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Mutation of the BiP/GRP78 gene causes axon outgrowth and fasciculation defects in the thalamocortical connections of the mammalian forebrain

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Abstract

Proper development of axonal connections is essential for brain function. A forward genetic screen for mice with defects in thalamocortical development previously isolated a mutant called *baffled*. Here we describe the axonal defects of *baffled* in further detail and identify a point mutation in the *Hspa5* gene, encoding the endoplasmic reticulum chaperone BiP/GRP78. This hypomorphic mutation of BiP disrupts proper development of the thalamocortical axon projection and other forebrain axon tracts, as well as cortical lamination. In *baffled* mutant brains, a reduced number of thalamic axons innervate the cortex by the time of birth. Thalamocortical and corticothalamic axons are delayed, overfasciculated, and disorganized along their pathway through the ventral telencephalon. Furthermore, dissociated mutant neurons show reduced axon extension *in vitro*. Together, these findings demonstrate a sensitive requirement for the ER chaperone BiP/GRP78 during axon outgrowth and pathfinding in the developing mammalian brain.

Keywords

brain development; axon guidance; cortical lamination; corticothalamic axons; chaperone; Hspa5; mouse; ENU mutant; protein misfolding; UPR; endoplasmic reticulum; heat shock protein

INTRODUCTION

Proper development of the cerebral cortex and its reciprocal axonal connections with the dorsal thalamus are critical for sensory perception and motor control. Changes in the functional connectivity of this system have been implicated in schizophrenia, autism, and epilepsy (Huguenard and McCormick, 2007; Kim et al., 2008; Mizuno et al., 2006; Welsh et al., 2008). In the mammalian neocortex, the generation and migration of neurons into six neuronal layers must be coordinated with their different patterns of axon connections. The first neurons born form a transient layer called the preplate on top of the progenitor layer. The preplate is split into the superficial marginal zone and deep subplate layer by invasion from the next cohort of neurons born. As development progresses, each successive cohort of

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Conflict of Interest Statement

The authors declare that they have no known or potential conflicts of interest.

newborn neurons must migrate past the existing layers and stop beneath the marginal zone. Neurons of layer 6 project axons to the dorsal thalamus, at the same time that dorsal thalamic neurons send their axons to the cortex. These reciprocal, topographic axon connections between thalamus and cortex grow as ordered fascicles in opposite directions along the same pathway, through the internal capsule and ventral telencephalon (Bicknese et al., 1994; Miller et al., 1993; Molnar et al., 1998a). Some molecular cues underlying their pathfinding are known, but the processes remain poorly understood (for review see Maroof and Anderson, 2006; Price et al., 2006).

To identify genes involved in these processes, we previously performed a genetic screen for mutations causing defects in cortical morphogenesis and thalamocortical axons in perinatal mice (Dwyer et al., 2011). One of the mutants isolated, called *baffled*, displayed a severe thalamocortical axon (TCA) defect with reduced innervation of the cortex. Here we describe the development of this phenotype in more detail, and report that the *baffled* mutant gene is Hspa5, which encodes the BiP protein.

BiP, also known as GRP78, is an Hsp70 family chaperone that assists in translocation and folding of secretory proteins in the endoplasmic reticulum (ER) (Hendershot, 2004). Additionally, BiP regulates the unfolded protein response (UPR) (Schroder and Kaufman, 2005), and is protective against cellular stress, apoptosis, and neurodegeneration in adult neurons (Ni and Lee, 2007). While much is known about these cellular functions of BiP and its protective role in adult tissues, very little is known about its requirements during animal development. Double mutants for the two *C.elegans* BiP homologs, *hsp-3* and *hsp-4*, are embryonic lethal (Sasagawa et al., 2007; Shen et al., 2001). Not surprisingly, deletion of BiP in the mouse also causes very early embryonic lethality, precluding developmental analysis (Luo et al., 2006). However, a targeted hypomorphic mouse mutant of BiP survives until birth and shows subtle organ defects including a smaller cerebral cortex (Mimura et al., 2007; Mimura et al., 2008). Since none of these studies examined the requirement for BiP in axon tract development, we used the *baffled* mutant as a tool to address this question.

MATERIALS AND METHODS

Animals and breeding

For timed pregnancies, the morning on which the vaginal plug was found was considered embryonic day (E) 0.5. Embryos were harvested by caesarean section following euthanasia of pregnant females. Littermate embryos were used as controls for all experiments. The *baffled* mutation was induced on C57BL/6 and maintained for greater than 10 generations of backcrossing on the FVB/N background. Animals were genotyped by PCR for microsatellite markers flanking the mutation (Dwyer et al., 2011). Mouse colonies were maintained in accordance with NIH guidelines, and protocols were approved by the IACUC of UVA.

Dye tracing

Brains were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) overnight. For dye tracing thalamic axons, a razor blade was used to make a coronal cut in fixed brains caudal to the thalamus. To label thalamic axons and corticofugal fibers, single crystals of DiI-C18 or DiA (Molecular Probes) were placed into equivalent positions in control and mutant brains in the cerebral cortex or the dorsal thalamus from the caudal side, using a binocular dissecting microscope. Dye was allowed to transport in the fixed tissue for 2–4 weeks in the dark at 37°C. The brains were embedded in 3% agar in PBS, and sectioned coronally at 150 μ m on a Leica VTS1000 vibratome. Examples shown are representative of at least 8 hemispheres of mutant and control brains for ages E15–18.5, and 6 hemispheres for ages E13–14.5.

X-GAL staining

E18.5 and P0 embryos were collected, numbered, decapitated, and tailed. DNA was extracted from tail for genotyping. Skulls were removed in PBS and the brains fixed for 30 minutes in 4% paraformaldehyde in PBS. Brains were then cut coronally with a razor blade in the approximate position of the internal capsule, and fixed for five more minutes. Brains were stained in 0.8 mg/ml X-GAL staining solution overnight at room temperature, and examined under low magnification. Some brains were sectioned by vibrating microtome (Leica, VT1000s) before staining.

Immunohistochemistry and Histology (H&E staining)

Paraffin sections (5 μ m), cryosections (16–25 μ m), and mouse embryonic fibroblasts (MEFs) on glass coverslips were preincubated for 30 min at room temp in blocking solution (PBS containing 2% BSA or normal goat or horse serum and 0.1% Triton X-100) and then incubated at 4°C overnight with primary antibody diluted in blocking solution. Sections were then rinsed three times for 5 minutes each in PBS and incubated for 1 hour at room temperature with the appropriate species secondary antibody (Biotin conjugated for DAB immunohistochemistry and Alexa fluorophore conjugated for immunofluorescence) diluted in blocking solution. Sections were again rinsed three times for 5 minutes each and coverslipped with appropriate mounting media (Cytoseal for DAB immunohistochemistry and Gel-mount for immunofluorescence). For DAB immunohistochemistry, sections were additionally quenched in 0.5% hydrogen peroxide diluted in PBS prior to the blocking step. Biotinylated secondary antibodies were further reacted with avidin-biotinylated enzyme complex (ABC) using the Vectastain Elite kit and diaminobenzidine (DAB) according to the manufacturer's instructions. The antibodies used in IHC were as follows: rat monoclonal anti-L1 (1:200, Chemicon), rabbit polyclonal anti-TAG-1 (1:10,000, gift of Jane Dodd), mouse monoclonal anti-Islet1 (antigen retrieval (AR), 1:100, Developmental Studies Hybridoma Bank (DSHB)), rabbit polyclonal anti-surfactant-C (AR, 1:400, Santa Cruz Biotechnology), mouse monoclonal anti-BrdU (AR, 1:100, BD Biosciences), mouse monoclonal anti-reelin (AR, 1:500, Chemicon), goat polyclonal anti-GRP78 N-20 (1:50, Santa Cruz), goat polyclonal anti-Cux1 (1:500, Santa Cruz), rat polyclonal anti-Ctip2 (1:500, Abcam), rabbit polyclonal anti-Foxp2 (AR, 1:2000, Abcam), and mouse IgM monoclonal anti-CSPG (AR, 1:500, Sigma). Paraffin sections and some antibodies required antigen retrieval (AR) by incubating slides with 10mM citrate buffer pH 6 at 95°C for 20 minutes followed by three rinses in PBS for 5 min each.

For H&E staining, paraffin or cryosections were incubated with hematoxylin and eosin Y according to manufacturer's instructions (Electron Microscopy Sciences). Briefly, sections were rinsed with PBS for 5 min, fixed in 4% PFA for 10 min, and again rinsed with PBS for 5 min. Sections were dipped three times in dH₂O and rinsed in dH₂O for 5 min. Sections were incubated with a 1:3 dilution of hematoxylin in dH₂O for 1–2 min followed by a rinse in dH₂O for 5 min with agitation. Sections were dipped in bluing solution (0.5% ammonium hydroxide diluted in dH₂O, pH 10) followed by a 5 min rinse in dH₂O. Sections were rinsed in 70% ethanol (EtOH) for 2 min and then incubated in eosin Y for 10 sec – 1 min. Sections were incubated in 95% EtOH for 2 min, 100% EtOH for 5 min, and two changes of Xylene for 5 min each. Finally, sections were coverslipped with Cytoseal.

Primary antibody characterization

The specifications of the primary antibodies used in this study are summarized in Table 2. Three different antibodies against BiP/GRP78 were used. GRP 78 (N-20) is an affinity purified goat polyclonal antibody raised against a peptide mapping at the N-terminus of GRP 78 of human origin (manufacturer's information). The N-20 antibody showed a decrease in the amount of GRP78 on immunoblots from mice heterozygous for a deletion of

Hspa5/BiP (tissue from the homozygous knockout could not be tested because they die at day E3.5 (Luo et al., 2006). It has been used as a marker of the ER in immunocytochemistry in many papers, e.g. (Khan et al., 2004; Zhao et al., 2005). GRP78 (SPA-826) specifically detects GRP78 protein (a single band at 78 kDa molecular weight) in mouse, rat, and hamster cells on immunoblot (Lee, 2005; Reddy et al., 2003). SPA-826 was also used to detect BiP on immunoblots and immunohistochemistry of brain tumor cell lines and brain tumor tissue (Graner et al., 2007). The anti-BiP antibody from Cell Signaling is a rabbit polyclonal directed against the N-terminal region of human BiP. It recognizes a single band of 78 kDa molecular weight on immunoblot from several cell types (manufacturer's information, and (Nicoletti-Carvalho et al., 2010; Xu et al., 2009).

Anti-surfactant protein C (SP-C) is a rabbit polyclonal raised against the first 197 residues of human SP-C protein (manufacturer's information). It has been used in immunohistochemistry (Mimura et al., 2007). This antibody recognizes a 21kDa doublet and 16kDa proforms of SP-C on immunoblots from mouse fetal lung, and immunostains alveolar Type II cells of fetal lung tissue (Salminen et al., 2009).

Mouse monoclonal anti-BrdU (clone 3D4 from BD Biosciences) is used to label cells during DNA replication and thus for birthdating. This antibody was raised against the immunogen BrdU, has been reported to be highly specific for BrdU, and does not cross-react with thymidine (Gratzner, 1982). It did not stain cells in control animals that did not receive a BrdU injection.

The anti-Islet-1 antibody is a mouse monoclonal raised against the C-terminal residues 178–349 of rat Islet-1 protein. It was shown to label corridor cells in (Lopez-Bendito et al., 2006), and specific immunostaining with this antibody is abolished by knockdown (Ganz et al., 2012).

Anti-CSPG antibody was made as a mouse monoclonal directed against membranes of chicken gizzard fibroblasts (manufacturer information), and CSPG has been used for many years as a marker for the preplate (Sheppard et al., 1991).

The anti-L1-CAM rat monoclonal antibody from Chemicon (now Millipore) reacts with the 220–240 kDa glycoprotein L1, and the antibody was raised against homogenate of 16-week human fetal brain cells (manufacturer information). L1 has been used in numerous manuscripts as an axonal marker for many types of axons, and labels both TCAs and CTAs (Chung et al., 1991).

The anti-reelin monoclonal antibody (clone G10) was raised against amino acids 164–496 of reelin. It recognizes full-length (400kDa) and two additional fragments (300 and 180kDa) of recombinant reelin on Western blot (Hu et al., 2007; Zhao et al., 2004). It has been used by many investigators as a marker for reelin and Cajal-Retzius cells in the cortical marginal zone (de Bergeyck et al., 1997).

TAG-1 (transient axonal glycoprotein, gene name CNTN2) was shown to be a marker for cortical axons (Denaxa et al., 2001), and has been used as such by several investigators, e.g., (Lopez-Bendito et al., 2002; Ma et al., 2008). The rabbit polyclonal anti-TAG-1 (TG3) used here was raised against a recombinant, tail-truncated, secreted TAG-1 protein (i.e., the entire extracellular portion of the protein), collected from supernatants of Tag-1-transfected 293 cells, and affinity purified with anti-TAG1 mAb (1C12). It gives identical staining on embryonic rat spinal cord as the monoclonal 4D7, and also labels a similar size band on immunoblot (Buttiglione et al., 1998; Dodd et al., 1988; Felsenfeld et al., 1994). It was generously donated to us by Jane Dodd's lab.

Cux1 (also known as CDP) is a homeodomain protein expressed in postmitotic neurons of the upper layers (2–4) in the cortical plate (Nieto et al., 2004). The goat polyclonal antibody from Santa Cruz Biotechnologies used here (C-20, sc-6327) was raised against the C-terminus of CDP of mouse origin. It recognizes a single major band of 200 kDa that is absent in homozygous mutant tissue immunoblot (Luong et al., 2002).

Ctip2 is a transcription factor highly expressed by corticospinal neurons in the deep layers (5–6) of the cortical plate. The rat monoclonal antibody 25B6 from AbCam used here was raised against a human fusion protein containing amino acids 1–150 of CTIP2, and recognizes a doublet corresponding to two isoforms of Ctip1 at about 120kDa that is absent in homozygous knockout mice (Arlotta et al., 2005; Chen et al., 2005; Wakabayashi et al., 2003).

Foxp2 (forkhead box protein P2) is a transcription factor expressed by projection neurons in layer 6 of the cortical plate (Ferland et al., 2003). The rabbit polyclonal from AbCam used here was raised against a synthetic peptide conjugated to KLH derived from within residues 703–715 of the C-terminus of human FOXP2, and recognized a band of 80 kDa on Western Blots of human 293T cell lysates and showed similar expression patterns to previous publications (Stillman et al., 2009 and Manufacturer's information).

Satb2 is a DNA-binding protein expressed in cortical plate neurons that extend callosal axons (Alcamo et al., 2008; Britanova et al., 2005). The mouse monoclonal from AbCam used here was raised against a human recombinant fragment of the C-terminus. It recognizes a single band on immunoblot that is abolished upon shRNA knockdown (Asanoma et al., 2012).

Tau1 is a microtubule associated protein that has long been known to segregate into axons of cultured cortical neurons (Kosik and Finch, 1987). The mouse monoclonal antibody from Chemicon used here was raised against purified denatured bovine microtubule associated proteins.

Tbr1 is a T-box transcription factor that regulates the fate of preplate neurons and deep layer neurons within the cortical plate (Hevner et al., 2001). The rabbit polyclonal from AbCam used here was raised against a synthetic peptide conjugated to KLH derived from within residues 50–150 of mouse TBR1 and the layer 6 staining is not present in Tbr1 mutant mice (McKenna et al., 2011).

In situ hybridization

In situ hybridization analysis was performed on cryosections (16–25µm). Sections were fixed with 4% PFA diluted in PBS for 10 min followed by three PBS rinses, 3 min each. Sections were treated with 10µg/ml of proteinase K diluted in PBS for 10 min followed by three quick rinses in PBS. Sections were again fixed with 4% PFA diluted in PBS followed by three PBS rinses for 3 min each. Sections were acetylated for 10 min followed by three PBS rinses, 5 min each. Sections were incubated with 0.85% NaCl followed by a 5 min wash with 70% EtOH/0.85% NaCl. Sections were washed with 95% EtOH for 5 min and air dried for at least 10 min. Sections were hybridized overnight at 66°C with a digoxigenin-labeled riboprobes at a final concentration of 500 ng/ml. P75, *Pax6*, *Dlx5*, *Sema6a*, and *Gbx2* riboprobes were synthesized from cDNA template. Post-hybridization washes were performed at 65°C before treatment with 2 µg/ml RNase A at 37°C for 15 min. Samples were then incubated with anti-digoxigenin-alkaline phosphatase (1:4000, Roche) at 4°C overnight. Signals were detected with BM purple (Roche). After being post-fixed with 4% paraformaldehyde/0.1% glutaraldehyde, samples were rinsed in PBS three times for 5 min.

each followed by a three quick rinses in dH₂O before being dried in a 55°C. Sections were coverslipped with Glycergel (Dako).

BrdU birthdating and lamination analysis

Pregnant mice were injected intraperitoneally with a single dose of bromodeoxyuridine (BrdU) (50 mg/kg) at gestational day E11.5, E13.5, or E15.5. E18.5 brains were collected and embedded to create cryo- or paraffin sections for BrdU immunostaining (BD Biosciences). To analyze the laminar position of BrdU+ cells, the cortex was partitioned with a grid of 10 equally sized horizontal bins from the pial surface to the ventricular surface. The number of cells in each bin were counted and the percent of total in each bin was averaged between sections.

Ex vivo electroporation

Ex vivo electroporation was performed as previously described (Hand et al., 2005). Electroporation of dorsal telencephalic progenitors was performed by injecting pCIG2 plasmid DNA (1 µg/ul endotoxin-free plasmid DNA; Qiagen kit; gift of Franck Polleux) plus 0.05% Fast Green (Sigma; 1:15 ratio) using a glass micropipette and plunger into the lateral ventricles of E15.5 mouse embryo heads. Heads were then decapitated and placed in complete HBSS. Electroporations were performed on the whole head (skin and skull intact) with gold-coated electrodes (GenePads 5 × 7 mm, BTX) using an ECM830 electroporator (BTX) and the following parameters: three 50 ms long pulses separated by 100 ms long intervals at 20V. Immediately after electroporation, the brain was removed and sectioned with a vibrating microtome at 250 µm (Leica VT1000S). The resulting slices were maintained in organotypic slice cultures for 5 days *in vitro* (5 DIV), fixed in 4% PFA, and immunostained with chicken polyclonal anti-GFP (1:1000, Upstate) as previously described (Polleux and Ghosh, 2002).

Dissociated neuron culture and axon tracing

Dissociated neuron cultures were prepared as described previously (Dotti et al., 1988). Embryos from timed pregnant females were collected at E16.5, and a portion of cortex was removed for dissociation. Neurons were cultured on poly-L-lysine-coated glass coverslips, fixed with 4% PFA and methanol, and immunostained with the axonal marker Tau-1 (mouse monoclonal anti-Tau1, 1:200, Millipore), rabbit polyclonal anti-Tbr1 (1:500, Abcam), or mouse monoclonal anti-Satb2 (1:200, Abcam), and DAPI. Secondary antibody was Alexa-488 anti-mouse or Alexa-568 anti-rabbit. Axons were traced on photomicrographs using the NeuronJ plugin of ImageJ. The longest Tau-1-stained neurite was considered the axon, and measured from the edge of the soma to the tip of the growth cone. The average lengths were compared with a two variable t-test.

Mouse embryonic fibroblast (MEF) culture

Embryos from timed pregnant females were collected at E12.5-E14.5. Tails were saved for genotyping. Embryos were decapitated, organs were removed from bodies, and resulting “skin flap” was deaggregated with an 18-gauge needle and incubated in 2ml of 0.25% trypsin-EDTA for 2 min at 37°C. The tissue was triturated and transferred to a 15ml tube filled with 1ml fetal bovine serum (FBS). Tubes were spun at 1000 rpm for 5 min and the medium was aspirated off. The pellet was washed with 2ml sterile HBSS or PBS, wash was aspirated, and pellet was resuspended in DMEM/10% FBS with pen/strep added. The resulting cells from each embryo were plated in two 10 cm plates. The next day, media was replaced.

Immunoblotting

Protein was extracted from mouse embryonic fibroblast (MEF) cells or brains using Triton lysis buffer (1% Triton X-100, 20 mM Tris pH 7.5, 150 mM NaCl, and 1mM EDTA with added protease inhibitor cocktails (Sigma)). For tissue lysates, a 20 gauge syringe was used to homogenize tissue. Lysates were cleared by centrifugation at 12,000g for 10 min at 4°C. An aliquot of cleared lysate was used to determine protein content using BCA Protein Assay according to manufacturer instructions (Pierce). For analysis, cleared supernatant was diluted 1:2 with 2X Laemmli sample buffer. Lysates were boiled for 5 min just prior to gel electrophoresis. Proteins were resolved on 10% SDS-polyacrylamide gels. In some cases, hamster GRP78 protein was loaded as a positive control. Nitrocellulose membranes containing transferred proteins were blocked in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween-20 for 1 h at room temperature. Nitrocellulose membranes were probed with primary and secondary antibodies diluted in Tris-buffered saline containing 5% BSA and 0.1% Tween 20. BiP was detected with two N-terminal antibodies (Cell Signaling BiP at 1:1000, or Santa Cruz N-20 at 1:50), or an anti-GRP78 rabbit polyclonal antibody directed against the carboxyl ten amino acids of rat GRP78 (SPA-826, StressGen at 1:5000). Similar results were found with two additional C-terminal antibodies (data not shown, anti-BiP from BD Biosciences, and anti-KDEL SPA-827 from StressGen). Respective horseradish peroxidase-conjugated secondary antibodies were used, and the protein bands were visualized by the ECL method (Pierce).

Photomicrograph production

Images were collected using either a Leica MZ16F stereoscope equipped with a Leica DFC300FX color digital camera, or a Zeiss Axio Imager. Z1 upright compound fluorescent microscope with a Zeiss AxioCam MRm black-white digital camera. Adobe Photoshop was used to crop and save images, and brightness or contrast were increased on some images at equivalent levels for control and mutant samples. Photo panels and graphs were labeled and assembled into figures in Canvas.

RESULTS

***baffled* mutants have defects in thalamocortical axons and other cortical axon projections at age E18.5**

Previously, we performed an ENU screen for mouse mutants with defects in TCA development, and showed that the *baffled* mutant is recessive and dies at birth with reduced thalamic innervation of the cerebral cortex. At E18.5, TCAs appeared disorganized and tangled in rostral cortex near the corticostriatal boundary (CSB) (Dwyer et al., 2011). Now we further characterize the axonal defects in this mutant at E18.5 and earlier ages, and address the underlying cause of these phenotypes.

The TCA defect is present in every *baffled* mutant examined, and appears to affect the entire projection. However, some TCAs do innervate caudal cortex. Using the TCA-TLZ reporter, which labels thalamocortical axons specifically (Dwyer et al., 2011), axon segments in ventral telencephalon (vTel) appear wavy and crossed in mutants rather than straight and parallel as in controls (Figure 1A–D). However, whereas the rostral portion of the TCA projection appears stalled in the CSB region (Figure 1B, arrow), in the caudal half of the hemisphere, some TCAs have grown into the cortex (Figure 1D). Axonal dye tracing by placing a crystal of the fluorescent lipophilic dye, DiI, into the dorsal thalamus of E18.5 brains (Figure 1E, F) confirms these findings, and also reveals that the axons in the intermediate zone (iz) of the mutant cortex are dispersed more widely (Figure 1F). Magnified views of the CSB region on thin sections stained with L1-CAM (which marks both thalamic and cortical axons) show that in control brains, thalamic and cortical axons

cross the CSB in straight, evenly dispersed fascicles (Figure 1G). However, in the *baffled* mutant, the fascicles are thicker and non-parallel in this region (Figure 1H). These defects suggest that the mutant axons overfasciculate rather than dispersing appropriately in lateral vTel and the CSB region.

Nissl stained sections of perinatal brains likewise show an abnormal internal capsule and smaller forebrain in *baffled* mutants compared to normal littermates (Figure 2). The internal capsule, comprised of TCAs and other axons, appears to contain fewer, thicker fascicles in mutants (Figure 2D, “ic” and black arrowheads). The intermediate zone of the cortex, which contains cortical efferents and afferents, is disorganized in mutants, with larger bundles (Figure 2B, D, brackets). Other forebrain axon tracts also show abnormalities in the mutants: the corpus callosum was abnormally thin or displayed Probst bundles (Pb, Figure 2B) in most mutant brains (13/17) but not in littermate controls (0/21). Additionally, the external capsule was abnormal (7/7 mutants, 0/9 controls examined), with some fascicles splitting off to an abnormal ventral pathway (Figure 2D, open arrowheads).

***baffled* is a hypomorph mutant of the ER chaperone BiP/GRP78 and phenocopies a previous targeted BiP hypomorph mutant**

To gain a molecular understanding of the anatomical defects in the *baffled* mutant brain, we positionally cloned the mutant gene. Previously, we had mapped the *baffled* mutation to a ~10Mb interval on mouse chromosome 2 containing 10 genes (Dwyer et al., 2011). Subsequently, sequencing of genomic DNA of all the exons and flanking sequences in the interval identified only one change from the C57BL6 reference sequence: a single base mutation in exon1 of Hspa5, which encodes the BiP/GRP78 ER chaperone protein (Figure 3A, red asterisk). The A to G mutation is predicted to convert residue 26 from Glutamic acid (E) to Glycine (G). This residue falls in a conserved hydrophilic motif of alternating acidic-basic-acidic residues that normally forms the N-terminus after signal peptidase cleavage (Figure 3B) (Normington et al., 1989). This missense point mutation does not disrupt BiP protein expression or localization, since BiP protein is still detected at comparable levels in mutant tissue lysates and in the ER of mutant cells (Figure 3C–E).

The finding that BiP was the *baffled* mutant protein was initially a surprise, since BiP is ubiquitously expressed and essential. Null mutant mice for BiP die before implantation (Luo et al., 2006). However, we found that *baffled* phenocopies a targeted hypomorphic mutant of BiP (Mimura et al., 2007; Mimura et al., 2008). First, *baffled* is runted and dies at birth with respiratory failure and reduced secretion of pulmonary surfactant (Figure 3F–K). Second, cerebellar morphology at birth is abnormal with reduced lobular definition (Figure 3L, M).

A third way that *baffled* phenocopies the targeted BiP mutant is that the cerebral cortex of both mutants is smaller with disordered lamination. In *baffled* mutants, neuronal birthdating at E15.5 with BrdU or electroporated GFP labels deeper scattered cells instead of the strong superficial band of cells as it does in controls (Figure 4A–D). Quantification with the BrdU birthdating method confirmed the lamination defect (Figure 4E, H). To further characterize this defect, we utilized markers of specific cortical layers. Immunostaining with these markers showed that in mutant brains, layers were present in the correct order, but were more scattered with less distinct edges (Figure 4I–P). For example, superficial layer marker Cux1 labels a dense band of superficial neurons in control cortex (Figure 4I, line) (Nieto et al., 2004), but in *baffled* mutants, the dense band is thinner and labeled cells spread deeper (4J, dotted line). Ctip2 marks layers 5 and 6 (Figure 4K) (Chen et al., 2005); in mutant brains, these layers are present but appear ragged and with a few labeled cells scattered in upper layers (4L). Similarly, layer 6 marker Foxp2 (Ferland et al., 2003) shows more superficial spread in *baffled* mutant brains than in control E18.5 cortex (Figure 4M, N). Reelin protein is present in the marginal zone but appears to be reduced, providing a

plausible explanation for the lamination defect (Figure 4O, P and Mimura et al., 2008). Thus, although the *baffled* mutation affects the N-terminus of BiP while the targeted mutation alters the C-terminal ER retention signal (see discussion), these two mutants appear to cause highly similar phenotypes, via partial reduction of BiP function.

Thalamocortical and corticothalamic axons are delayed and overfasciculated

Since the effects of BiP loss of function on axon growth or tract formation had not been previously examined, we decided to more carefully analyze the thalamocortical axon trajectory. To understand the origins of the TCA pathfinding defect seen at E18.5, we examined TCAs at earlier stages of brain development. Axonal dye tracing using lipophilic dyes was performed for these experiments, since the beta-galactosidase of the TCA-TLZ reporter does not reach the tips of the axons. At E15.5, in normal brains, dye crystals placed into dorsal thalamus label neat parallel arrays of TCAs that have already grown into the cortex at rostral through caudal levels (Figure 5A, C, E). However, in *baffled* mutant brains, very few TCAs have reached rostral vTel (Figure 5B). Many TCAs have successfully grown through middle and caudal levels of the vTel (closer to thalamus), so the initial trajectory through the internal capsule appears normal. However, the axons in lateral vTel appear disorganized in mutants (Figure 5D, F). Only a few TCAs have crossed the CSB, in middle regions (Figure 5D, arrowhead). The disorganization suggests that the thalamic axons have trouble not only crossing the CSB, but also reaching it. To confirm this, TCAs were examined in even younger brains (Figure 6A–D). At E13.5, control TCAs have extended into vTel, whereas mutant TCAs have not yet (Figure 6A, B). In normal E14.5 brains, TCAs have almost reached the CSB, but in mutant brains they are still in medial vTel (Figure 6C, D). These analyses suggest that in the *baffled* mutant, thalamocortical axons are delayed at early steps in their pathway from thalamus to cortex.

Corticothalamic axons follow the same route as TCAs but in the opposite direction, either in adjacent but separate tracts (Miller et al., 1993), or co-fasciculating (Molnar et al., 1998a; Molnar et al., 1998b). Therefore, we tested whether the corticothalamic axons were normal in *baffled* mutants (Figure 6E–H). Placing small DiI crystals in the E13.5 cortex showed that by this age, many cortical axons had crossed the CSB, and a few had reached the middle of vTel (Figure 6E, arrow). However, the farthest mutant cortical axons were still in lateral vTel near the CSB (Figure 6F, arrow). By E14.5, control cortical axons have reached medial vTel (Figure 6G, arrow), but the farthest extending mutant cortical axons have only reached as far as lateral vTel (Figure 6H, arrow). To confirm this apparent delay in extension of cortical axons, immunohistochemical staining for L1-CAM was used (Figure 7A, B). L1-CAM, which labels both thalamocortical and corticothalamic axons, shows that control brains at E15.5 have a thick parallel array of axons crossing the CSB and bending rostrally into the cortex. However, in *baffled* brains at E15.5, there is a consistent gap in L1 staining at the CSB (Figure 7B, arrow), demonstrating that neither thalamic nor cortical axons have crossed the CSB in significant numbers yet. Thus, corticothalamic axons also appear to be delayed in growth along their pathway in *baffled* mutant brains.

To examine whether cortical axons were not only delayed, but also disorganized and overfasciculated at E18 in a similar manner as TCAs, the TAG-1 antibody, which distinguishes cortical axons, was used (Figure 7C, D) (Denaxa et al., 2001). In normal brains, TAG-1 labels the intermediate zone layer (bracket, Figure 7C) and a neat fan-shaped array of cortical axon fascicles descending toward the internal capsule. In mutant brains, however, TAG-1 labeled a wider intermediate zone with jagged edges (brackets and open arrowhead, Figure 7D) and thicker round fascicles that were unevenly dispersed (black arrowhead). These data indicate that mutant cortical axons are disorganized and overfasciculated, in a similar manner as TCAs, as they grow out of the cortex. Taken together, these data suggest that in *baffled* mutant brains, both thalamocortical and

corticothalamic pioneer axons are delayed by about one day at early steps in their trajectories, and some TCAs may be delayed by two or more days in crossing the CSB. Further, both TCAs and CTAs are disorganized and overfasciculated in the CSB and vTel regions.

Forebrain patterning and guide cell populations for TCAs appear intact in *baffled* mutants

To address the underlying cause of the defects in the thalamocortical axons, we considered two main possibilities: a deficiency in the axon pathway (non-cell-autonomous), or a deficiency in the axons themselves (cell-autonomous). As a first step, and since *baffled* mutant brains were slightly smaller than littermates', we tested whether forebrain regions were properly specified by examining some gene expression patterns. RNA *in situ* hybridization on E18.5 mutant brains showed normal expression patterns in telencephalon and diencephalon for *Pax6*, *Dlx5*, *Gbx2*, and *Sema6a* (Figure 8 and data not shown) (Jones and Rubenstein, 2004; Kawano et al., 1999; Little et al., 2009; Nakagawa and O'Leary, 2001; Simpson et al., 2009).

Since the major divisions of telencephalon and diencephalon appeared to be patterned normally, we looked for more subtle deficits in the thalamo-cortical pathway. The factors that mediate TCA defasciculation in the vTel and TCA crossing of the CSB are not understood. However, there are three different populations of guiding cells thought to help establish the TCA pathway (Grant et al., 2012; Molnar et al., 2012). We tested for the survival and localization of these three types of guide cells in *baffled* mutants. First, the internal capsule guidepost cells, which may aid the initial part of the TCA trajectory, sit in the internal capsule region and project axons to the dorsal thalamus (Metin and Godement, 1996; Mitrofanis and Baker, 1993; Molnar et al., 1998a; Molnar and Cordery, 1999; Tuttle et al., 1999). With no known specific marker, they can only be labeled by retrograde axon tracing from the thalamus. Using this method we found that internal capsule guidepost cells were still present in *baffled* mutants (Figure 9A–B'). Although the detected cells appeared fewer in number and in a slightly more caudal position, it is not distinguishable whether this signifies fewer guidepost cells in the mutant, or means that the normal number of guidepost cells were present, but could not all be back-labeled due to delay in growth of their own axons to dorsal thalamus. However, at least some guidepost cells were consistently labeled in mutants by the retrograde tracing method.

A second population of guiding cells, the corridor cells, is thought to provide a permissive channel for TCAs through the vTel (Lopez-Bendito et al., 2006). As shown by the corridor marker *Islet1*, the corridor is still present with normal borders in *baffled* mutants (brackets, Figure 9C, D). The third population of guiding cells are the subplate and layer 6 cortical cells that project to the dorsal thalamus, and whose axons may help guide TCAs to innervate the cortex (De Carlos and O'Leary, 1992; Ghosh et al., 1990; Hevner et al., 2002; Molnar et al., 1998a). In *baffled* mutant brains the disorganized intermediate zone and cortical layers make it difficult to distinguish the subplate by Nissl staining (Figure 2B). However, the subplate splits normally from the marginal zone in mutants by E14.5 as revealed by chondroitin sulfate proteoglycan (CSPG) immunostaining (Figure 9E, F) (Sheppard et al., 1991). The subplate and layer 6 are still present in deep cortical plate in E18.5 mutant brains, as indicated by the p75 marker (Figure 9G, H) (Mackarechtschian et al., 1999). They may be present in a slightly wider band, concomitant with the spread in layer organization. On the whole, these three types of guiding cells appear grossly normal in *baffled* mutants, and the possible slight displacements would not account for the severity of the defects in thalamic or cortical axon outgrowth, fasciculation, and CSB crossing.

baffled* mutant cortical neurons have an axon extension defect *in vitro

To test the second main possibility, that *baffled* mutant neurons have a cell-autonomous defect in axon outgrowth, we cultured dissociated cortical pyramidal neurons *in vitro*. In this system, the dissociated neurons must grow new neurites, passing through a multipolar stage (Stage 2), before one neurite is specified as the single axon and grows rapidly (Stage 3) (Dotti et al., 1988). Such a standardized system has not yet been established for dissociated thalamic neuron culture, and in our own attempts dissociated thalamic neurons had poor survival. Since cortical axons in *baffled* mutants showed similar delay and overfasciculation defects as TCAs, we used cortical neuron culture to test cell autonomy. Cortical neurons from control and *baffled* mutant brains were dissociated and plated at low density on polylysine-coated coverslips. Cultures were fixed at different time points and immunostained for Tau1, a microtubule-associated protein enriched in axons. Tracing and measuring the axons of Stage 3 neurons revealed that *baffled* mutant neurons had significantly shorter average axon length (Figure 10A–C).

The shorter mutant axons could represent an axon extension defect, or a shift in neuron types in the cultures. To control for this, the subsets of neurons expressing the transcription factor Tbr1 were directly compared (Figure 10D–F). Tbr1 marks glutamatergic, early-born neurons including layer 6 corticothalamic projection neurons (Hevner et al., 2001). We found that even when only the Tbr1⁺ neurons were considered, the mutant neurons still had significantly shorter axons than controls (Figure 10F). Moreover, the relative proportions of layer subtypes did not differ significantly in the *baffled* mutant neuron cultures: the percentage of Tbr1⁺ neurons was similar (34% of total Stage 3 control neurons vs. 31% of the mutant); and, in a separate set of experiments, double immunostaining for Tbr1 and Satb2 (a marker of upper layer callosal neurons) labeled similar percentages in both cultures (Figure 10G–I) (Alcamo et al., 2008; Britanova et al., 2005; Britanova et al., 2008). These data strongly suggest that *baffled* mutant cortical neurons have an intrinsic deficiency in axon extension. Further, they suggest that the delayed outgrowth of both thalamic and cortical axons observed in the mutant brains *in vivo* at early stages of their pathfinding is due at least in part to cell-autonomous requirements for BiP in both of these projection neurons.

DISCUSSION

We have identified a mouse mutant in the Hspa5 gene that demonstrates a sensitive requirement for the ER chaperone BiP/GRP78 during axon outgrowth in the embryonic brain. Specifically, our *in vivo* analyses of the thalamocortical and corticothalamic projections in *baffled* mutants suggest that these axons are delayed in their extension and overfasciculated, particularly as they cross the corticostriatal boundary. Furthermore, *in vitro* analysis suggests that reduction of BiP function causes a cell autonomous decrease in axon growth. Together, these data lead to a hypothesis that the *baffled* mutation of BiP compromises the ability of neurons to rapidly grow an axon. *In vivo*, delays in axon extension or fasciculation may cause secondary defects in axon guidance or organization. This is the first analysis of the vulnerability of growing axons to disruption of BiP function.

The importance of BiP/GRP78 in development as well as disease

BiP's role as a major ER chaperone suggests that the *baffled* mutant axon phenotypes could be due to reduced secretion of any number of proteins. BiP is highly conserved from yeast (KAR2) to human, and its function has been well studied in single cell and cell-free systems. It is an integral part of all ER functions (Hendershot, 2004). It aids the translocation, folding, and assembly of many proteins. It also acts as a monitor for unfolded proteins and ER stress, regulating the unfolded protein response (UPR). Further, BiP is protective against apoptosis.

These critical functions of BiP have associated it with a wide array of human diseases (Lee, 2007; Ni and Lee, 2007). For example, it protects adult neurons against degeneration, and has been implicated in several neurodegenerative diseases (Matus et al., 2011; Penas et al., 2011; Wang et al., 2010; Yuan et al.). However, this cell protective function can also enhance tumor growth and cancer progression, and BiP is highly expressed in brain tumors (Graner et al., 2007; Li and Lee, 2006; Luo and Lee, 2012). Despite the vast literature on BiP's cellular functions and therapeutic potential for adult degenerative diseases, its roles in tissue development have scarcely been examined. Since BiP is a drug target for cancer therapy, and is ubiquitously expressed, it is extremely important to know whether diminishing BiP function will have effects on nervous system development or repair.

Fortunately, now there are two mouse mutants in which partial loss of BiP function enables developmental studies. The previously published BiP hypomorph was a targeted alteration of the C-terminal ER retention signal (KDEL), so that BiP would not be efficiently retained in the ER (Mimura et al., 2007). By contrast, the *baffled* mutation was found in a random ENU mutagenesis screen and disrupts an N-terminal acidic motif; its effects on the protein are still unclear. In *baffled* mutant cells, the level of BiP protein and localization to the ER is not detectably different. The point mutation could affect the efficiency of cleavage of the signal peptide and its translocation into the ER. Alternatively, the mutation could alter the interaction of the N-terminus of BiP with substrates or the ER translocon. Thus the two different mutations probably have different specific effects on the protein, but both serve ultimately to slightly reduce the function of BiP. It appears that even this slight reduction is nevertheless devastating for certain cell types with high secretory activity. The respiratory defects of both hypomorphic mutants cause death soon after birth. Perhaps future creation of conditional hypomorphic alleles of BiP will allow postnatal survival and analysis of BiP requirements in adult brain function or repair.

The requirement for efficient BiP function in axon growth and tract formation

Although the protective role of BiP against adult neurodegeneration is well established, the vulnerability of growing neurons to reduction of BiP function had not previously been shown. Nevertheless, a few findings hinted at its importance for neural development. BiP is expressed at higher levels in embryonic than adult brain (Zhang et al., 2007). BiP and ER are present in neurites (Vuppalachchi et al., 2010; Willis et al., 2007), and BiP enhances neurite outgrowth in the PC12 cell line (Satoh et al., 2000). During polarized neuron morphogenesis, ER exit site localization has been linked to early axon growth, while the Golgi apparatus and outposts appear to be more influential for dendrite growth (Aridor and Fish, 2009; Litterman et al.,; Ye et al., 2007). Thus, while there was circumstantial evidence suggesting BiP's importance for axon growth, the data described herein provide *in vivo* evidence that full BiP function is required for early axon outgrowth and proper axonal wiring of the mammalian brain. It also adds to growing support for the idea that regulation of secretory dynamics could play an important role in neuronal morphology changes during development, regeneration, or pathological conditions (Renvoise and Blackstone, 2010).

Our developmental analysis of the thalamocortical projection in *baffled* mutant brains suggests that reduction of BiP function affects these axons in three ways: it delays axon extension through the pathway, causes overfasciculation, and impairs crossing of the corticostriatal boundary. To examine possible non-cell autonomous causes for the TCA phenotypes, we assessed *baffled* mutant brains for three types of guiding cells thought to aid TCAs at different points along their pathway. In *baffled* mutants, the internal capsule guidepost cells, corridor cells, and subplate cells all appear to reside in their normal locations. Even possible slight displacements would not account for the severity of the TCA defects. The disorganization of the cortical layers could explain the spread of TCAs within a thicker intermediate zone in the final leg of their trajectory, but not the earlier defects of

axon delay and overfasciculation. However, it remains possible that the guide cells themselves have axon outgrowth delays, or that they secrete guidance cues less efficiently in *baffled* mutants.

We favor a cell autonomous origin of the TCA phenotypes. Both thalamic and cortical axons are delayed at early points in their pathways, prior to other defects. This delay may be explained by the intrinsic defect in axon extension seen in our dissociated neuron cultures. Building a long axon requires rapid addition of new membrane and proteins to the growth cone, and therefore places enormous demands on the biosynthetic pathway of a young neuron. It may be that many neuron types extend axons more slowly in the *baffled* mutant, but that most can still reach their targets. Axons with complex trajectories that cross boundaries, such as TCAs, may be more vulnerable, requiring dynamic turnover of growth cone receptors and membrane.

In vivo, delays in TCA outgrowth could cause secondary defects in axon organization and guidance by preventing appropriate interactions with time-limited intermediate cues important for defasciculation in the vTel or crossing the CSB. Alternatively, the overfasciculation and disorganization defects seen in *baffled* mutant TCAs may be independent from the axon outgrowth delay. The overfasciculation phenotype suggests that reduction of BiP function could affect axonal secretion of adhesion molecules that mediate the balance of adhesion of TCAs to each other and to substrates. An increased affinity of TCAs for each other, when they should normally fan out, may inhibit other interactions required for CSB crossing. Indeed, their failure to defasciculate may preclude TCAs from interacting with descending subplate axons that may facilitate entry to the cortex (the “handshake hypothesis”) (Molnar et al., 1998a; Molnar and Blakemore, 1991).

While the *baffled* mutant analysis does not prove or disprove the handshake hypothesis, the CSB crossing defect does provide support for the idea that the CSB is non-permissive for TCA crossing. Whatever normally facilitates CSB crossing, whether it is a cortical efferent axon scaffold (Chen et al., 2012), a physical early “open window” that shuts later, or secretion of boundary-busting molecules by the TCA growth cones, is lacking in *baffled* mutants. Rigorous testing of whether TCAs must interact with internal capsule guidepost cells or subplate axons in order to defasciculate and cross the CSB would require selectively genetically ablating or blocking axon outgrowth from each of these neuron types separately, or blocking the adhesion molecules they use to recognize one another. Unfortunately, selective markers and adhesion molecules for these guiding cells at early ages are not known, making these types of experiments an ongoing challenge.

In conclusion, these findings indicate that efficient BiP function is required for normal axon outgrowth and defasciculation of both thalamocortical and corticothalamic axons, and provide a basis for further analyses of BiP function in brain development, disease, and repair.

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Role of Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: CBF and NDD. Acquisition of data: CBF, RNH, CMG, AS, and NDD. Analysis and interpretation of data: CBF, RNH, CMG, AS, DRB, and NDD. Drafting of the manuscript: CBF and NDD. Critical revision of the manuscript for important intellectual content: NDD. Statistical analysis: CBF, RNH, AS, and NDD. Obtained funding: DRB and NDD. Administrative, technical, and material support: RNH, AS, and DRB. Study supervision: NDD.

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Figure 1. *baffled* mutants have defects in thalamocortical axons (TCAs)

A–B. Rostral halves of embryonic day (E)18.5 forebrains expressing the TCA-TLZ reporter. In *baffled* brains (right), TCAs are disorganized in lateral ventral telencephalon (vTel) and stalled at the corticostriatal boundary (B, red arrow).

C–D. In the caudal forebrain, the TCA-TLZ reporter shows that some TCAs do innervate the cortex in *baffled* mutants, but the axons are disorganized and non-parallel (D, red arrow). Scale bar for A–D, 500 μm.

E–F. Caudal sections of E18.5 brains in which a lipophilic carbocyanine dye (DiI) crystal was placed into dorsal thalamus (dTh) reveal similar phenotypes as with TCA-TLZ reporter. Unlike control axons (left), the mutant axons (right) do not cross straight through the corticostriatal boundary (CSB). In addition, the intermediate zone (iz) of the cortex is wider and disorganized in *baffled* mutant brains. Scale bar, 500 μm.

G–H. Close-up of CSB region in postnatal day (P)0 control and *baffled* brains immunostained with anti-L1, which labels both TCAs and corticothalamic axons (CTAs), shows *baffled* fascicles are thicker and not parallel. All sections are coronal, left panels are control and right panels are mutants. Abbreviations: CSB, corticostriatal boundary; ctx, cortex; dTh, dorsal thalamus; iz, intermediate zone; lv, lateral ventricle; TCAs, thalamocortical axons; vTel, ventral telencephalon. Scale bar, 100 μm.

Figure 2. *baffled* mutants exhibit cortical dysplasia and defects in major fiber tracts of the forebrain

A–B. Rostral coronal sections of control (left) and *baffled* (right) E17.5 brains stained with hematoxylin and eosin (H&E) show the *baffled* brain is slightly smaller and has Probst bundles (Pb) in the corpus callosum (CC). The mutant also has a thickened, irregular intermediate zone (brackets).

C–D. Caudal sections show that the *baffled* brain is smaller with a rounder hippocampus (hip). The mutant's cortical intermediate zone (brackets) is disorganized with abnormally large axon bundles. *baffled* mutants have fewer but thicker fascicles in the region of the internal capsule (ic) and at the corticostriatal boundary (black arrowheads). In the mutant some axons from the external capsule project in an abnormal ventral pathway (open arrowheads). Abbreviations: cc, corpus callosum; CSB, corticostriatal boundary; hip, hippocampus; Pb, Probst bundle; dTh, dorsal thalamus; ic, internal capsule; vTel, ventral telencephalon. Scale bar for A–D, 500µm.

Figure 3. The *baffled* mutant carries a missense mutation in Hspa5, encoding BiP/GRP78, and phenocopies reported phenotypes of a targeted BiP hypomorph mutant
A. Sequencing of genes in the *baffled* mutant interval revealed an A to G mutation in exon 1 of Hspa5 (red asterisk).

B. The mutation is predicted to convert a conserved acidic residue (Glutamic Acid, E, red asterisk) to a neutral Glycine in a hydrophilic region between the signal peptide and the ATPase domain. Inverted triangle indicates the signal peptidase cleavage site in mouse BiP.
C. Immunoblotting was used to detect the 78 kDa protein BiP/GRP78 in control and *baffled* mutant cell lysates. Top row: Using a C-terminal anti-BiP antibody (SPA-826), BiP protein is detected in both heterozygote littermate control (+/-) and *baffled* mutant (-/-) brain lysates. Hamster recombinant protein (R) is included as a positive control. Middle row: using an N-terminal antibody (Cell Signaling), BiP protein is detected in homozygote littermate control (+/+), heterozygote littermate control (+/-), and *baffled* mutant (-/-) mouse embryonic fibroblast (MEF) lysates. The same result was found by immunoblot with the anti-BiP (N-20) antibody (data not shown).

D–E. Immunostaining of primary MEFs cultured from homozygous *baffled* mutants (-/-) and littermate controls (+/+) with anti-BiP (N-20) antibody confirms that BiP has normal ER localization and level of expression in mutant cells. Scale bar, 10µm.

F–G. *baffled* mutants (bottom row) are runted with a hunched posture at age E16 through birth. Scale bar, 100 µm.

H–K. Immunostaining of P0 lung sections shows *baffled* mutants have decreased alveolar airspace (I) and reduced surfactant protein-C (K). Scale bar, 100µm.

L–M. *baffled* mutant cerebellum (M) is smaller and underdeveloped with less lobular definition at birth. Scale bar, 500µm.

Figure 4. *baffled* mutants display disordered cortical lamination

A–B. Electroporation of CAG:GFP plasmid into cortical progenitors of E15.5 control brains, followed by slice culture for 5 days *in vitro* (5DIV), labels neurons that inherited the GFP plasmid and migrated into superficial layers (A, bracket), as well as deeper progenitors. By contrast, in *baffled* mutant brains, few GFP-expressing neurons are detected in the superficial layer (B, bracket).

C–D. Uptake of BrdU into dividing progenitors at day E15.5 followed by immunostaining with anti-BrdU at E18.5 results in a strongly labeled band of neurons in superficial layers of a control brain (C, bracket). However, in mutant brains the darkly labeled cells are spread more deeply through the cortical plate (D, bracket).

E. Quantification shows a statistically significant shift of E15.5 BrdU-labeled cells to deeper layers in *baffled* mutants. Histogram quantifies the average percent ($\pm$ SEM) of BrdU⁺ cells in each of ten bins of equal depth between the ventricle and pial surface ($n = 3$ mutant, 4 control brains; 5–7 fields per brain. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

F–H. BrdU uptake at E13.5 labels deep cortical plate neurons most darkly in control E18.5 brains (F, bracket). Labeled cells appear more spread out in *baffled* mutant brain sections (G, bracket), but the bin shift was not statistically significant when data from multiple samples were pooled (H, $n = 4$ mutant, 4 control brains; 2 fields per brain; $p > .05$).

I–P. Control (ctrl) cortical sections are shown in top row, and *baffled* mutant brain sections on bottom row, stained with cortical layer markers.

I–J. Superficial layer marker Cux1 labels a tight band of superficial neurons in control P0 cortical sections (I, line), but labels neurons scattered more deeply into the cortical plate in *baffled* mutants (J, dotted line).

K–L. Ctip2 marks layers 5 strongly and layer 6 weakly in control E18.5 brains (K). In mutant brains (L), the layers are present in the correct order but appear less organized, with a few scattered labeled cells.

M–N. Layer 6 marker Foxp2 shows more superficial scatter in *baffled* mutant brains (N, dotted line) than in control E18.5 cortex (M, line).

O–P. Immunostaining for reelin protein in E18.5 cerebral cortex suggests reelin is present but reduced in the marginal zone (mz) of *baffled* mutants, similar to the targeted hypomorph BiP mutant.

All scale bars represent 200 μ m.

Figure 5. *baffled* mutant TCAs fail to extend into cortex at E15.5

DiI crystal was placed into dorsal thalamus of E15.5 control (left) and *baffled* mutant (right) brains.

A–B. Rostral sections of control brains show many axons entering the cortex while *baffled* brains show sparse axons and most have not entered the cortex (ctx).

C–D. In middle sections of control brains, axons cross the corticostriatal boundary (CSB) in a neat array and extend dorsally into cortex. In contrast, only a few *baffled* mutant axons have crossed the CSB (arrow).

E–F. In caudal sections, *baffled* mutant axons appear disorganized rather than parallel within the ventral telencephalon.

Scale bar for A–F, 500 μ m. Abbreviations: CSB, corticostriatal boundary; dTh, dorsal thalamus; vTel, ventral telencephalon.

Figure 6. Reciprocal connections between the thalamus and cortex are delayed in *baffled* mutants at E13.5 and E14.5

DiI crystal was placed into either dorsal thalamus (top row) to label TCAs, or cortex (bottom row) to label corticothalamic axons (CTAs) in control (ctrl) and *baffled* brains. Arrows mark the farthest extended axons detectable in each panel.

A–B. At E13.5, control TCAs have extended into ventral telencephalon, whereas *baffled* mutant TCAs remain stalled just past the diencephalon-telencephalon boundary.

C–D. At E14.5, control TCAs have reached the lateral vTel, but *baffled* mutant TCAs remain in medial vTel.

E–F. At E13.5, corticothalamic axons (CTAs) of controls have reached the lateral vTel, whereas only a few *baffled* mutant CTAs have reached lateral vTel.

G–H. At E14.5, many control CTAs have reached the medial vTel but *baffled* mutant CTAs are only starting to accumulate in lateral vTel.

All scale bars represent 500 μm.

Figure 7. *baffled* mutants have disorganized, overfasciculated corticothalamic axons

A–B. Coronal sections of E15.5 control and *baffled* mutant brains immunostained with anti-L1-CAM, which labels both thalamic and cortical axons (Chung et al., 1991), show that whereas controls have a neat interdigitated and parallel array of axons crossing the CSB, *baffled* mutants display a gap in axons at the CSB (arrow), indicating neither cortical nor thalamic axons have crossed in significant numbers.

C–D. Sagittal sections of P0 control (left) and *baffled* mutant (right) brains were immunostained with anti-TAG-1, which specifically labels cortical axons, not thalamic axons. Control brains exhibit a fan-like array of cortical axon fascicles, but in *baffled* brains there are fewer fascicles and they are enlarged (e.g., black arrowhead, D). *baffled* mutants also display thicker, jagged intermediate zone staining (bracket, D), indicating axon bundles sometimes invade layer 6 (open arrowhead, D). Bars in B and D represent 500 μ m.

Figure 8. Specification of dorsal telencephalon, ventral telencephalon, and diencephalon appears normal in *baffled* mutants

Coronal cryosections of E18.5 brains were hybridized with DIG probes for mRNA markers. Expression patterns of *Gbx2*, *Pax6*, and *Sema6a* appeared normal.

A–B. In rostral sections of control and *baffled* mutant brains, *Pax6* is detected strongly in the ventricular zone of the dorsal telencephalon, less strongly in the ventricular zone of the ventral telencephalon, and in cells near the anterior commissure.

C–D. In caudal sections, *Pax6* is detected in the ventricular zone of the cortex, in the diencephalon as a V-shaped zone in ventral thalamus (prethalamus), and in a ventral cluster of cells in ventral telencephalon.

E–F. *Gbx2* expression is restricted to the diencephalon in both control (top) and *baffled* (bottom) mutant brains. It was detected in the paraventricular nucleus of epithalamus, a subset of nuclei in dorsal thalamus, and was absent in ventral (pre-)thalamus.

G–H. *Sema6a* mRNA is detected in a similar pattern in control (top) and mutant (bottom) brains, including in the ventricular zone and deep cortical plate of cortex, as well as in the amygdala and mantle zone of ventral telencephalon.

Scale bar for A–G, 1 mm.

Figure 9. Guiding cells for TCAs appear present in *baffled* mutant brains, but some may be displaced

A–B. Internal capsule (ic) guidepost cell bodies were retrogradely labeled by DiI crystal placement into the dorsal thalamus (dTh) at E14.5. Ic guidepost cells were sometimes detected in more caudal sections in *baffled* mutant brains as compared to controls, indicating that they could be slightly displaced caudally, or that axons of rostral ic cells had not reached dorsal thalamus yet. Scale bar, 1 mm.

A'–B'. Higher magnification of boxed regions in A and B show that fewer ic guidepost cells were able to be retrogradely labeled in *baffled* mutants as compared to controls. Scale bar, 500 μ m.

C–D. Immunostaining of E14.5 coronal brain sections with anti-Islet1 reveals corridor cells (bracketed) are present in both control (left) and *baffled* mutant brains (right). Arrows indicate the unstained axon bundles traveling through the corridor. Scale bar, 500 μ m.

E–F. Immunostaining of E14.5 cortical sections with preplate marker CSPG shows preplate splitting occurs in both control (left) and *baffled* mutant cortex (right). Scale bar, 50 μ m.

G–H. RNA *in situ* hybridization with subplate/layer 6 marker p75 on E18.5 sagittal sections of control (left) and mutant (right) cortex suggests that the subplate and layer 6 neurons,

which project axons to dorsal thalamus, remain correctly positioned in deep cortical plate in *baffled* mutant brains. Scale bar, 500 μ m.

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Figure 10. Dissociated *baffled* mutant neurons show an axon extension defect in culture

A–C. A, B show representative fields from control and *baffled* mutant cortical neuron cultures fixed after two days *in vitro* and immunostained for Tau1 and DAPI. C, Average axon lengths ($\pm$ SEM) of mutant neurons are significantly shorter. 311 control neurons (gray bar), and 265 mutant neurons (black bar) with Stage 3 morphology (having a single longer process enriched for Tau1) were traced from independent dissociations of three control and mutant littermate brains each. $p = 3.8 \times 10^{-17}$, t-test.

D–F. D, E show example neurons from control (D) and mutant (E) cultures with nuclei stained red for Tbr1 (arrowheads), a transcription factor marker of layer 6 corticothalamic projection neurons. A Tbr1⁻ neuron is indicated with an arrow. F, Stage 3 neurons expressing Tbr1 have shorter axons, on average, in *baffled* mutant cultures (black bar) than in control cultures (gray bar). For Tbr1⁺ neuron comparison between control and mutant, $p = 4.0 \times 10^{-8}$, t-test ($n = 105$ control neurons, 83 mutant neurons). For Tbr1⁻ neuron comparison, $p = 5.2 \times 10^{-11}$, t-test ($n = 206$ control neurons, and 182 mutant neurons). As a side note, no significant difference was found between average axon lengths of Tbr1⁺ and Tbr1⁻ neurons in control or mutant cultures.

G–I. Dissociated cortical neuron cultures from control (left) and mutant (right) brains have similar proportions of upper and deep layer marker expression. Cultures were fixed, immunostained for transcription factors Satb2 (green, arrows) and Tbr1 (red, arrowheads), and scored for expression. Graph in I shows average percent of cells ($\pm$ SEM) labeled by each marker on six coverslips from three brains each of control (gray) and mutant (black), with 4174 total control cells scored and 3635 total mutant cells. For Tbr1⁺ cells, $p = 0.77$; for Satb2⁺ cells, $p = 0.98$; for cells expressing neither, $p = 0.11$, t-test. All scale bars represent 100 μ m.

Table 1

Table of Abbreviations

CC	Corpus Callosum
CSB	Corticostriatal Boundary
CTA	CorticoThalamicAxons
ctx	cortex
DIV	Days in vitro
dTh	Dorsal Thalamus
E	Embryonic day
ic	Internal capsule
lv	Lateral ventricle
mz	Marginal zone
P	Postnatal day
Pb	Probst bundle
TCA	Thalamocortical Axons
TCA-TLZ	ThalamoCortical Axon Tau-LacZ
vTel	Ventral Telencephalon

Table 2

Primary Antibodies used in this study

Antigen	Immunogen	Manufacturer, Species antibody was raised in, mono- vs polyclonal, Catalog Number	Dilution used
BrdU, clone 3D4	Bromo-deoxy Uridine	BD Biosciences, Mouse monoclonal, #555627	1:100
CDP (Cux1), C-20	epitope mapping at the C-terminus of CDP of mouse origin	Santa Cruz Biotechnology, Goat polyclonal, #sc-6327	1:500
CSPG, clone CS-56	ventral membranes of chicken gizzard fibroblasts	Sigma, Mouse IgM monoclonal, #C8035	1:500
Ctip2	Fusion protein (human); between amino acids 1-150 of CTIP2	AbCam, Rat monoclonal, #18465	1:500
Foxp2	Synthetic peptide conjugated to KLH derived from within residues 700 to the C-terminus of Human FOXP2.	AbCam, Rabbit polyclonal, #16046	1:1000 cryo, 1:2000 paraffin
GRP78, N-20	epitope mapping at the N-terminus of human GRP 78	Santa Cruz Biotechnology, Goat polyclonal, #sc-1050	1:50
GRP78, SPA-826	Synthetic peptide corresponding to 10aa's at the C-terminus of rat Grp78 (BiP). The sequence is completely conserved in mouse and hamster.	Stressgen, Rabbit polyclonal, #SPA- 826	1:5000
GRP78/BiP	synthetic peptide corresponding to the sequence of human BiP from N-terminal region. Purified by protein A and peptide affinity chromatography.	Cell Signaling, Rabbit polyclonal, #3183	1:1000
Islet1	C-terminal portion of rat islet-1 and 2, bacterial expression	Developmental Studies Hybridoma Bank, Mouse monoclonal, #39.4D5-s	1:100
L1, clone UJ127.11	Homogenate of 16 week human foetal brain cells.	Chemicon (Millipore), Rat monoclonal, #CBL275	1:200
Reelin, clone G10	Recombinant reelin amino acids 164–496	Chemicon (Millipore), Mouse monoclonal, #MAB5364MI	1:500
Satb2	Recombinant fragment C-terminal. (Human)	AbCam, Mouse monoclonal, #51502	1:200
Surfactant-C, FL-197	full length (197aa) pulmonary surfactant protein C (SP-C) of human origin	Santa Cruz, Biotechnology, Rabbit polyclonal, #13979	1:400
TAG-1	Recombinant TAG-1	gift of Jane Dodd (TG3), Rabbit polyclonal	1:10,000
Tau1	Purified denatured bovine microtubule associated proteins.	Chemicon, Mouse monoclonal, #3420	1:200
Tbr1	Synthetic peptide conjugated to KLH derived from within residues 50 – 150 of Mouse TBR1.	AbCam, Rabbit polyclonal, #31940	1:500