ANY-maze software (Stoelting Co), target hole nose pokes and errors defined as non-target nose pokes were quantified by handwriting of collected videos. Forty-eight hours after the probe test, mice were re-introduced to the testing apparatus in which the escape chamber had been moved from its original position to hole +8 (new target test). Mice were permitted to explore the maze until they located the new escape for up to 3 min, at which point they were guided to the new escape (see learning phase). Four subsequent trials were performed and latency to escape and errors were quantified as described above.

2.9 | Experimental design and statistical analyses

Statistical analyses were performed using Graph Pad Prism 8 and JASP (Version 0.11.1). All data represent the mean ± SEM and statistical significance was set at p < 0.05. Outliers (ROUT, Q = 1%) were excluded from data analyses. All data sets were examined for normality using Shapiro–Wilks test and for equality of variance using Levene’s/ Mauchly’s test and, if necessary, data were corrected using Welch’s/ Geisser–Greenhouse correction Statistical analyses were performed using one-way ANOVA/Welch’s ANOVA/Kruskal-Wallis test and Tukey’s/ Dunnett’s multiple comparisons test (Figures 1c–e; 3g, h; 4a; 5b; 6a, d, e); paired nonparametric Wilcoxon test (Figure 2c, d), or unpaired t-test (Figure 7c) for comparison of two groups only and two-way ANOVA/ mixed-effects analysis and Tukey’s multiple comparison test for comparisons of multiple variables (Figure 3i, j; Figure 4b–d, Figure 6b, c, f–h; Figure 7b, d, e). Simple randomization was performed to assign animals to a group, groups were balanced for sex and within cages when applicable (Kim & Shin, 2014). Given that Cyt-1 is a splice variant of ErbB4, requisite sample sizes were determined based on previous work in our lab studying animals with mutations in the Nrg/ErbB signaling pathway that had sufficient power to identify biologically significant phenotypes using approaches similar to those employed here (Shamir et al., 2012; Skirzewski et al., 2020; Wen et al., 2010); a priori power calculations were not performed.

3 | RESULTS

3.1 | Generation of Cyt-1 KO mice

Numerous studies have investigated the functions of ErbB4 receptors during early neurodevelopment and in adult using heart-rescued ErbB4 null mice (Flames et al., 2004; Shamir et al., 2012; Tan et al., 2018; Tidcombe et al., 2003). However, because the specific in vivo functions of different receptor isoforms are presently unknown, we targeted exon 26 by Cre/loxP homologous recombination to investigate the role of ErbB4 Cyt-1 isoforms (see Materials & Methods, Figure 1a). Initially, the frt-flanked neomycin cassette was removed by crossing founder mice to B6SJL-Tg(ACI-FPLPe)9205Dym/J (Figure 1a). Germline ablation of exon 26 from Cyt-1fl/fl mice was achieved by crossing neomycin-deleted offspring to Ella-Cre mice and verified by PCR (Figure 1b). ErbB4 Cyt-1 KOs are viable and have normal litter sizes (6.4 pups ± 0.1, N = 2 cohorts; C57BL/6J 6.2 ± 0.2 pups [Nagasawa et al., 1973]) and exhibit no obvious anatomical, developmental or behavioral abnormalities. These observations were unexpected because germline deletion of ErbB4 results in embryonic lethality (Gassmann et al., 1995) and Cyt-1 transcripts account for 80% of all ErbB4 transcripts in the heart (Chung et al., 2016; Erben et al., 2018; Veikkolainen et al., 2011), suggesting that ErbB4 Cyt-2 isoforms can functionally compensate for Cyt-1-containing receptors during cardiac development.

Using exon-specific primers and Taqman qRT-PCR, we quantified how the removal of exon 26 affects the relative transcript levels of different ErbB4 Cyt-1/Cyt-2 and JMa/JMb receptor isoforms in brains of Cyt-1 heterozygote and KO mice (Figure 1c). We found that Cyt-1 transcripts account for 40% of ErbB4 Cyt variants in whole-brain RNA from controls and that their levels are reduced to approximately half in Cyt-1 heterozygotes and undetectable in KOs (Figure 1c, left). On the contrary, the relative levels of JMa to JMb receptor isoforms were similar across the three genotypes (Figure 1c, right), indicating that splicing of ErbB4 transcripts at the two loci (JM and Cyt) are regulated independently. Importantly, the removal of exon 26 and its splicing acceptor/donor sites did not affect overall...
receptor expression, as the total levels of ErbB4 transcripts relative to GAPDH in whole brain (Figure 1d) are unchanged in Cyt-1 heterozygote and KO mice relative to controls, and only a small increase in ErbB4 transcripts was observed in the hippocampus of Cyt-1 KOs (Figure 1e). Lastly, we investigated if the expression of ErbB3 is affected in ErbB4 Cyt-1 KOs. This is important because Cyt-2 subunits...
could potentially heterodimerize with ErbB3 to activate downstream PI3K/Akt, as reported previously (Gambarotta et al., 2004), to compensate for the loss of Cyt-1 isoforms. As expected, the relative levels of ErbB3 are lower than ErbB4, and we found that ErbB3 expression is unchanged in the whole-brain (Figure 1d) and hippocampus (Figure 1e) of Cyt-1 heterozygote and KO mice relative to controls. Taken together, these results show that ErbB4 Cyt-1 KOs exclusively express Cyt-2 receptor isoforms that are not compensated by ErbB3.

3.2 Cyt-1 is expressed on cortical and hippocampal GABAergic interneurons, but dispensable for GABAergic interneuron development

ErbB4 Cyt-1 variants are highly expressed at ganglionic eminences and in migrating GABAergic neuroblasts during embryonic and postnatal development (Fornasari et al., 2016; Fregnan et al., 2014; Rakic et al., 2015), and adult ErbB4 KO mice have reduced cortical and

**Figure 3** GABAergic interneurons in the hippocampus and primary somatosensory cortex are unaltered in Cyt-1 mutant mice. Representative images of GFP-expressing GABAergic interneurons in the (a–c) dorsal hippocampus and (d–f) primary somatosensory cortex of control (a, d), Cyt-1 heterozygote (b, e), and KO (c, f) adolescent mice (P30). GABAergic neurons were labeled by breeding ErbB4 Cyt-1 KOs to transgenic GAD67-GFP mice (see Methods). Quantification of GFP-labeled GABAergic interneurons/mm² in the (g) hippocampus (genotypes +/+; 180.8 ± 7.73, +/-; 178.1 ± 8.21 and -/-; 180.1 ± 3.54 cells/mm²; n = 4, Kruskal-Wallis test, p = 0.9410) and (h) in SSCtx (genotypes +/+; 407.7 ± 10.71, +/-; 385.5 ± 7.10 and -/-; 392.0 ± 11.99 cells/mm²; n = 4, one-way ANOVA F(2, 9) = 1.264, p = 0.3283). (i, j) Graphs showing (i) the Hpp subregional and (j) SSCtx layer-specific distribution of GFP-labeled GABAergic interneurons in ErbB4 Cyt-1 KO (orange), heterozygote (green) and control (black) mice (n = 4; two-way ANOVA). Scale bars 500 μm in (c), 100 μm in (f). DG, dentate gyrus; sub, subiculum; CA1-CA3, cornu ammonis regions.
hippocampal GABAergic interneurons (Flames et al., 2004; Neddens & Buonanno, 2010). To test whether ErbB4 Cyt-1 is involved in the migration and/or maturation of GABAergic interneurons, using exon-specific ISH (i.e., BaseScope) we initially characterized the expression of Cyt-1 and Cyt-2 ErbB4 splice variants in the adult mouse dorsal hippocampus (Hpp) and somatosensory cortex (SSCtx). As shown in the representative micrographs, Cyt-1 (Figure 2a) and Cyt-2 (Figure 2b) isoforms were detected in a scattered pattern in the Hpp (left) and SSCtx (right), consistent with the distribution of GABAergic interneurons (Lai & Lemke, 1991; Yau et al., 2003). Cyt-1 ErbB4 variants accounted for ~40% of total ErbB4 in both areas (Figure 2c, d), similar to the relative levels previously reported (Chung et al., 2016; Erben et al., 2018; Neddens et al., 2011; Veikkolainen et al., 2011). These observations suggest that a significant portion of ErbB4 signaling in cortical and hippocampal GABAergic interneurons is mediated through ErbB4 Cyt-1 receptors.

FIGURE 4 Behaviors altered in ErbB4 null mice are not affected in Cyt-1 KOs. Performances on motor, affective (anxiety) and sensory-motor behavioral tasks known to be altered in ErbB4 null and PV-Cre; ErbB4fl/fl mice were analyzed in cohorts of adult Cyt-1 KO (orange), heterozygote (green) and control (black) mice. (a) Open field: mice showed similar habituation to a novel environment and total distance traveled in 30 min (genotypes +/+: 101.7 ± 6.2, +/-: 91.3 ± 3.8, −/−: 85.6 ± 2.4 meters, Welch's ANOVA, F(2, 21.4) = 3.197, p = 0.0610, Dunnett’s multiple comparisons test +/+ vs. −/− p = 0.0791, +/- vs. +/+ p = 0.4167, +/+ vs. −/− p = 0.5085; n = 10–14/genotype). (b) Elevated plus maze: Cyt-1 KO, heterozygotes and control mice spent the same time (sec) in the open (genotypes +/+: 27.04 ± 3.87, +/-: 28.59 ± 6.41 and −/−: 25.07 ± 4.36) and closed (genotypes +/+: 194.5 ± 9.82, +/-: 187.8 ± 9.81, −/−: 189.7 ± 11.21; mixed-effects analysis, F(2, 69) = 0.1420, p = 0.8679, 1 outlier value removed) arms of the plus maze. (c) Startle response (arbitrary units; AU) to increasing auditory stimuli intensities (70–120 dB) in both sexes (left panel: n = 17–18/genotype, mixed-effects analysis, F(22, 532) = 1.191, p = 0.2494, 10 outlier values removed), male ErbB4 Cyt-1 KOs only (center panel: n = 8–9/genotype, mixed-effects analysis, F(2, 23) = 5.321, p = 0.0126*), and female KO mice only (right panel: n = 9/genotype, mixed-effects analysis, F(2, 24) = 0.01569, p = 0.9844). (d) PPI: sensory-motor inhibitory responses were not significantly different between Cyt-1 KO, heterozygote and control mice (n = 17–18/genotype, two-way ANOVA, F(2, 49) = 0.1582, p = 0.8541)
Based on the aforementioned observations, we investigated if germine ablation of ErbB4 Cyt-1 receptors alters GABAergic interneuron migration by assessing interneuron numbers and distribution in the dorsal postnatal Hpp and SSCtx of WT, Cyt-1 heterozygote, and Cyt-1 KO mice. For these experiments, GFP-labeled GABAergic interneurons were analyzed in mice derived from crosses between GAD-GFP transgenic (Tamamaki et al., 2003) and Cyt-1 KO mice (see Methods). Unexpectedly, GABAergic interneuron number was unchanged in the Hpp (Figure 3a–c, g) and SSCtx (D–F, H) of young adult Cyt-1 heterozygote and KO mice relative to controls (GAD-GFP cells/mm²; Cyt-1 +/+ 180.8 ± 7.73 cells/mm², Cyt-1 +/- 178.1 ± 8.21 cells/mm², Cyt-1 −/− 180.1 ± 3.54 cells/mm²; n = 4, Kruskal-Wallis test, p = 0.9410; in SSCtx: Cyt-1 +/+ 407.7 ± 10.71 cells/mm², Cyt-1 +/- 385.5 ± 7.10 cells/mm², Cyt-1 −/− 392.0 ± 11.99 cells/mm²; n = 4, one-way ANOVA F(2, 9) = 1.264, p = 0.3283). Further analysis of the distribution of GAD-GFP expressing neurons in hippocampal areas CA1-CA3, dentate gyrus, and subiculum (Figure 3i), as well as layers I-VI of the SSCtx (Figure 3i), failed to detect differences between WT and Cyt-1 heterozygote and KO mice. Based on these observations, we conclude that ErbB4 Cyt-1 isoforms are dispensable for GABAergic interneuron migration and postnatal viability.

3.3 | Cyt-1 mutant mice exhibit minor behavioral abnormalities compared to ErbB4 null mice

ErbB4 null and conditional KO mice lacking ErbB4 selectively in PV-interneurons (PV-Cre; ErbB4fl/fl) exhibit numerous overlapping behavioral deficits, such as hyperactivity in the open field, reduced anxiety on the elevated plus maze, and impaired PPI (Shamir et al., 2012; Skirzewski et al., 2020; Wen et al., 2010). To determine if ErbB4 Cyt-1 receptors contribute to these phenotypes, we subjected Cyt-1 KOs to a similar battery of behavioral tests (Figure 4). We found that Cyt-1 heterozygote and KO mice traveled similar distances in the open field compared to their littermate WT controls, with a trend toward hypoactivity and faster habituation (Figure 4a). In the elevated plus maze, Cyt-1 KOs and their controls spent similar time in the open and closed arms, suggesting that Cyt-1 KOs are not more anxious than controls (Figure 4b). Next, we investigated sensory-motor responses of Cyt-1 mutants by measuring PPI to startle, a response that is altered in PV-Cre; ErbB4fl/fl and ErbB4 null mice and is an endophenotype associated with Scz. Initially, we measured startle responses to increasing intensities of sound and found that, although the overall startle responses were similar between controls, ErbB4 Cyt-1 heterozygote, and KO mice (Figure 4c, left), there was a significant interaction of sex and genotype in male (Figure 4c, center; F(2, 23) = 5.321, p = 0.0126) but not female mice (Figure 4c, right; F(2, 24) = 0.01569, p = 0.9844). We then proceeded to analyze PPI and observed no differences in sensorimotor gating between controls, ErbB4 Cyt-1 heterozygote, and KO mice (Figure 4d; two-way ANOVA, F(2, 49) = 0.1582, p = 0.8541). These results suggest that ErbB4 Cyt-1 isoforms are unlikely to account for the behavioral phenotypes previously reported in ErbB4 null and PV-Cre; ErbB4fl/fl mice and that their functions can be compensated by Cyt-2 receptor isoforms.

3.4 | Extracellular DA levels are augmented in ErbB4 Cyt-1 mutants

We (Skirzewski et al., 2018; Skirzewski et al., 2020) and others (Kato et al., 2011; Mizuno et al., 2013; Namba et al., 2016) previously reported that ErbB4 is expressed in approximately 90% of
Cyt-1 mutant mice have normal working and reference memory, and cognitive flexibility. (a) Working memory, plotted as percentage for novel arm preference in the Y-maze, is normal in Cyt-1 mutant mice (−/−; orange) compared to heterozygote (+/−; green) and wildtype (+/++; black) littermates (n = 12–14/genotype, % alternation +/+ 47.58 ± 2.00%, +/− 44.66 ± 2.32%, +/+ 46.54 ± 2.12%, one-way ANOVA, F(2, 36) = 0.4677, p = 0.6302). (b–g) Spatial reference memory of Cyt-1 KO mice in the Barnes maze is comparable to heterozygote and wild-type littermates during (b, c) initial training over four days, (d–f) testing on day five (n = 8–11/genotype) and (g) adaption to a new target location. (b) Latency to escape during training (n = 8–11/genotype, two-way ANOVA, F(6, 81) = 1.580, p = 0.1635). (c) Number of errors committed during training (n = 8–11/genotype, mixed-effects analysis, F(6, 80) = 1.158, p = 0.3373). (d) Number of errors during testing (+/+: 17.5 ± 3.5, +/−: 24.8 ± 3.5, −/−: 20.8 ± 3.8, one-way ANOVA, F(2, 27) = 0.9498, p = 0.3994). (e) Time spent in the correct quadrant/zone during testing (+/+: 33.6 ± 4.7 s, +/−: 36.0 ± 3.0 s, −/−: 37.4 ± 3.4 s, one-way ANOVA, F(2, 27) = 0.2508, p = 0.7799). (f) Nose pokes in individual holes during testing (0 – target, 10 – opposite; two-way ANOVA (no outlier exclusion), F(38, 513) = 0.8407, p = 0.7396, Tukey’s multiple comparisons test main column effect, +/+ vs. +/− p = 0.1450, +/+ vs. −/− p = 0.8096, +/− vs. −/− p = 0.3263) (g) Latency to escape (n = 8–11/genotype, mixed-effects analysis, F(6, 79) = 0.5949, p = 0.7335, 2 outlier values) and (h) number of errors after moving target to a new position (cognitive flexibility; n = 8–11/genotype, mixed-effects analysis, F(6, 78) = 1.906, p = 0.0903, 3 outlier values removed).
DAergic neurons in the mesencephalon, where it regulates the function of the DA reuptake transporter (Skirzewski et al., 2018; Skirzewski et al., 2020). Moreover, Th-Cre;ErbB4fl/fl mice exhibit an imbalance of extracellular DA levels in the mPFC that is rescued by microinjection of AAV-ErbB4 into the mesencephalon of adult Th-Cre;ErbB4fl/fl mice (Skirzewski et al., 2018; Skirzewski et al., 2020). To evaluate the role of Cyt-1 in modulating extracellular DA levels, we performed no-net-flux microdialysis in the mPFC of freely moving Cyt-1 KOs (Figure 5a). Unexpectedly, we found that ErbB4 Cyt-1 heterozygote mice, but not KOs, have increased basal extracellular DA levels (Figure 5b; ++ 0.546 ± 0.080 nM, +/- 0.977 ± 0.103 nM, +/− 0.689 ± 0.085 nM, n = 6–7 genotype, one-way ANOVA, F(2, 17) = 5.863, *p = 0.0116; ++ vs. -/- p = 0.4876, +/- vs. ++ *p = 0.0094, +/- vs. -/- p = 0.089). The reasons why ErbB4 Cyt-1 heterozygotes but not KOs exhibit differences in DA levels are presently unknown; potential reasons are discussed below (see Discussion).

### 3.5 ErbB4 Cyt-1 KOs and WT controls perform similarly on the Y- and Barnes-mazes

The alterations in extracellular DA levels in the mPFC of heterozygous Cyt-1 mice prompted us to investigate cognitive performance in mutant mice, which were previously reported to be impaired in ErbB4 null (Skirzewski et al., 2020; Tan et al., 2018) and...
Th-Cre;ErbB4<Sup>fl/fl</Sup> mice (Skirzewski et al., 2018). Mice were tested on a Y-maze spontaneous alternation task to assess spatial working memory, and in a Barnes maze to assess learning abilities and spatial reference memory. Novel arm preference in the Y-maze was unaltered in Cyt-1 KO mice compared to controls (Figure 6a). Next, we tested the acquisition and recall of spatial reference memory in the Barnes maze. Cyt-1 KO mice learned the task equally well compared to heterozygotes and controls, as evidenced by similar latency to escape (Figure 6b) and the number of errors committed during the 4-day training phase (Figure 6c). Cyt-1 heterozygote and KO mice performed similarly in controls in the probe test that measures the number of errors, time spent in the correct zone and the number of nose pokes/hole, including the target escape hole (Figure 6d–f).

Finally, we tested cognitive flexibility and perseverance by moving the escape target in the Barnes maze to a new location. All three genotypes adapted equally to the new paradigm as indicated by similar latencies to escape (Figure 6g) and errors committed during the four trials (Figure 6h).

To circumvent possible developmental compensation in germline deletion of ErbB4 Cyt-1 isoforms does not affect cognitive behaviors previously shown to be altered in ErbB4 null and targeted Th-Cre;ErbB4<Sup>fl/fl</Sup> mice, and they suggest that the functions of ErbB4 Cyt-1 receptors are potentially compensated during development by Cyt-2 isoforms.

3.6 | Behavioral effects are observed when ErbB4 Cyt-1 is acutely targeted in the adult VTA

To circumvent possible developmental compensation in germline deletion of ErbB4 Cyt-1 KO mice, we employed an acute loss-of-function paradigm in adulthood by injecting AAV-Cre into the VTA of ErbB4 Cyt-1<Sup>fl/fl</Sup> mice (Figure 7a). Because direct detection of Cyt-1 recombination is technically challenging, we injected control littermate mice with AAV9-hSynl-GFP and acutely targeted the floxed Cyt-1 exon in ErbB4 Cyt-1<Sup>fl/fl</Sup> mice (hereafter referred as “acute Cyt-1 KO”) by co-injecting AAV9-hSynl-Cre and AAV9-hSynl-DIO-GFP. Cre-mediated recombination was effective in mesencephalic DAergic neurons, as judged by the presence of numerous TH-positive neurons that co-label with GFP (Figure S2). Importantly, we used low AAV-Cre titers that were sufficiently high to recombine loxP sites but that did not alter the expression of TH or DAT non-specifically as others (Rezai Amin et al., 2019) and we (unpublished) observed when using higher AAV-Cre titers (DAT ctrl vs. Cre <i>p</i> = 0.7531; TH ctrl vs. Cre <i>p</i> = 0.7471, <i>n</i> = 8–11/group, Welch’s test).

After allowing time for recovery and recombination (see Methods), cohorts of acute Cyt-1 KO and controls were tested on different behavioral tasks. In the elevated plus maze, acute Cyt-1 KO mice spent less time in the closed arms compared to controls (Figure 7b; two-way ANOVA, F(1, 17) = 4.421, <i>p</i> = 0.0507, Sidak’s multiple comparison test controls vs. Cyt-1 KO <i>p</i> = 0.0234<Sup>+</Sup>), suggesting reduced anxiety levels in acute Cyt-1 KO mice. Next, we used Y-maze spontaneous alternation to evaluate hippocampal and mPFC function because we recently reported that re-expression of ErbB4 in the VTA of adult Th-Cre; ErbB4<Sup>fl/fl</Sup> mice restores their performance in this task (Skirzewski et al., 2018). We found that alternation in the Y-maze, which evaluates working memory, did not differ between acute Cyt-1 KO mice and controls (Figure 7c).

Interestingly, acute Cyt-1 KO mice showed increased startle responses relative to controls (Figure 7d; mixed-effects analysis, F(11, 181) = 1.874, <i>p</i> = 0.454*, ctrl vs. Cre <i>p</i> = 0.0490<Sup>+</Sup>), but PPI was unaffected (Figure 7e; two-way ANOVA, F(3, 51) = 0.9446, <i>p</i> = 0.454*, ctrl vs. Cre <i>p</i> = 0.0601). When the results of acute exon 26 ablations are taken together with the effects on DA levels and behaviors we observed in ErbB4 Cyt-1 heterozygote mice, they suggest that Cyt-2 receptor isoforms can effectively compensate for the lack of Cyt-1 isoforms during development and to a lesser extent when deleted acutely in adults (see Discussion).

4 | DISCUSSION

We were interested in studying the effects of germline and acute ablation of the ErbB4 Cyt-1 receptor isoform in adults because numerous studies have underscored the importance of ErbB4 function during development and continued expression in mature mice for regulating DA levels, neuronal plasticity, and behaviors (Del Pino et al., 2013; Shamir et al., 2012; Skirzewski et al., 2018; Wang et al., 2018; Wen et al., 2010). Moreover, polymorphisms regulating Cyt-1 splicing (Law et al., 2007; Silberberg et al., 2006) and Cyt-1/Cyt-2 transcript ratios in the postmortem DLPFC of patients are both associated with risk for Scz (Chung et al., 2016; Joshi et al., 2014; Law et al., 2007; Silberberg et al., 2006). We undertook the present studies to investigate the functional role of ErbB4 Cyt-1 receptors in vivo, by generating a novel conditional Cyt-1<Sup>fl/fl</Sup> mouse line used to excise exon 26 either constitutively in the germline or acutely in the adult brain by microinjecting AAV-Cre in the VTA.

4.1 | Developmental loss of ErbB4 Cyt-1 receptors in germline KO mice is partially compensated

Although Cyt-1 is the major cardiac ErbB4 isoform (~80%), we unexpectedly found that germline Cyt-1 KO mice are viable. Similarly, although Cyt-1 comprises ~40% of the receptor in the brain, germline ablation of Cyt-1 receptors does not result in a loss of GABAergic interneurons during neurodevelopment, as had been previously observed in ErbB4 null mice (Li et al., 2012; Perez-Garcia, 2015), and Cyt-1 KO mice exhibit minor behavioral impairments relative to ErbB4 KO mice (Fazzari et al., 2010; Flames et al., 2004; Shamir et al., 2012; Skirzewski et al., 2018). Nevertheless, we observed that heterozygote Cyt-1 mutants exhibit elevated basal extracellular DA levels in the mPFC (Figure 5), suggesting that mutation of both ErbB4 exon 26 alleles may be required to trigger alternative mechanisms to compensate for the loss of Cyt-1-mediated PI3K/Akt signaling during cardiac and neuronal development.
While it is unlikely that Cyt-1 and Cyt-2 ErbB4 receptors exert completely redundant functions in vivo, given that expression of both isoforms has been conserved through evolution, our data strongly suggest that loss of Cyt-1 can be compensated on a cellular or systemic level in both the heart and the brain. Of note, total ErbB4 transcript levels are unchanged in Cyt-1 KO mice and, instead, removal of exon 26 directs all splicing to generate exclusively the Cyt-2 isoform (Figure 1c, d). The loss of Cyt-1-containing receptors could arguably be compensated functionally by the heterodimerization of ErbB4 Cyt-2 with ErbB3 receptors to indirectly activate PI3K (Elenius et al., 1999; Gambaretta et al., 2004). However, this possibility seems remote for the following reasons: 1) ErbB3 mRNA expression is generally undetectable in the developing heart and central neurons (Pinkas-Kramarski et al., 1997; Steiner et al., 1999), 2) ErbB3 expression does not increase in the brain of Cyt1 KOs (Figure 1e) and 3) ErbB4 functions were not compensated in mice harboring germline mutations of the receptor (Shamir et al., 2012) or in conditional mice with targeted ErbB4 ablation in either GABAergic (Fazzari et al., 2014; Flames et al., 2004; Mei & Nave, 2014; Shamir et al., 2012; Skirzewski et al., 2018) or D/Aergic (Skirzewski et al., 2018) neurons, indicating that continued ErbB4 function is required in the adult and cannot be compensated by ErbB3.

Other potential compensatory mechanisms are that MAPK signaling downstream of ErbB4 Cyt-2 positively regulates PI3K/Akt/mTOR, as previously reported (Mendoza et al., 2011), or that it is the structural roles of the receptor, rather than its kinase activity, that regulate many of the functions attributed to ErbB4. For example, NRG modulates the expression of GABA alpha1 receptors in an ErbB4-dependent, but kinase-independent mode (Mitchell et al., 2013), and NRG3 regulates ErbB4-containing glutamatergic synapses on GABAergic interneurons without activating ErbB4 kinase activity (Mueller et al., 2018). Hence, several of the behavioral abnormalities observed in ErbB4 KO mice could have resulted from the physical loss of the receptor and not its kinase and downstream signaling activities.

4.2 | Some functions of Cyt-1 isoforms are not compensated when acutely targeted in adult mice

The deficits in neurotransmission, neuronal plasticity, and behaviors observed in mice that develop in the absence of ErbB4 (Del Pino et al., 2013; Shamir et al., 2012; Skirzewski et al., 2018; Wang et al., 2018; Wen et al., 2010) can be rescued in adult mice by re-expressing ErbB4 (Skirzewski et al., 2018; Wang et al., 2018) or pharmacologically reactivating (Skirzewski et al., 2018; Wang et al., 2018) receptor function, indicating a continued requirement for ErbB4 in the adult. Interestingly, we found that these findings are consistent with our experiments using acute ablation of Cyt-1 receptors in the mouse VTA that resulted in increased startle responses and reduced time in the closed arms of an elevated plus maze, which was not observed in germline ErbB4 Cyt-1 KOs, thus uncovering functions for these receptor variants in adult mice that cannot be compensated by ErbB4 Cyt-2 isoforms.

4.3 | Potential importance of ErbB4 Cyt isoforms in schizophrenia

Polymorphisms in ERBB4 have been linked to an increased risk for Scz. While the question of whether total ErbB4 levels are unchanged or increased in Scz is controversial (see Chung et al., 2016; Joshi et al., 2014), five independent studies have identified an increased ratio of JMa and Cyt-1 to JMb and Cyt-2 transcript levels in the DLPFC of Scz patients (Chung et al., 2016; Chung et al., 2018; Joshi et al., 2014; Law et al., 2007; Law et al., 2012; Silberberg et al., 2006). The increased ratio of JMa/Cyt-1 to JMb/Cyt-2 transcripts is associated with a selective decrease of synaptic inputs and parvalbumin expression, indicative of reduced function, in postmortem DLPFC GABAergic interneurons of persons diagnosed with Scz and mood disorders (Chung et al., 2016; Chung et al., 2018). Moreover, ErbB4 polymorphisms were shown to correlate with increased Cyt-1 and PI3KCD expression (Law et al., 2007; Law et al., 2012; Silberberg et al., 2006), and a link between Cyt-1 and PI3K/Akt signaling has been viewed as a potentially novel target for the treatment of Scz (Law et al., 2007; Law et al., 2012). Until this work, no study to date has directly targeted Cyt splice variants to investigate their functional roles in vivo. Our findings demonstrate that downstream PI3K/Akt functions mediated by ErbB4 Cyt-1 receptors can be partially compensated by Cyt-2 receptor isoforms in inbred captive mice, particularly in GABAergic interneurons. Although it is presently unknown if ErbB4 Cyt-1 functions in distinct environmental situations or in different species can be compensated by ErbB4 Cyt-2 isoforms to the same extent, the ErbB4 Cyt1<sup>19</sup> mouse line provides a novel tool to test the specificity of drugs that target the coupling between Cyt-1 and the PI3K/Akt signaling pathway and represent a potentially novel therapeutic strategy for the treatment of Scz (Law et al., 2007; Law et al., 2012). This study represents an important initial step for understanding the distinct and unique functions of different ErbB4 receptor isoforms in vivo. Given the consistent findings showing that the relative ratios of ErbB4 JMa/JMb and Cyt-1/Cyt-2 splice variants are affected in Scz (Chung et al., 2016; Joshi et al., 2014; Law et al., 2007; Law et al., 2012; Silberberg et al., 2006) and mood disorders (Chung et al., 2018), in the future it will be important to continue assessing the effects of ErbB4 Cyt-1 receptor loss- and gain-of-function in other neuronal subtypes, as well as the specific functions attributable to either JMa or JMb receptor subtypes.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST
Andrés Buonanno is a senior editor for the Journal of Neurochemistry. The authors have no additional conflicts of interest to report.

AUTHOR CONTRIBUTIONS
LE and AB designed this study. Funding is provided by AB and SLC. LE, MEC, RM, and JPW carried out surgical and behavioral experiments. LE, DV, and MS carried out chemical and biochemical experiments. Mice were generated by DV and SLC. LE, MEC, MS, JPW, and AB analyzed the data. LE, JPW, and AB wrote the manuscript. All authors provided critical feedback and contributed to the final manuscript.

DATA AVAILABILITY STATEMENT
Data are available from the authors upon reasonable request.

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