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Disturbances in the positioning, proliferation, and apoptosis of neural progenitors contribute to subcortical band heterotopia formation

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Abstract

Cortical malformations are commonly associated with intractable epilepsy and other developmental disorders. Our studies utilize the tish rat, a spontaneously occurring genetic model of subcortical band heterotopia (SBH) associated with epilepsy, to evaluate the developmental events underlying SBH formation in the neocortex. Our results demonstrate that Pax6⁺ and Tbr2⁺ progenitors are mislocalized in tish^{+/−} and tish^{−/−} neocortex throughout neurogenesis. In addition, mislocalized tish^{−/−} progenitors possess a longer cell cycle than wildtype or normally-positioned tish^{−/−} progenitors, owing to a lengthened G₂+M+G₁ time. This mislocalization is not associated with adherens junction breakdown or loss of radial glial polarity in the ventricular zone, as assessed by immunohistochemistry against phalloidin (to identify F-actin), aPKC-λ, and Par3. However, vimentin immunohistochemistry indicates that the radial glial scaffold is disrupted in the region of the tish^{−/−} heterotopia. Moreover, lineage tracing experiments using *in utero* electroporation in tish^{−/−} neocortex demonstrate that mislocalized progenitors do not retain contact with the ventricular surface and that ventricular/subventricular zone progenitors produce neurons that migrate into both the heterotopia and cortical plate. Taken together, these findings define a series of developmental errors contributing to SBH formation that differs fundamentally from a primary error in neuronal migration.

Keywords

Tbr2; Pax6; epilepsy; intermediate progenitor; migration; radial glia

Normal development of the mammalian neocortex is a complex process of precisely timed molecular and cellular events, the details of which continue to be elucidated and refined.

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Considering this complexity, it is not surprising that developmental errors are common, with an incidence of cortical malformation of ~1% in the general population, ~14% in epileptic patients, and ~40% in patients with intractable epilepsy (Farrell et al., 1992; Hardiman et al., 1988; Meencke and Veith, 1992). One of the major categories of cortical malformation, classical lissencephaly, results in a neocortex that exhibits agyria, pachygyria, or subcortical band heterotopia pathologically, and some degree of mental retardation and epilepsy (Dobyns et al., 1996; Dobyns et al., 1993). Research into the genetic causes of this disease spectrum has identified mutations in genes such as doublecortin, lissencephaly-1, aristaless-related, α -tubulin, reelin, very-low density lipoprotein receptor, and ApoE receptor 2, which are critical for the effective migration of newborn neurons into the cortical plate (des Portes et al., 1998; Hirotsune et al., 1995; Keays et al., 2007; Kitamura et al., 2002; Reiner et al., 1993; Trommsdorff et al., 1999). These findings have reinforced the concept that most lissencephaly-spectrum malformations of the neocortex result from a primary defect in neuronal migration. Despite these advances in our understanding, the causative gene(s) and cellular mechanisms underlying some human cases of this disorder remain elusive (Delatycki and Leventer, 2009).

The present studies utilize a novel model of subcortical band heterotopia (SBH), the tish rat, to investigate the cellular mechanisms underlying SBH formation during embryonic development. The tish rat is a spontaneously occurring genetic model of SBH in which the malformation is inherited in an autosomal recessive manner (Lee et al., 1997). A unique feature of the developing tish^{-/-} neocortex is the presence of an abnormally located (heterotopic) band of proliferating cells in the intermediate zone and cortical plate in addition to the normally-positioned (normotopic) band of cells in the ventricular and subventricular zones (Lee et al., 1998). Our current studies evaluate the identities of these cells and the mechanism underlying their mislocalization. Our findings define a form of developmental error contributing to SBH formation that differs fundamentally from a primary error in neuronal migration and that has broader applicability for understanding other neurodevelopmental disorders that are hypothesized to result from neuronal migration defects.

Experimental Procedures

Animals and breeding

Animals were housed at 22°C on a standard 12h:12h light–dark schedule with free access to food and water. Animals were handled according to NIH guidelines and a protocol approved by the University of Virginia Animal Care and Use Committee. The tish phenotype is expressed on a Sprague-Dawley background, and the heterotopia are inherited in an autosomal recessive manner, requiring two mutated alleles in order to display the SBH phenotype. Therefore, timed pregnant litters of tish^{-/-} pups were generated by mating a tish^{-/-} male with a tish^{-/-} female. Wildtype Sprague-Dawley control litters were generated by mating a wildtype male to a wildtype female. Tish^{+/-} litters were generated by mating a tish^{-/-} male to a wildtype female. In all cases, the morning of vaginal plug discovery was designated as embryonic day E0.5.

Bromodeoxyuridine administration

For those animals used in the immunohistochemical characterization of tish progenitor cells and the *in utero* electroporation experiments, bromodeoxyuridine (BrdU) was administered as previously described (Lee et al., 1998). Briefly, pregnant dams were given an intraperitoneal injection of BrdU (50 mg/kg, Sigma) and euthanized 2h later under deep anesthesia. The brains of the embryos were then removed and prepared for sectioning. This administration protocol was employed to label only those progenitor cells within S phase or

about to exit S phase at the time of administration, since a two-hour survival is insufficient time for these cells to complete mitosis and pass BrdU on to their progeny (Takahashi *et al.*, 1995).

Tissue processing and Immunohistochemistry

Dams with timed-pregnancies were anesthetized with isoflurane and decapitated, and embryos were removed between embryonic days E13 and E20. Embryonic brains were rapidly dissected in 0.1M PBS and fixed for 1h in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose until sinking. Cryostat sections were cut at 10, 20, or 60 μ m and mounted on Superfrost Plus slides (Fisher Scientific).

Slide mounted sections were boiled briefly in 10mM sodium citrate pH 6 to enhance antigen recognition, followed by incubation in 2N HCl for 30 min to expose BrdU or IdU. Sections were then blocked in 5% normal goat serum (Vector Laboratories, Burlingame CA) with 0.3% Triton X100 in 0.1M PBS for 2h before overnight incubation at 4°C with primary antibody diluted in blocking serum. The following primary antibodies were used: anti-Pax6 (rabbit, 1:300, Covance), anti-Tbr2 (rabbit, 1:300, Millipore), anti-phosphorylated vimentin 4A4 clone (mouse, 1:500, Assay Designs), anti-BrdU (mouse, 1:10, BD Biosciences), anti-BrdU (rat, 1:40, Abcam), anti- β III tubulin (mouse, 1:100, Sigma-Aldrich), anti-cleaved caspase 3 (rabbit, 1:200, Cell Signaling Technology), anti-aPKC- λ (mouse, 1:100, BD Transduction Labs), anti-PAR3 (rabbit, 1:100, Millipore), anti-GFP (rabbit, 1:500, Invitrogen), anti-vimentin (mouse, 1:40, Sigma). Incubation with secondary antibody diluted in blocking serum was performed for 1h at room temperature. For cell counting of Pax6⁺ and Tbr2⁺ cells, a goat anti-rabbit biotinylated antibody (1:250, Vector Laboratories) was used, followed by detection via the ABC method (Vector Laboratories, Burlingame CA) with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) as chromagen. For immunofluorescence, the following secondary antibodies were used: goat anti-rabbit Alexa 633, goat anti-rabbit 488, goat anti-mouse Alexa 488, goat anti-mouse Alexa 594, or goat anti-rat Alexa 594 (all at 1:250, Invitrogen), followed by incubation with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. For phalloidin immunohistochemistry, sections were not exposed to sodium citrate; rather, they were blocked for 20 minutes in 0.1% Triton in 0.1M PBS, followed by 10% normal goat serum in 0.1M PBS, before incubation with Alexa 488 conjugated phalloidin diluted in blocking serum (5U/mL, Invitrogen) and then DAPI. Finally, in the case of the DAB-developed sections, slides were dehydrated in graded ethanols, cleared in Xylenes, and coverslipped with Permount (Fisher Scientific), or in the case of fluorescent sections, slides were air dried, coverslipped with ProLong Gold anti-fade reagent (Invitrogen), and stored at -20°C. Light microscopic images of DAB labeled sections were captured by a Zeiss Axiocam camera mounted on a Zeiss Axioplan2 microscope. Fluorescent images were captured on a Zeiss LSM510 confocal microscope.

Cell counting and cell cycle assay

Quantification of Pax6⁺ and Tbr2⁺ cell density was performed on sections of wildtype, tish^{+/-}, and tish^{-/-} embryos from E15 through E20. Pax6⁺ and Tbr2⁺ nuclei were counted within a defined area that spanned the entire cortical depth from ventricular to pial surface in two non-consecutive sections from five embryos for each genotype and developmental day. Data are expressed as the number of cells/mm² and p-values are the result of a Kruskal-Wallis One-way ANOVA with a Tukey's multiple comparisons test or a One-way ANOVA with a Holm-Sidak multiple comparisons test comparing wildtype, tish^{+/-}, and tish^{-/-} animals at each embryonic age. All statistical analyses were performed using SigmaStat software (SYSTAT Software, Inc.).

In order to assess cell cycle parameters within Pax6⁺ and Tbr2⁺ progenitor populations, an IdU/BrdU labeling method was used as previously described (Quinn et al., 2007). Briefly, at E17 or E20, timed-pregnant dams were injected i.p. with IdU followed by BrdU (both at 50mg/kg) 1.5h later. Dams were sacrificed 2h after the first injection and embryos were removed, fixed, and sectioned as detailed above. Triple immunofluorescence was performed as indicated utilizing either an anti-Pax6 or anti-Tbr2 antibody to label the total population of progenitor cells, in conjunction with an anti-BrdU antibody which recognizes both IdU and BrdU (mouse, BD Biosciences) and an anti-BrdU antibody which only recognizes BrdU (rat, Abcam). Confocal images were imported into ImageJ (National Institutes of Health), and the proportions of IdU/BrdU labeled cells in the proliferative zones of the dorsal neocortex were counted in two non-consecutive sections from 3–5 embryos for each genotype and developmental day. Cell cycle lengths were calculated as follows: cells that left S-phase during the 1.5 hr interval between the two injections (IdU⁺ only) were designated the leaving fraction (L_{cells}). Cells that remained in S phase for the entire 2 hr duration (IdU and BrdU⁺) or that entered S phase after the second injection (BrdU⁺ only) were designated the S-phase cells (S_{cells}). Cell cycle parameters were then calculated as follows: S phase length (T_s) → $T_s/1.5 = S_{cells}/L_{cells}$; cell cycle length (T_c) → $T_c/T_s = P_{cells}/S_{cells}$, where P_{cells} represents the total number of proliferating cells as indicated by Pax6 or Tbr2 immunoreactivity; percentage of time spent in S phase → T_s/T_c ; length of G₂+M+G₁ → $T_c - T_s$. Data are expressed as mean ± SEM and p-values are the result of either a One-way ANOVA with the Holm-Sidak multiple comparisons test or a Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons testing.

***In utero* electroporation**

In order to assess the mechanisms underlying the progenitor cell mislocalization in the *tish*^{-/-} neocortex, a pCAGGS plasmid expressing the GFP gene was electroporated into radial glial cells to allow for visualization of these cells and their progeny through expression of GFP (Stuhmer et al., 2002). Briefly, a timed-pregnant wildtype or *tish*^{-/-} dam was anesthetized via an intraperitoneal injection of a ketamine/xylazine mixture (67/10 mg/kg) and the uterine horns were exposed via an abdominal incision. Embryos were visualized by backlighting the uterus with a fiberoptic light source, and a pulled borosilicate glass electrode (1.0mm OD/0.78mm ID, Sutter Instruments, Novato CA) containing 4mg/ml pCAGGS-GFP plasmid (a kind gift from S. Anderson) in a 0.1% solution of Fast Green dye (Sigma-Aldrich) was lowered into the lateral ventricle of the embryos and 1 µL of solution was injected using an MPPI-2 pressure injector (Applied Scientific Instrumentation, Eugene OR). The plasmid was electroporated using an ECM830 square wave electroporator (BTX, Harvard Biosciences) using 5 pulses of 50–75V, 50ms duration, and 950ms interval. After electroporation, the dam was allowed to survive for 12, 24, or 72h before embryos were harvested and their brains were processed for immunohistochemistry as described above.

Results

Cortical progenitor cells are incorrectly positioned in the *tish*^{+/-} and *tish*^{-/-} neocortex

Given recent evidence that radial glial cells (RGCs) and intermediate progenitor cells (IPCs) are neurogenic (Noctor et al., 2001; Noctor et al., 2002; Noctor et al., 2004), we sought to characterize the abnormally-positioned, proliferative cells that have been previously identified in the intermediate zone (IZ) and normally-positioned cortical plate (CP) of the developing *tish*^{-/-} neocortex (Lee *et al.*, 1998). Toward this end, we utilized immunohistochemistry to visualize Pax6⁺ RGCs and Tbr2⁺ IPCs (Englund et al., 2005). Examination of wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices at time points corresponding to early, mid, and late cortical plate neurogenesis demonstrated that the heterotopic proliferative zone of the *tish*^{-/-} cortex contains both Pax6⁺ and Tbr2⁺ cells (Fig. 1). Early in

cortical plate neurogenesis at E15, small groups of inappropriately located Pax6⁺ and Tbr2⁺ cells were found in the *tish*^{-/-} preplate region just beneath the pial surface (Fig. 1C arrows, compare with Fig. 1A–B; Fig. 1F arrows, compare with Fig. 1D–E). These mislocalized cells were present in addition to the normally-positioned Pax6⁺ cells in the ventricular zone (VZ) and Tbr2⁺ cells in the subventricular zone (SVZ), but they were not observed in the corresponding preplate region in either wildtype or *tish*^{+/-} cortex. As development proceeded, these small groups of heterotopic Pax6⁺ and Tbr2⁺ cells coalesced into a diffuse band in the IZ and CP by E17 (Fig. 1I arrows, compare with Fig. 1G–H; Fig. 1L arrows, compare with Fig. 1J–K). This band of mislocalized cells persisted through E20 (Fig. 1O arrows, compare with Fig. 1M–N; Fig. 1R arrows, compare with Fig. 1P–Q). Interestingly, at E17 in the *tish*^{+/-} neocortex, a few small clusters of Pax6⁺ and Tbr2⁺ cells were present in the IZ, suggesting a partial gene dosage effect for the *tish* gene in the positioning of Pax6⁺ and Tbr2⁺ progenitor cells during development (Fig. 1E, arrowheads). Despite this partial positioning defect in *tish*^{+/-} animals, subcortical band heterotopia are observed only in *tish*^{-/-} animals and not in *tish*^{+/-} animals, suggesting that a greater number of mislocalized cells are required before the adult structural malformation can be established.

In order to establish whether the abnormally located Pax6⁺ or Tbr2⁺ cells were, in fact, mitotic progenitors or whether they were simply post-mitotic cells that had not yet downregulated a marker of an earlier stage of development (Hevner, 2006), we employed co-labeling for Pax6 or Tbr2 and BrdU, β -III tubulin (β -tub, an early neuronal marker), and phosphorylated vimentin (pvim, a marker of mitotic cells). Cycling Pax6⁺ and Tbr2⁺ cells (i.e., Pax6⁺/BrdU⁺ or Tbr2⁺/BrdU⁺ cells) were observed in an appropriate position in the VZ or SVZ of wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices at E17 (Fig. 2A–C and Fig. 3A–C). In addition, inappropriately positioned Pax6⁺/BrdU⁺ and Tbr2⁺/BrdU⁺ cells were present in the *tish*^{-/-} IZ/CP, indicating that these cells were also actively engaged in the cell cycle (Fig. 2D–E and Fig. 3D–E). Similarly, mitotic Pax6⁺/pvim⁺ and Tbr2⁺/pvim⁺ cells were observed in the VZ or SVZ in wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices (Fig. 3F–H); however, double-positive cells were also found in the *tish*^{-/-} IZ/CP, indicating that these mislocalized Pax6⁺ and Tbr2⁺ cells were undergoing mitosis (Fig. 2I–J and Fig. 3I–J).

The positioning of immature neurons relative to RGCs and IPCs was examined using double labeling for Pax6 or Tbr2 and β -III tubulin. Appropriately positioned Pax6⁺/ β -III tubulin- and Tbr2⁺/ β -III tubulin- cells were found in the VZ or SVZ of wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices (Fig. 2K–M and Fig. 3K–M). Notably, the mislocalized Pax6⁺ and Tbr2⁺ cells in the *tish*^{-/-} IZ/CP were also β -III tubulin⁻, indicating that these cells were indeed progenitor cells and not immature neurons (Fig. 2N–O and Fig. 3N–O). Although these cells were located in a region of dense β -III tubulin⁺ neuronal axons, three-dimensional reconstruction of confocal image stacks failed to reveal any colocalization between Pax6 or Tbr2 and β -III tubulin. Rather, β -III tubulin⁺ processes appeared to course around these Pax6⁺/ β -III tubulin- and Tbr2⁺/ β -III tubulin- cells (Fig. 2O and Fig. 3O). A similar phenotype was observed for the more limited population of Pax6⁺ and Tbr2⁺ cells in the IZ of *tish*^{+/-} neocortex, indicating that these cells were also progenitor cells and not immature neurons (data not shown).

Taken together, these results indicate that a combination of mitotic RGCs and IPCs are present in the region of heterotopic proliferation in the *tish*^{-/-} neocortex and, to a lesser extent, in the corresponding region of the neocortex of *tish*^{+/-} animals as well. Furthermore, these results demonstrate that the number of heterotopic progenitors in the developing neocortex is a key determinant of SBH formation.

Progenitor cell density is increased in the *tish*^{-/-} neocortex

Qualitative observations of the Pax6 and Tbr2 immunohistochemistry results suggested that *tish*^{-/-} neocortex contained more progenitor cells than wildtype cortex. We therefore sought to quantify these observations by measuring Pax6⁺ and Tbr2⁺ cell density in radial columns extending from the ventricular surface to the pial surface at each embryonic day from E15-E20. Pax6⁺ and Tbr2⁺ cell density across the depth of the cortical wall was increased in *tish*^{-/-} animals compared with wildtype or *tish*^{+/-} animals beginning on E17 and proceeding through E20 (Fig. 4A–B). It is known that alterations in the size of the progenitor population can lead to changes in the size of the adult neocortex (Caviness et al., 2003); however, previous work from our laboratory has shown that adult *tish*^{-/-} neocortex (normotopic cortex + SBH) is similar in volume to control neocortex (Lee et al., 1999). We therefore hypothesized that the observed increase in progenitor cell density in *tish*^{-/-} neocortex must be offset by an increased amount of apoptosis during development. Indeed, activated caspase 3 immunohistochemistry at E17 revealed a significant increase in apoptotic cell death in the IZ/CP of *tish*^{-/-} neocortex compared with either *tish*^{+/-} or wildtype cortex. There was also a trend toward an increase in apoptosis in the *tish*^{-/-} VZ/SVZ (Fig. 4C), although this effect did not achieve statistical significance. These data suggest that, in addition to a defect in progenitor cell positioning, a disruption in progenitor cell density also occurs in the developing *tish*^{-/-} neocortex. This alteration in cell density is offset during development by an increased level of cell death, leading to an adult neocortex whose volume is similar to that of wildtype neocortex.

Cell cycle kinetics are altered in *tish*^{-/-} neocortical progenitor cells

In an effort to identify an underlying basis for the increase in progenitor cell density at E17 and E20 in the *tish*^{-/-} neocortex, we employed an IdU/BrdU labeling assay to assess the cell cycle kinetics of normally- and abnormally-positioned progenitor populations at these times. This assay has been shown previously to yield values for cell cycle kinetic parameters that are comparable to those calculated using serial BrdU injection methods (Quinn *et al.*, 2007). We reasoned that, inasmuch as longer cell cycle times are associated with neurogenic rather than self-renewing divisions (Calegari et al., 2005; Calegari and Huttner, 2003), an increase in progenitor cell density at E17 and E20 could result from a shortening of the cell cycle in these populations and, thus, an increase in self-renewing divisions.

We considered first the population of Pax6⁺ progenitors, which corresponds to RGCs. Pax6⁺ cells were identified in wildtype, normotopic *tish*^{-/-} (n-*tish*^{-/-}) and heterotopic *tish*^{-/-} (h-*tish*^{-/-}) neocortices. This analysis allows a differential assessment of cell cycle kinetics in the appropriately- and inappropriately-positioned proliferative cells in *tish*^{-/-} neocortex. Unexpectedly, at E17, no significant differences were detected among groups for Pax6⁺ cells in terms of the percentage of time spent in S phase (T_s/T_c), the total cell cycle length (T_c), or the lengths of S phase (T_s) or G₂+M+G₁ phases (T_c-T_s) (Fig. 5A–D, Table 1). In contrast, at E20, multiple differences were noted in the Pax6⁺ cell populations among the various groups. Pax6⁺ cells in the n-*tish*^{-/-} neocortex did not differ from wildtype in terms of their cell cycle kinetics, demonstrating that, even in *tish*^{-/-} neocortex, appropriate positioning of Pax6⁺ progenitors serves as a prerequisite for proper control of cell cycle behavior. However, E20 Pax6⁺ cells in h-*tish*^{-/-} neocortex demonstrated an increased T_s/T_c and a decreased T_c-T_s compared with wildtype or n-*tish*^{-/-} Pax6⁺ cells (Fig. 5A–D, Table 1). Interestingly, T_c was not significantly different among groups. Although a trend toward a decreased T_c was observed in h-*tish*^{-/-} cells, this effect did not achieve statistical significance (Fig. 5B, Table 1). Taken together, these results indicate that heterotopic Pax6⁺ progenitors in *tish*^{-/-} neocortex at E20 possess shorter G₂+M+G₁ phases than wildtype cells with a trend toward a decrease in the length of the cell cycle.

We next considered the possibility that changes in cell cycle kinetics could be occurring in the IPC population. For Tbr2⁺ cells at E17 and E20, n-tish^{-/-} progenitors possessed cell cycle kinetics that were not significantly different from wildtype, highlighting that, even in tish^{-/-} neocortex, appropriate positioning of Tbr2⁺ progenitors may contribute to proper control of cell cycle behavior. In contrast, T_s/T_c was increased in h-tish^{-/-} cells compared to wildtype, while both T_c and T_s-T_c were significantly decreased (Fig. 5E-H, Table 1). Taken together, these results indicate that heterotopic Tbr2⁺ progenitors in tish^{-/-} neocortex at E17 and E20 possess a shorter cell cycle length than wildtype cells due to shortened G₂+M+G₁ phases.

Radial glial adherens junctions and apical polarity domains are intact in tish^{-/-} neocortex

In light of the finding that some RGCs and IPCs are located heterotopically in the tish^{-/-} neocortex, we sought to identify a mechanism by which these cells might become mislocalized. Considering that the RGC population appeared to be affected most severely at E15 (Fig. 1), we hypothesized that the observed positioning defect might result from a population of VZ RGCs losing its attachments to the ventricular surface and migrating into the IZ/CP to seed the heterotopic proliferative zone. In order to test this possibility, we employed immunohistochemistry and *in utero* electroporation techniques to assess the status of adherens junctions and apical polarity markers at the ventricular surface. We reasoned that if RGCs were losing their attachments to the ventricular surface and seeding a new proliferative zone, then we would observe disruptions in the F-actin components of VZ adherens junctions and in the apical polarity proteins aPKC-λ and PAR3 (Cappello et al., 2006; Costa et al., 2008). We also reasoned that we would observe a greater percentage of RGCs with retracted apical processes following *in utero* electroporation of a pCAGGS-GFP construct.

Examination of adherens junctions using Alexa 488 conjugated phalloidin to identify F-actin demonstrated no obvious differences between wildtype and tish^{-/-} neocortices at E13, E15, or E17 (Fig. 6A-F). Had a loss of adherens junctions been responsible for the heterotopic mitoses in tish^{-/-} neocortex, one would have anticipated an interruption in phalloidin staining at the ventricular surface as has been described previously (Cappello et al., 2006). Such an interruption was not observed. Similarly, aPKC-λ and PAR3 staining revealed no obvious disruptions of apical polarity within the endfeet of RGCs at the ventricular surface at E13, E15, or E17 (Fig. 6G-R). Moreover, examination of RGC apical processes at E17, 12h after electroporation with a pCAGGS-GFP construct, revealed that the percentage of electroporated GFP⁺ cells maintaining an apical process with ventricular contact did not differ between wildtype and tish^{-/-} neocortex (wt 67.2±2.84%, tish^{-/-} 69.3±1.23%, *p* > 0.05). Thus, we conclude that adherens junctions and apical polarity within RGC endfeet are maintained in tish^{-/-} neocortex and that progenitor cells do not become mislocalized as a result of losing their apical attachments to the ventricular surface.

Mislocalized tish^{-/-} neocortical progenitor cells are not produced by the pallial ventricular zone at mid-neurogenesis

Based on our finding that tish^{-/-} RGCs maintain their ventricular attachments during a time of extensive heterotopic proliferation, it appears that a different causative mechanism underlies the mislocalization of progenitor cells in the tish^{-/-} IZ/CP. One possibility is that a population of VZ RGCs could have suffered a disruption in interkinetic nuclear migration such that their nuclei failed to return to the ventricular surface to undergo mitosis. Instead, the nuclei of these cells may have continued toward the pial surface after completing S phase, dividing at some location within the IZ/CP while maintaining a ventricle-contacting radial process. Alternatively, daughter cells produced by RGC mitoses in the VZ might

initiate migration and fail to undergo cell cycle arrest, instead continuing to cycle as they migrate into the IZ/CP.

In order to test these possibilities, we electroporated a pCAGGS-GFP construct into the lateral ventricles of E14.5 and E16.5 wildtype and *tish*^{-/-} embryos and examined the neocortex 3 days later, after a single BrdU pulse 2h prior to embryo collection. We chose a 3 day survival period after initial experiments demonstrated no GFP⁺ cells in the heterotopic proliferative zone at 24 hrs post-electroporation (data not shown). At this earlier time point, GFP⁺ cells were found only in the VZ/SVZ, consistent with the latent period during which radial glial progeny reside in the SVZ for a 24 hr period before resuming migration toward the CP (Noctor *et al.*, 2004). Due to a high rate of post-electroporation mortality of E14.5 *tish*^{-/-} embryos secondary to amniotic fluid leakage caused by polyhydramnios and increased intra-amniotic pressure, we were unable to assess whether heterotopic progenitors were produced by the VZ/SVZ at the onset of cortical plate neurogenesis. Consequently, we present here the data from embryos electroporated at E16.5, an age corresponding to the middle stages of cortical plate neurogenesis.

In wildtype neocortex electroporated at E16.5, GFP⁺ cells were detected in the IZ and CP 3 days post-electroporation. Those cells in the IZ maintained a migratory morphology with a long pial-directed process characteristic of migrating neurons (Fig. 7A). GFP⁺ cells in the CP elaborated branched apical dendrites and a basal axonal projection, which, in some cases, could be followed into the IZ (Fig. 7B). While there were some BrdU incorporating cells in these regions, our immunohistochemical analysis revealed no colocalization between BrdU and GFP, indicating that VZ daughter cells born at E16.5 in wildtype neocortex were able to arrest their cell cycles before migrating into the IZ.

In *tish*^{-/-} neocortex electroporated at E16.5, GFP⁺ cells were also detected in the IZ and CP 3 days post-electroporation. Some cells within the IZ maintained a morphology characteristic of migrating neurons; however, other cells appeared to be extending dendrites and axons as part of the growing SBH (Fig. 7C and Fig. 8b', c', d'). Similar to wildtype, GFP⁺ cells in the CP elaborated apical dendrites and basal axons. Surprisingly, despite the abundance of BrdU⁺ cells in the IZ and CP of *tish*^{-/-} neocortex, no GFP⁺ cells were observed to incorporate BrdU (Fig. 7C–D, n = 3–5 sections from each of 4 embryos). These results indicate that VZ daughter cells born at E16.5 in *tish*^{-/-} neocortex were able to arrest their cell cycles before migrating into the IZ, suggesting that VZ born cells at this age do not seed the heterotopic proliferative zone in the IZ/CP. These data also suggest that errors in interkinetic nuclear migration at mid-neurogenesis do not underlie the progenitor cell positioning defect, because BrdU⁺ proliferative cells in the *tish*^{-/-} IZ do not maintain ventricle-contacting processes that can be electroporated at E16.5. Instead, heterotopic proliferative cells are either produced from a source other than the pallial VZ, or they are produced by the pallial VZ early in neurogenesis (before E16.5) and migrate into the IZ/CP without exiting the cell cycle, thus seeding the new proliferative zone.

The normally-positioned proliferative zone in *tish*^{-/-} neocortex produces neurons for both the cortical plate and heterotopia

In light of the finding that GFP⁺ cells were found in the CP of *tish*^{-/-} neocortex at 3 days post-electroporation, we decided to examine the distribution of electroporated cells across the cortical wall in greater depth. In previous work from our laboratory, we had hypothesized that normally-positioned progenitors in the *tish*^{-/-} neocortex produce neurons destined for the heterotopia, while abnormally-positioned progenitors produce neurons for both the CP and the heterotopia (Lee *et al.*, 1998). Our goal was to test this hypothesis using *in utero* electroporation to trace the origins of CP and SBH neurons.

Embryos were electroporated at E16.5 and examined three days post-electroporation. In wildtype embryos, GFP⁺ cells were detected in developmental zones across the depth of the neocortex, and many cells could be identified largely on the basis of their morphology. GFP⁺ cells in the VZ maintained a radial morphology with apical and basal processes characteristic of parental RGCs. GFP⁺ cells in the SVZ possessed a multipolar morphology, indicative of neurons in phase two of radial migration, which are known to arrest in the SVZ before continuing toward the CP (Noctor *et al.*, 2004), and IPCs (Fig. 8A and data not shown). Within the IZ, GFP⁺ cells possessed a bipolar morphology with a long leading process, indicative of migrating neurons (Fig. 8A, a', open arrowheads). In the CP, GFP⁺ cells were arranged in laminae beneath the pial surface, and they extended a ramified apical dendrite as well as a basal axon that could, in many cases, be traced into the IZ (Fig. 8A, a'', arrows).

In the *tish*^{-/-} neocortex, similar to wildtype, GFP⁺ cells were detected across the depth of the neocortex. GFP⁺ cells within the VZ maintained a radial morphology with apical and basal processes characteristic of parental RGCs. GFP⁺ cells within the SVZ possessed a multipolar morphology, indicative of either neurons in phase two of radial migration or IPCs (Fig. 8B and data not shown). Interestingly, GFP⁺ cells were also located within the developing heterotopia amid the axons of the IZ and within the normally positioned CP (Fig. 8B, b', b''). Many of these cells in both locations could be identified as neurons based on the presence of a ramified apical dendrite and a basal axonal projection that, in some cases, could be traced into the IZ (Fig. 8b', b'', c', c'', arrows). Some GFP⁺ neurons within the heterotopia deviated from a normal orientation. Instead, these cells were oriented at angles which, in the most severe cases, caused them to align themselves parallel to the ventricular surface (Fig. 8b', c', closed arrowheads). In one instance, a misaligned, heterotopic neuron was observed to project a ramified dendrite medially, while its axon coursed laterally before looping back medially and entering the white matter directed toward the contralateral hemisphere (Fig. 8D, d', d'', a * indicates the cell body, closed arrowheads trace the axon). Moreover, GFP⁺ cells that resembled migrating cells with a long leading process and trailing cell body were observed in the heterotopia and CP (Fig. 8b', c', open arrowheads). In some instances, these cells appeared to be migrating as closely apposed clusters rather than as individual, spread-out entities. Taken together, these data suggest that the normally-positioned proliferative zone produces neurons that are destined for both the CP and the heterotopia. Given that abnormally-located *tish*^{-/-} progenitors are not generated by the VZ compartment at E16.5 and thus could not have produced neurons for the CP and heterotopia, it seems most plausible to conclude that, at E16.5, normally-positioned RGCs produced daughter cells either directly or indirectly via IPCs that differentiated into neurons and migrated into both locations.

Radial glial fibers are disrupted in the *tish*^{-/-} SBH

In light of the finding that the normally-positioned proliferative zone in *tish*^{-/-} neocortex produces neurons that can arrive successfully in the CP, we reasoned that the radial glial scaffold must remain at least partially intact in order for neurons to traverse the growing SBH and reach the CP. In order to investigate the integrity of the radial glial scaffold, we utilized immunohistochemistry against the intermediate filament protein vimentin, which is present in progenitor cells of the neocortex as well as in the long radial fibers of radial glial cells. We utilized sections from E19.5 neocortex from wildtype and *tish*^{-/-} animals for examination, which corresponds to the same developmental timepoint examined in the previous electroporation experiments (see Figure 8). In wildtype neocortex, vimentin positive cells were concentrated in the VZ/SVZ and their radial fibers extended through the IZ to the pial surface (Figure 9A, a', a''). These fibers were organized into dense, radial arrangements in the IZ (Figure 9a'), which terminated in punctate endfeet at the pial surface

(Figure 9a''). In *tish*^{-/-} neocortex, vimentin positive cells were concentrated in the VZ/SVZ as in wildtype; however, radial fibers were less dense in the region of the SBH. Vimentin-positive fibers within the SBH also lacked the tight radial arrangement of wildtype fibers, appearing more wavy and disorganized, with interspersed vimentin-positive cell bodies corresponding to mislocalized progenitor cells (Figure 9B, b', cell bodies indicated by arrows). Interestingly, despite the disorganization present in the region of the SBH, radial fibers in the *tish*^{-/-} CP appeared more similar to wildtype in terms of density, organization, and endfoot formation (Figure 9b''). These results indicate that radial fibers in *tish*^{-/-} neocortex are capable of penetrating the SBH and reaching the pial surface, albeit after a disorganized course through the developing SBH (Figure 9b'').

Discussion

During neocortical development, daughter cells born in the VZ/SVZ must migrate toward the pial surface, exit the cell cycle, and initiate differentiation. While the mechanisms coordinating these processes are still being elucidated, multifunctional proteins such as Neurog2, p27^{kip1}, p57^{kip2}, and Rb play important roles (Britz et al., 2006; Ferguson et al., 2002; Heng et al., 2008; Itoh et al., 2007; Kawauchi et al., 2006; Miyata et al., 2004; Nguyen et al., 2006). Despite this coordination, these processes can be uncoupled, suggesting that cells can migrate out of proliferative compartments without exiting the cell cycle (Lobjois et al., 2008; Zindy et al., 1999). Our results demonstrate that: 1) the *tish* mutation disrupts the positioning and number of neural progenitor cells in the developing neocortex, 2) the mislocalized progenitors in *tish*^{-/-} neocortex exhibit altered cell cycle kinetics, and 3) migration out of the VZ/SVZ is partially disrupted by the *tish* mutation such that daughter cells are incorporated into both the SBH and CP. Taken together, these results highlight the importance of proper regulation of neural progenitor cell positioning and number during neocortical development and indicate that errors in the regulation of these processes can contribute to SBH formation.

The *tish* mutation dysregulates progenitor cell positioning and number

Our findings demonstrate that the developing *tish*^{-/-} neocortex contains mislocalized progenitors of the RGC and IPC lineage in the IZ/CP. These cells are present during early CP neurogenesis and persist through E20. Moreover, supernormal numbers of these progenitors are produced from E17 to E20 in the *tish*^{-/-} neocortex. Inasmuch as mislocalized proliferating cells are not found in animal models of SBH caused by mutations in neuronal migration genes, we further investigated the potential cellular mechanisms underlying progenitor cell mislocalization.

Previous studies have indicated that the loss of function of adherens junctions in RGCs leads to heterotopic mitoses, although SBH formation has not been reported in these studies (Cappello *et al.*, 2006; Imai *et al.*, 2006; Lien *et al.*, 2006). Our results indicate that apical adherens junctions and polarity are maintained in the RGCs of *tish*^{+/-} and *tish*^{-/-} neocortices, suggesting that adherens junction loss is not responsible for progenitor cell mislocalization in the *tish* rat. Thus, we employed *in utero* electroporation of a GFP plasmid at E14.5 and E16.5 in order to assess the birthplace and morphology of *tish*^{-/-} progenitors.

With regard to the birthplace of mislocalized progenitors, we found that these cells are not produced in the VZ/SVZ at E16.5; however, we were unable to determine whether they are produced in the VZ/SVZ at an earlier timepoint (E14.5) due to the high mortality rate at this age. It is known that *tish*^{-/-} rats suffer from hydrocephalus (Lee *et al.*, 1998; Lee *et al.*, 1997). Related to this hydrocephalus is prominent polyhydramnios, or increased amniotic fluid levels and intra-amniotic pressure, during development. It is known that *in utero* electroporation using similar methodological parameters is safe and effective in E15 rat

embryos (Bai et al., 2003; Rosen et al., 2007). Therefore, we believe that the increase in E14.5 *tish*^{-/-} embryo mortality is due to the injection process itself, causing excessive amniotic fluid leakage in *tish*^{-/-} amniotic sacs that contain a higher intra-amniotic pressure. Such an increase in mortality was not observed in E14.5 wildtype embryos, which supports this explanation. Future studies will consider alternative approaches for labeling E14.5 progenitors, including injection of replication incompetent retrovirus.

We chose the E14.5 and E16.5 timepoints on the basis of two hypothetical scenarios. First, it is possible that heterotopic *tish*^{-/-} progenitors are generated throughout development by the VZ/SVZ and migrate to the IZ/CP after birth. If so, we would expect to find BrdU⁺/GFP⁺ cells in the IZ/CP of *tish*^{-/-} neocortex after electroporation at both E14.5 and E16.5. On the other hand, heterotopic *tish*^{-/-} progenitors might be generated by the VZ/SVZ very early during neocortical neurogenesis and possess the ability to self-renew, which would support formation of the heterotopic proliferative zone without further contribution from the VZ/SVZ. In this case, we would expect to find BrdU⁺/GFP⁺ cells in the IZ/CP of *tish*^{-/-} neocortex after electroporation at E14.5 but not E16.5. Our results are more consistent with the latter hypothesis.

With regard to the morphology of mislocalized *tish*^{-/-} progenitors, we reasoned that if BrdU⁺/GFP⁺ nuclei were present in the *tish*^{-/-} IZ/CP and possessed apical and basal processes spanning the depth of the cortical wall, then we could conclude that an error in nuclear migration led to their mislocalization. Alternatively, if BrdU⁺/GFP⁺ nuclei were found in the *tish*^{-/-} IZ/CP without intact ventricle-contacting processes, then we could conclude that mislocalized *tish*^{-/-} progenitors were born from the VZ/SVZ and that they migrated to the IZ/CP while continuing to proliferate. However, it was not possible to directly assess the morphology of heterotopic progenitors because no GFP⁺/BrdU⁺ cells were observed in *tish*^{-/-} IZ/CP. This finding indicates that the mislocalized progenitors do not retain contact to the lateral ventricle. It also indicates that VZ daughter cells born at E16.5 in *tish*^{-/-} neocortex are able to arrest their cell cycles before migrating into the IZ, further supporting the concept that VZ born cells at this age do not seed the heterotopic proliferative zone in the IZ/CP.

When considering these birthplace and morphology data together, it is most parsimonious to conclude either that mislocalized progenitors are derived from a source other than the pallial VZ/SVZ or, more likely, that they originate from the pallial VZ/SVZ prior to E16.5, migrate into the IZ/CP, and retain mitotic competency. While our results cannot directly exclude the possibility of a non-pallial source for heterotopic progenitors, their expression of Pax6 and Tbr2, markers of pallial progenitors, renders this explanation unlikely (Hevner, 2006).

Mislocalized *tish*^{-/-} progenitors exhibit dysregulated cell cycle kinetics

The observed increase in progenitor cell density in *tish*^{-/-} neocortex raised the possibility that the cell cycle kinetics of RGCs and IPCs are disturbed. Our findings demonstrate a shortened cell cycle length in mislocalized *tish*^{-/-} progenitors, due primarily to a shortening of G₁+M+G₂ phases. A short cell cycle length, particularly a short G₁ phase, is associated with continued proliferation rather than cell cycle exit and differentiation (Calegari *et al.*, 2005; Calegari and Huttner, 2003). Thus, the shortened G₂+M+G₁ and total cycle lengths in mislocalized progenitors suggest an increase in self-renewing divisions, which would lead to more cells continuing to express Pax6 and Tbr2, as we observed. It is important to recognize that the IdU/BrdU assay does not allow the discrimination of changes in G₂+M, or G₁ as more detailed BrdU serial injection protocols would permit (Takahashi *et al.*, 1993). Nonetheless, the IdU/BrdU assay has been validated against serial BrdU injection methods (Quinn *et al.*, 2007), and the cell cycle values we obtained for wildtype rats in the present

study are similar to published values for wildtype mice at comparable developmental stages (Miyama *et al.*, 1997; Takahashi *et al.*, 1995; Tarui *et al.*, 2005).

Other questions raised by our results include how progenitor cell positioning regulates cell number and what role the environmental setting plays in this process. This is a particularly intriguing issue in light of our finding that normally-positioned Pax6⁺ and Tbr2⁺ progenitors in the *tish*^{-/-} neocortex behave similarly to wildtype progenitors in terms of their cell cycle kinetics, while mislocalized progenitors do not (see Figure 5). It is known that control of proliferation and coordination of cell cycle behavior is mediated by growth factors, Notch signaling, gap junction coupling, and purinergic signaling (Arsenijevic, 2003; Bittman *et al.*, 1997; Del Bene *et al.*, 2008; Mishra *et al.*, 2006; Weissman *et al.*, 2004). It is intriguing to postulate that the mislocalization of progenitor cells could disrupt critical cell-cell junctions and exposure to mitogenic signals, thus altering cell cycle kinetics. In this way, cell cycle dysregulation in mislocalized progenitors may reflect a consequence of cellular mislocalization rather than a cell-autonomous effect of the *tish* mutation.

Migration out of the VZ/SVZ is partially disrupted by the *tish* mutation

In previous work from our laboratory, we hypothesized that normally-positioned progenitors in the *tish*^{-/-} neocortex produce neurons destined for the heterotopia, while abnormally-positioned progenitors produce neurons for both the CP and the heterotopia (Lee *et al.*, 1998). Our current findings using *in utero* electroporation challenge this hypothesis by demonstrating that VZ/SVZ proliferative cells in the *tish*^{-/-} neocortex contribute offspring both to the developing SBH and to the CP. Importantly, this finding indicates that many daughter cells born in the VZ/SVZ are capable of migrating through the developing SBH and the heterotopic proliferative zone en route to an appropriate destination in the CP. In order to migrate through the SBH, young *tish*^{-/-} neurons must possess a substrate on which to migrate, contain functional migrational machinery, and interact with appropriate guidance cues. Our current findings using vimentin immunohistochemistry demonstrate that the migratory substrate in *tish*^{-/-} neocortex is moderately impaired in the SBH but is relatively preserved in the CP. Thus, young neurons may become mislocalized in the IZ secondary to a loss of migratory substrate; however, those neurons that succeed in finding a radial fiber that contacts the pial surface may succeed in reaching the CP.

Role of mislocalized proliferative cells in SBH formation

While we have demonstrated that VZ/SVZ progenitors contribute neurons to both the SBH and CP in *tish*^{-/-} neocortex, it remains to be clarified whether mislocalized progenitor cells also do, or whether they interfere with migration out of the VZ/SVZ in another way. Neuronal migration out of the heterotopic proliferative zone could be influenced by the migratory substrate and diffusible signals both superficial and deep to the proliferating cells. In addition, the absence of ventricular attachments in these progenitor cells, which we demonstrated in our *in utero* electroporation experiments, may disturb intrinsic signaling mechanisms required for proper orientation and/or appropriate directional migration. However, mislocalized *tish*^{-/-} progenitors may also interfere with existing guidance cues in the environment, or they may produce aberrant guidance cues of their own. Thus, mislocalized *tish*^{-/-} progenitors may serve a secondary role in SBH pathogenesis when they reach a critical number by disrupting otherwise normal migration out of the VZ/SVZ.

To what extent SBH formation in the *tish*^{-/-} neocortex is the result of a cell-autonomous effect of the *tish* mutation on neuronal migration or a non-cell-autonomous effect of heterotopic progenitors on otherwise normal migrating neurons remains to be determined. There exist precedents for both cell-autonomous and non-cell-autonomous effects on cellular positioning in animal models of cortical heterotopia in which migration-related

molecules have been manipulated (Bai *et al.*, 2003; Hammond *et al.*, 2001). Thus, an important remaining goal for future research will be to delineate the relative roles of cell-autonomous and non-cell-autonomous factors in tish SBH pathogenesis. Elucidation of these roles could both clarify potential mechanisms contributing to the formation of certain types of SBH in humans and further characterize fundamental mechanisms underlying neocortical development.

Abbreviations

CP	cortical plate
IPC	intermediate progenitor cell
IZ	intermediate zone
RGC	radial glial cell
SBH	subcortical band heterotopia
SVZ	subventricular zone
VZ	ventricular zone

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Figure 1.

Pax6⁺ and Tbr2⁺ cells are incorrectly positioned in the tish^{+/-} and tish^{-/-} neocortex. Light microscopic images were taken of coronal sections of embryonic neocortex during timepoints corresponding to early, middle, at late cortical plate neurogenesis. **A–C**, Early during cortical plate neurogenesis (E15), clusters of inappropriately positioned Pax6⁺ cells can be found in a superficial position in the vicinity of the preplate (PP) of the tish^{-/-} neocortex (arrows). These cells are normally restricted to the ventricular zone (VZ) in wildtype and tish^{+/-} animals. **D–F**, Similarly, scattered Tbr2⁺ cells can be found near the preplate (PP) of the tish^{-/-} neocortex (arrows) rather than in the developing subventricular zone (SVZ) as in wildtype and tish^{+/-} neocortex. **G–I**, During the middle stage of cortical plate neurogenesis (E17), a diffuse band of inappropriately positioned Pax6⁺ cells is observed in the intermediate zone (IZ) and cortical plate (CP) of tish^{-/-} neocortex (arrows). Some Pax6⁺ cells are also observed in the IZ/CP of tish^{+/-} neocortex, though their numbers are substantially fewer than in tish^{-/-} neocortex (arrowheads). These cells are normally restricted to the VZ in wildtype neocortex. **J–L**, Similarly, a diffuse band of inappropriately positioned Tbr2⁺ cells can be observed in the intermediate zone (IZ) and cortical plate (CP) of tish^{-/-} neocortex (arrows), while some Tbr2⁺ cells are also observed in the IZ/CP of tish^{+/-} neocortex, though their numbers are substantially fewer (arrowheads). **M–O**, Late in cortical plate neurogenesis (E20), the diffuse band of inappropriately positioned Pax6⁺ cells persists in the IZ/CP of tish^{-/-} neocortex (arrows). **P–R**, Similarly, the diffuse band of inappropriately positioned Tbr2⁺ cells persists in the IZ/CP of tish^{-/-} neocortex at E20 (arrows). Scale bars = 100μm.

Figure 2.

Incorrectly positioned Pax6⁺ cells in the *tish*^{-/-} neocortex are mitotic progenitor cells. Confocal images were taken of coronal sections of embryonic neocortex at E17. In **E**, **J**, and **O**, compressed stacks are presented. Vertical and horizontal lines correspond to optical planes of section through the depth of the stack. Three-dimensional reconstructions of these regions are shown in the sidebars of **E**, **J**, and **O**. **A–E**, Actively cycling Pax6⁺/BrdU⁺ cells can be observed in an appropriate position in the VZ of wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices, as well as in an inappropriate position in the *tish*^{-/-} CP (arrows indicate representative double labeled cells). **F–J**, Mitotic Pax6⁺/pvim⁺ cells can be found at the ventricular surface in wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices; however, Pax6⁺/pvim⁺ cells can also be found in the *tish*^{-/-} CP (arrows indicate representative double labeled cells). **K–O**, Appropriately positioned Pax6⁺/β-III tubulin⁺ cells can be observed in the VZ of wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices. Notably, the inappropriately positioned Pax6⁺ cells in the *tish*^{-/-} CP are also β-III tubulin⁺ confirming that they are not immature neurons. Scale bars = 100μm in **A–D**, **F–I**, **K–N**; 10μm in **E**, **J**, **O**.

Figure 3.

Incorrectly positioned $Tbr2^{+}$ cells in the $tish^{-/-}$ neocortex are mitotic progenitor cells. Confocal images were taken of coronal sections of embryonic neocortex at E17. In **E**, **J**, and **O**, compressed stacks are presented. Vertical and horizontal lines correspond to optical planes of section through the depth of the stack. Three-dimensional reconstructions of these regions are shown in the sidebars of **E**, **J**, and **O**. **A–E**, Actively cycling $Tbr2^{+}/BrdU^{+}$ cells can be observed in an appropriate position in the SVZ in wildtype, $tish^{+/-}$, and $tish^{-/-}$ neocortices, as well as in an inappropriate position in the $tish^{-/-}$ CP (arrows indicate representative double labeled cells). **F–J**, Mitotic $Tbr2^{+}/pvim^{+}$ cells can be found at the ventricular surface in wildtype, $tish^{+/-}$, and $tish^{-/-}$ neocortices; however, $Tbr2^{+}/pvim^{+}$ cells can also be found in the $tish^{-/-}$ CP (arrows indicate representative double labeled cells). **K–O**, Appropriately positioned $Tbr2^{+}/\beta$ -III tubulin $^{-}$ cells can be observed in the VZ of wildtype, $tish^{+/-}$, and $tish^{-/-}$ neocortices. Importantly, the inappropriately positioned $Tbr2^{+}$ cells in the $tish^{-/-}$ CP are also β -III tubulin $^{-}$ confirming that they are not immature neurons. Scale bars = 100 μ m in **A–D**, **F–I**, **K–N**; 10 μ m in **E**, **J**, **O**.

Figure 4.

Progenitor cell density and cellular apoptosis are increased in the *tish*^{-/-} neocortex. **A**, Pax6⁺ cell density, measured in radial columns from the ventricular to pial surfaces, is increased in *tish*^{-/-} neocortex as compared to wildtype and *tish*^{+/-} neocortex beginning on E17 and persisting through E20. **B**, Tbr2⁺ cell density, measured similarly, is also increased in *tish*^{-/-} neocortex as compared to wildtype and *tish*^{+/-} neocortex beginning on E17 and persisting through E20. **C**, Examination of activated caspase 3 positive cells in the VZ/SVZ and IZ/CP of wildtype, *tish*^{+/-}, and *tish*^{-/-} neocortices revealed that there was an increased amount of cell death in the IZ/CP of *tish*^{-/-} neocortex. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal-Wallis One-way ANOVA with Tukey's multiple comparisons testing or One-way ANOVA with Holm-Sidak multiple comparisons testing.

Figure 5.

Cell cycle kinetics are altered in *tish*^{-/-} neocortical progenitor cells. **A**, No significant differences in the percentage of time spent in S phase (T_s/T_c) were found among Pax6⁺ cells at E17 in the wildtype, n-*tish*^{-/-}, and h-*tish*^{-/-} neocortices. However, at E20, h-*tish*^{-/-} cells exhibited an increased T_s/T_c , while n-*tish*^{-/-} cells exhibited a T_s/T_c that was similar to wildtype. **B**, Pax6⁺ cell cycle length was similar among the groups at E17 and E20; however, at E20, h-*tish*^{-/-} cells exhibited a non-significant trend toward a shorter cell cycle time than the other groups. **C**, The length of S phase of Pax6⁺ cells was not significantly altered among groups at either E17 or E20, although there was a non-significant trend for a more prolonged S phase in h-*tish*^{-/-} cells at E20. **D**, The length of G₂+M+G₁ in Pax6⁺ cells was

unchanged at E17; however, at E20, h-tish^{-/-} cells had a significantly shorter G₂+M+G₁ length than both n-tish^{-/-} and wildtype. **E**, At E17 and E20, h-tish^{-/-} Tbr2⁺ cells exhibited an increase in T_s/T_c compared with wildtype and n-tish^{-/-} cells. **F**, Tbr2⁺ cell cycle length was shortened in h-tish^{-/-} cells compared with wildtype and n-tish^{-/-} cells both at E17 and E20. **G**, S phase length was similar among wildtype, n-tish^{-/-}, and h-tish^{-/-} Tbr2⁺ cells at E17 and E20, except for a single significant difference between wildtype and h-tish^{-/-} cells at E20. **H**, The length of G₂+M+G₁ was significantly decreased in h-tish^{-/-} Tbr2⁺ cells compared with wildtype and n-tish^{-/-} cells at E17 and E20. Data are presented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, One-way ANOVA with Holm-Sidak multiple comparisons test or Kruskal-Wallis One-way ANOVA with Dunn's multiple comparisons test.

Figure 6.

Radial glial adherens junctions and apical polarity domains are intact at the ventricular surface of E13, E15, and E17 *tish*^{-/-} neocortex. Confocal images were taken of coronal sections of embryonic neocortex at E13, E15, and E17. Immunohistochemistry against phalloidin to identify the adherens junction component F-actin (**A–F**) and against the apical polarity proteins aPKC-λ (**G–L**) and PAR3 (**M–R**) revealed no differences between wildtype and *tish*^{-/-} neocortex, suggesting that *tish*^{-/-} radial glia do not become mislocalized as a result of adherens junction or polarity loss. Scale bars = 10μm.

Figure 7.

Mislocalized *tish*^{-/-} progenitor cells are not produced by the pallial ventricular zone during the middle stage of cortical plate neurogenesis. Wildtype and *tish*^{-/-} embryos were electroporated with a pCAGGS-GFP construct at E16.5. The embryonic brains were examined at E19.5, i.e., 3 days after electroporation. The pregnant Dams were also administered a single injection of BrdU at 2 h prior to collecting the embryonic brains in order to label proliferating cells. Compressed confocal stacks of coronal sections of wildtype and *tish*^{-/-} neocortex are presented. No GFP/BrdU colocalization was observed in the IZ (A) or CP (B) of the wildtype neocortex, consistent with the idea that VZ/SVZ born cells exit the cell cycle before migrating into either location. Similarly, in *tish*^{-/-} neocortex, no GFP/BrdU colocalization was observed in the IZ (C) or CP (D), suggesting that the heterotopic proliferative cells are not generated by the pallial VZ at E16.5. A few profiles appeared to be double labeled in some sections (e.g. panel C); however, three-dimensional

reconstructions through the depth of the stack examining profiles of this type (c', c'') revealed that the BrdU and GFP staining were in adjacent cells rather than colocalized in the same cell. Scale bars = 50µm.

Figure 8.

The normally-positioned proliferative zone in $tish^{-/-}$ neocortex produces neurons for both the cortical plate and heterotopia. Wildtype and $tish^{-/-}$ embryos were electroporated with a pCAGGS-GFP construct at E16.5 and examined 3 days later. Compressed confocal stacks of coronal sections of wildtype and $tish^{-/-}$ neocortex are presented. **A**, Wildtype neocortex 3 days post-electroporation revealed GFP⁺ cells distributed across the depth of the cortex, with most cells clustered in the IZ and CP. **a'**, GFP⁺ cells in the wildtype IZ possessed the morphology of migrating neurons (open arrowheads). **a''**, GFP⁺ cells in the wildtype CP were primarily maturing neurons with an apical dendrite and basal axon that could, in many cases, be traced into the IZ (arrows). **B, C, D**, $Tish^{-/-}$ neocortex 3 days post-electroporation demonstrated GFP⁺ cells distributed across the depth of the cortex, with many cells clustered in the SBH in the IZ. **b', c'**, In the $tish^{-/-}$ IZ, some GFP⁺ cells were identified as neurons within the SBH (arrows), and some neurons were misaligned at angles that deviated from their expected orientation (closed arrowheads). Other GFP⁺ cells resembled migrating cells with a long leading process and a trailing cell body (open arrowheads). **b'', c''**. In the $tish^{-/-}$ CP, the majority of GFP⁺ cells were maturing neurons that were oriented appropriately, with apical dendrites and basal axons (arrows). **d', d''**, Some neurons in the $tish^{-/-}$ IZ were misaligned so severely that they were oriented parallel to the ventricular surface (* indicates soma, closed arrowheads trace an axon laterally then medially into an axon bundle, d' and d'' correspond to overlapping regions within the boxed area in D). Scale bars = 100 μ m in A–D, 50 μ m in a'–d', a''–d''.

Figure 9.

Radial glial fibers are disrupted in the *tish*^{-/-} neocortex. Confocal images were taken of 60μm thick coronal sections of wildtype and *tish*^{-/-} neocortex at E19.5, and compressed stacks are presented. **A**, Wildtype neocortex at E19.5 revealed vimentin⁺ cells concentrated in the VZ/SVZ with radial fibers extending through the IZ to the pial surface. **a', a''**, Vimentin⁺ fibers were organized into dense, radial arrangements in the wildtype IZ and terminated in punctate endfeet at the pial surface. **B**, *Tish*^{-/-} neocortex at E19.5 revealed vimentin⁺ cells concentrated in the VZ/SVZ as in wildtype; however, radial fibers appeared less dense in the *tish*^{-/-} SBH relative to the wildtype IZ (dashed lines outline the developing SBH). **b'**, Vimentin⁺ fibers in *tish*^{-/-} neocortex lacked the tight radial arrangement of wildtype fibers, appearing more wavy and disorganized in the SBH, with interspersed vimentin⁺ cell bodies corresponding to mislocalized progenitors (arrows). **b''**, Vimentin⁺ fibers appeared more normal in the CP and terminated in endfeet at the pial surface as in wildtype neocortex. Scale bars = 100 μm in A-B, 50 μm in a'-b', a''-b''.

Table 1

Cell cycle parameters (mean $\pm$ SEM) for RGC and IPC populations in wildtype, normotopic $tish^{-/-}$ (n- $tish^{-/-}$), and heterotopic $tish^{-/-}$ (h- $tish^{-/-}$) proliferative zones.

			T_f/T_c	T_c (h)	T_s (h)	T_s-T_c (h)
RGC	E17	wildtype	0.41 $\pm$ 0.015	22.30 $\pm$ 0.78	9.07 $\pm$ 0.42	13.22 $\pm$ 0.60
		n- $tish^{-/-}$	0.40 $\pm$ 0.010	22.07 $\pm$ 0.78	8.67 $\pm$ 0.34	13.39 $\pm$ 0.55
		h- $tish^{-/-}$	0.41 $\pm$ 0.009	20.42 $\pm$ 0.82	8.35 $\pm$ 0.35	12.06 $\pm$ 0.53
E20		wildtype	0.31 $\pm$ 0.012	35.31 $\pm$ 0.81	11.22 $\pm$ 0.53	24.08 $\pm$ 0.67
		n- $tish^{-/-}$	0.31 $\pm$ 0.026	33.07 $\pm$ 2.03	9.90 $\pm$ 0.62	23.17 $\pm$ 1.84
		h- $tish^{-/-}$	0.48 $\pm$ 0.026 *	28.40 $\pm$ 2.35	13.37 $\pm$ 1.31	15.02 $\pm$ 1.58 *
IPC	E17	wildtype	0.34 $\pm$ 0.013	41.61 $\pm$ 0.89	14.05 $\pm$ 0.42	27.56 $\pm$ 1.05
		n- $tish^{-/-}$	0.34 $\pm$ 0.015	45.12 $\pm$ 1.03 *	15.37 $\pm$ 0.49	29.76 $\pm$ 1.20
		h- $tish^{-/-}$	0.43 $\pm$ 0.019 ***	33.28 $\pm$ 1.32 ***	14.17 $\pm$ 0.66	19.11 $\pm$ 1.17 ***
		E20	0.29 $\pm$ 0.011	67.20 $\pm$ 1.09	19.27 $\pm$ 0.63	47.93 $\pm$ 1.38
		n- $tish^{-/-}$	0.24 $\pm$ 0.010	76.14 $\pm$ 1.83	18.00 $\pm$ 0.70	58.14 $\pm$ 1.18
		h- $tish^{-/-}$	0.47 $\pm$ 0.020 *	35.79 $\pm$ 2.05 *	17.04 $\pm$ 1.40 *	18.75 $\pm$ 1.19 *

*
p < 0.05
**
p < 0.01

p < 0.001