Neurological symptoms in tuberous sclerosis complex (TSC) and associated brain lesions are thought to arise from abnormal embryonic neurogenesis due to inherited mutations in \textit{Tsc1} or \textit{Tsc2}. Neurogenesis persists postnatally in the human subventricular zone (SVZ) where slow-growing tumors containing \textit{Tsc}-mutant cells are generated in TSC patients. However, whether \textit{Tsc}-mutant neurons from the postnatal SVZ contribute to brain lesions and abnormal circuit remodeling in forebrain structures remain unexplored. Here, we report the formation of olfactory lesions following conditional genetic \textit{Tsc1} deletion in the postnatal SVZ using transgenic mice or targeted single-cell electroporation. These lesions include migratory heterotopias and olfactory micronodules containing neurons with a hypertrophic dendritic tree. Most significantly, our data identify migrating glial and neuronal precursors that are re-routed and infiltrate forebrain structures (e.g. cortex) and become glia and neurons. These data show that \textit{Tsc1}-mutant cells from the neonatal and juvenile SVZ generate brain lesions and structural abnormalities, which would not be visible using conventional non-invasive imaging. These findings also raise the hypothesis that micronodules and the persistent infiltration of cells to forebrain structures may contribute to network malfunction leading to progressive neuropsychiatric symptoms in TSC.

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder that is associated with lesions in many different organs including in the kidney, skin, heart and brain. The incidence of TSC is estimated between 1:6000 and 1:10 000 individuals (1). TSC is caused by inactivating mutations in one of two tumor suppressor genes, \textit{Tsc1} and \textit{Tsc2}, which encode hamartin and tuberin, respectively, and lead to hyperactivity of the mammalian target of rapamycin (mTOR) (2). Although TSC affects many organ systems, the neurological symptoms (e.g. seizures, autism, psychiatric problems) cause the most significant disability and morbidity (3–6).

Neurological symptoms in TSC result from anomalies in brain development, in particular cortical neurogenesis, leading to an abnormal circuitry and the formation of brain lesions (7–16). These lesions are thought to occur \textit{in utero} when cortical neurons are generated and migrate to their final location (8). Indeed, cortical lesions have been detected as early as 19 weeks of gestation in humans (17) and were generated following \textit{Tsc1} deletion in embryonic neural progenitor cells and developing neurons (9). Neurogenesis, however, does not stop at birth, but persists in selective regions of the neonatal and adult brain in all species examined, including humans (18–21). This continual generation of new neurons confers plasticity at the cellular and circuit levels. It also raises the possibility that postnatal neurogenesis in individuals with TSC could contribute to specific brain lesions and abnormal circuit remodeling (7,22).

The subventricular zone (SVZ) along the lateral ventricle and the hippocampal subgranular zone of the dentate gyrus are sites of active neurogenesis throughout life (21,23). The SVZ contains the largest pool of neural progenitor cells in
the adult human brain and span the entire cerebrum (19,24–26). During the neonatal period, the SVZ generates both neurons and glia, and essentially neurons in adults (27). Newborn neuroblasts migrate to the olfactory bulb where they differentiate into interneurons (19,20,28). A subset of these neurons also migrates to cortical and subcortical structures most prominently during the neonatal period (29–31). Individuals with TSC display lesions (referred to as nodules or hamartomas) in the forebrain, such as the olfactory and basal ganglia structures (32–38). In addition, they display SVZ nodules and giant cell subependymal astrocytomas (SEGAs) that have been recently generated in mice by deleting Tsc1 in neonatal neurogenic progenitor cells (39–41). Collectively, these pieces of evidence suggest that Tsc1null neurons are continuously generated from the postnatal SVZ, but their contribution to olfactory lesions and alterations in surrounding neuronal circuits remain unexplored.

Using genetic tools including conditional transgenic mice and single-cell genetic targeting, we report that olfactory lesions (i.e. heterotopic and micronodules) are generated from the postnatal SVZ. In addition, we found a continual infiltration of cells, including neurons and glia, from the migratory stream to cortical and subcortical areas. This finding raises new questions regarding the contribution of micronodules and ectopic migration to progressive circuit disruptions during neonatal and perhaps adult life in patients with TSC and other genetic disorders associated with cortical malformation.

RESULTS

Neonatal Tsc1 deletion using nestin-CreERT2 mice leads to olfactory lesions and the presence of enlarged neurons in the cortex

To examine whether postnatal neurogenesis contributes to olfactory lesions, we used conditional transgenic mice as recently reported to induce SVZ nodules and SEGAs (41). Loss of heterozygosity in SVZ cells was achieved using mice expressing Tsc1 alleles flanked by LoxP sites (floxed, fl) crossed with nestin-CreERT2/R26R-YFP mice to generate Tsc1fl/fl/nestinCreERT2 mice (Fig. 1A) (12,42). These nestin-CreERT2 mice have been used to induce selective recombination in cells of the neurogenic zones in adults (43). In neonates, nestin is also expressed in astrocytes and progressively disappears as they mature and acquire glial fibrillary acidic protein (GFAP) in rodents. Upon tamoxifen injection in Tsc1fl/fl/nestinCreERT2, nestin-expressing cells, including SVZ cells and astrocytes, are expected to lose Tsc1 and express YFP. Control mice were Tsc1fl/wt/nestinCreERT2 (wild-type, wt).

Tamoxifen was injected at P7 (two injections) and brains were collected at postnatal day (P) 28. To examine whether recombination at the Tsc1 allele occurred, we prepared genomic DNA from the P28 cortex from Tsc1fl/fl/nestinCreERT2 mice. We detected a Tsc1-mutant allele band, suggesting successful recombination (n = 3, Fig. 1B). In addition, as recently reported, Tsc1fl/fl/nestinCreERT2 mice displayed GFAP- and RC2-rich SEGA-like lesions in the ventral SVZ (Fig. 1D, GFAP not shown, locations shown on the diagram in Fig. 1C) (41). RC2 is a radial glial cell marker (44).

Every Tsc1fl/fl/nestinCreERT2 mouse displayed small YFP+ lesions along the migratory tract taken by newborn neurons to reach the olfactory bulb, the rostral migratory stream (RMS) (n = 5, Fig. 1C and E, red circle). These lesions consisted of the accumulation of 5–20 YFP+ cells that were not observed in Tsc1fl/fl/nestinCreERT2 mice (Fig. 1E). Strikingly, YFP+ cells were also visible throughout the cortex in Tsc1fl/fl/nestinCreERT2 mice (Fig. 1F), in the AON, and around the anterior commissure (data not shown). Closer examination of these cells revealed the presence of bushy cells with an astrocytic morphology and cells with a pyramidal neuron morphology (Fig. 1G for neurons). While YFP+ astrocytes were also observed in the cortex of Tsc1fl/wt/nestinCreERT2 mice, YFP+ neurons were rarely detected (1–2 per section, data not shown).

These data suggest that lesions in the olfactory migratory tract can form postnatally. In addition, ectopic neurons were found in the cortex of Tsc1fl/fl mice. To identify whether cells in the lesions and the cortex were generated in the SVZ, we used an alternative approach, neonatal electroporation. This method also allows genetic manipulations of a small group of cells as opposed to global modification using transgenic mice. In addition, we used mice expressing an fl and a mutant Tsc1 allele (Tsc1fl/mut) because individuals with TSC inherit a viable and a mutant Tsc1 (or Tsc2) allele. Tsc1fl/wt mice (wt for wild-type) were control littermates.

Targeted Tsc1 deletion and mTOR activation in newborn neurons via neonatal electroporation

Neonatal electroporation allows precise targeting of plasmids into neural progenitor cells lining the lateral ventricle (45). These progenitor cells generate neurons that migrate to the olfactory bulb via the RMS and are synaptically mature by 3–4 weeks after birth (46). We used Tsc1 mice crossed with R26R-Stop-RFP mice (RFP, red fluorescent protein). In Tsc1fl/mut/RFP mice, Cre recombinase (Cre) expression is expected to lead to TSC1 loss and RFP expression (Fig. 2A). RFP+ neurons are thus expected to be Tsc1null while surrounding RFP− neurons are Tsc1haplo (haplo for haploinsufficient).

Cre- and GFP-encoding plasmid were electroporated into SVZ progenitor cells at P0–1 resulting in visible GFP fluorescence 1-day post-electroporation (Fig. 2B and C) (45,47). GFP allows to birth-mark neurons born during the first 7–10 days post-electroporation because GFP is diluted as cells divide while RFP is permanently expressed (45) (Fig. 2D). As a result, RFP+ but not GFP+ newborn neurons continuously accumulate in the circuit (Fig. 2E). To test for recombination at the Tsc1 allele, we prepared genomic DNA from P7 ipsilateral (i.e. containing RFP+ cells) and contralateral olfactory bulbs from Tsc1fl/fl/RFP mice. A Tsc1 mutant allele band was detected only in the ipsilateral olfactory bulb, suggesting that Tsc1 was removed in RFP+ cells (n = 3, Fig. 2F). Next, we performed reverse transcription polymerase chain reaction (RT-PCR) for Tsc1 and western blot analysis from P28 olfactory bulb (OB) when Tsc1null neurons have accumulated in the OB.
for over 3 weeks, which increase our chance to detect changes in mRNA and protein levels. End-point and quantitative RT–PCR for \( Tsc1 \) illustrated that there was a 30–40% decrease in \( Tsc1 \) mRNA in the ipsilateral versus the contralateral \( Tsc1^{fl} \) olfactory bulb (\( n = 3, \) P28, Fig. 2G). Western blot illustrates a decrease in TSC1 (hamartin) expression in \( Tsc1^{fl/mut} \) compared with \( Tsc1^{fl/wt} \) ipsilateral olfactory bulbs (Fig. 2H).

mTOR hyperactivity results in elevated phosphorylation of ribosomal protein S6 (pS6) and 4E-BP. We immunostained for pS6 (Ser 240/244) in P28 olfactory bulb sections. In \( Tsc1^{fl/mut} \) mice, RFP+ neurons displayed a significant 115% increase in pS6 staining intensity compared with surrounding RFP- neurons (\( P < 0.001, \) Fig. 3A–D). In contrast, 4E-BP phosphorylation was not altered (data not shown). There was

Figure 1. Postnatal deletion of \( Tsc1 \) using inducible transgenic mice. (A) The diagram illustrating the inducible transgenic mouse line used to delete \( Tsc1 \) (i.e. null) and express YFP in nestin-expressing cells and their progeny following tamoxifen injection at P7. (B) PCR gels from genomic DNA obtained from a P28 olfactory bulb following tamoxifen at P7. A mutant band in addition to the fl band is visible. (C) The diagram illustrating the locations of the images shown in (D) (ventral SVZ, green square) and in (E) (RMS, green square, for \( Tsc1^{B^{fl}} \) and black square for \( Tsc1^{B^{wt}} \)) on a sagittal section. LV, lateral ventricle; SVZ, subventricular zone; OB, olfactory bulb; RMS, rostral migratory stream. (D) The P28 image of the RC2 (red)-rich SVZ nodule containing YFP+ \( Tsc1^{fl/mut} \) cells following tamoxifen-induced recombination at P7. (E) Confocal images of YFP-immunopositive cells in the RMS/RMS-OB of \( Tsc1^{B^{wt}/nestin^{CreERT2}} \) and RMS of \( Tsc1^{B^{Fl}/nestin^{CreERT2}} \) following tamoxifen injections at P7. The dotted orange lines highlight small clusters of misplaced cells in the RMS. (F and G) Confocal images of YFP+ cells (low and high magnifications) in the cortex of \( Tsc1^{B^{wt}/nestin^{CreERT2}} \) mice. Scale bars: 70 \( \mu \)m (D, E and G) and 200 \( \mu \)m (F).
no difference in pS6 staining intensity in RFP+ compared with RFP− neurons from Tsc1fl/wt mice (data not shown). Another well-known consequence of mTOR hyperactivity is an increase in cell size that was visible in Tsc1null neurons (Fig. 3C). RFP and 4′,6-diamidino-2-phenylindole (DAPI) counterstain were used to quantify soma and nuclei size in Tsc1fl/mut mice (Fig. 3E and F). RFP+ Tsc1null neurons were ≈3 times larger than RFP− neurons and their nuclei 55% larger (P < 0.001, Fig. 3G). Collectively, neonatal electroporation is an effective method to induce Tsc1 deletion in newborn neurons leading to mTOR pathway activation and cytomegally.

**Tsc1null cells form the migratory heterotopia in and out of the RMS**

Gross examination of sagittal sections from P28 Tsc1fl/mut mice revealed the presence of the migratory heterotopia, defined by the ectopic location or misplacement of groups of Tsc1null cells. Examination of sagittal sections at low magnification led us identification of the heterotopia in all animals examined (n = 13) that were found at three major locations: in the RMS both at its entry point caudally and at the interface RMS and RMS-OB, in the RMS-OB and adjacent to the RMS in the AON as well as near the anterior commissure olfactory limb (Fig. 4A and B, black rectangles indicate the location of the heterotopia). No migratory heterotopias were visible in electroporated Tsc1fl/wt/R26R mice (data not shown).

Closer examination of the heterotopia revealed two categories, some with an apparent mixed neuroglial phenotype (Fig. 4C–G) and some with a neuronal phenotype (Fig. 4H and I). The mixed neuroglial phenotype was visible due to the presence of a meshwork of lamelipodia-like processes giving a nodular appearance or the presence of cells with neuronal or glial morphology (Fig. 4C). Heterotopia with a lamelipodia-like structure was preferentially found in the AON as shown in Figure 4B and C. Heterotopia in the RMS and RMS-OB displayed cells with an astrocytic (bushy), a neuronal morphology or an undifferentiated morphology.
Some cells stained for the astrocytic marker GFAP (data not shown). Cells with a neuronal morphology displayed extensive dendrites and a thin process identified as an axon by the presence of varicosities (white arrows, Fig. 4E–G). Cells forming the heterotopia displayed enlarged cells body compared with surrounding cells, in particular doublecortin (DCX)-immunopositive neuroblasts in the RMS (Fig. 4F and G, DCX staining not shown).

Mixed phenotype–heterotopia comprised GFP and GFP−/RFP+ cells. In wt mice, GFP+ cells, which are born between P0 and P10, should have reached the olfactory bulb by P28 (see 45 for more details). Indeed in Tsc1fl/wt/R26R mice, only 8% of the RFP+ cells were GFP+ in the RMS (23/250 cells, n = 3 mice). In contrast, we found that 22% of Tsc1fl null RFP+ cells were GFP+ cells in the RMS (280/1092 cells, n = 3, P < 0.0001, Fisher’s exact test). Thus, heterotopia contained early born GFP+ cells that are stalled in the RMS and may thus trap late born cells as well as disrupt their migratory path.

Heterotopia comprised of 3−15 cytomegalic neurons is also visible along the RMS-OB and more specifically, in the anterior commissure olfactory limb (Fig. 4H and I). These cells displayed a neuronal morphology and were NeuN and pS6 immunopositive (data not shown). The majority of these ectopic neurons were GFP+ and thus early born (Fig. 4H).

**Tsc1null cells in the RMS display abnormal morphology and are slow or stalled**

Outside the heterotopia, the RMS of P28 Tsc1fl/mut/R26R mice appeared more disorganized compared with that of Tsc1fl/wt/R26R mice (Fig. 5A and B). This appearance was due to the presence of cells with a more complex morphology and enlarged soma scattered along the RMS (arrows, Fig. 5B). As a mean, Tsc1null cells had one more process than Tsc1haplo cells (a mean of 2.2 ± 0.2 versus 1.4 ± 0.1, P < 0.05, Fig. 5C). Complex Tsc1null cells were DCX−, while morphologically simpler cells were DCX+ identifying them as neuroblasts (data not shown).

Next, we performed a migration assay in acute sagittal slices from the RMS of P21 mice (45,47). Tsc1haplo neuroblasts migrated at a 54.4 ± 2.2 μm/h, which is similar to a previously reported value for neuroblast migration in wt mice (47–49). Tsc1null neuroblasts migrated at 37.1 ± 2.5 μm/h and were thus 33% slower than Tsc1haplo cells (128 and 83 cells, P < 0.0001, Fig. 5D). Tsc1null cells with a complex morphology were stationary.

We also found scattered Tsc1null giant cells characterized by a soma size 3−4 times larger than that of other Tsc1null cells (Fig. 5E). These cells had a disheveled morphology, were NeuN negative and contain multiple nuclei (Fig. 5E−G). Two nuclei can be appreciated in Figure 5G and H. A mean of five giant cells were found per RMS of Tsc1fl/mut/R26R mice, while none was seen in the RMS of Tsc1fl/wt/R26R mice.

**Micronodules throughout the olfactory bulb containing neurons with a hypertrophic dendritic tree**

Upon entering the olfactory bulb, the majority of newborn neurons integrate in the granule cell layer. Ultimate circuit formation depends on proper cell placement and morphogenesis. We thus examined the overall organization of granule cells and their dendritic arbor. In coronal olfactory bulb sections, we performed nearest neighbor calculations in which the overall cell distribution was disrupted in Tsc1fl/mut/R26R compared with Tsc1fl/wt/R26R mice (Fig. 6A and B). This is in addition to obvious increase in the size of Tsc1null cells as measured in Figure 2. Perhaps, more visible in sagittal sections was the apparent clumping of cytomegalic Tsc1null cells (Fig. 6D), while Tsc1haplo cells were equally distributed (Fig. 6C). To quantify the disorganization of granule cells, we performed nearest neighbor calculations in which the...
density of cells and the distance between each were measured (Fig. 6E). These measurements were compared with a theoretical Poisson distribution. The difference between the theoretical and experimental values in \( Tsc1^{fl/mut} \) mice was significantly lower than that in \( Tsc1^{fl/wt} \) mice (\( R = 4.2 \pm 1.2, n = 10 \) versus \( R = 9.0 \pm 1.5, n = 12 \) sections, Fig. 6F), suggesting a decreased organization in the \( Tsc1^{fl/mut} \) olfactory bulb (\( P < 0.05 \)).

Another observation in coronal sections was the hypertrophic dendritic tree of \( Tsc1^{null} \) granule neurons (Fig. 7A). Using Scholl analysis of the proximal dendrites, we found that the number of dendritic crossings was increased in \( Tsc1^{null} \) compared with \( Tsc1^{haplo} \) neurons (Fig. 7B).

### Newborn neurons are rerouted to cortical and subcortical areas

Considering that cortical neurons were observed in the \( Tsc1^{fl/wt} \) brain, we examined for the presence of neurons in cortical and subcortical structures in coronal sections. In all \( Tsc1^{fl/mut} \) brains examined, we found GFP+ neurons in the nucleus accumbens and in the cortex (Fig. 8). The majority of neurons were GFP+ suggesting that they were born during the neonatal period. Such neurons were absent in the \( Tsc1^{fl/wt} \) brain or very rare (1 per section occasionally). In the nucleus accumbens, neurons were located at the base of the lateral ventricle and around the anterior commissure.
(Fig. 8B and C). GFP+ cells were identified as neurons based on NeuN immunostaining. In the cortex, 10–15 neurons were found per sections. Neurons occurred in clusters of 2–6 and were located in the deep cortical layers and in layer II (Fig. 8D–I). Cortical neurons display an extensive dendritic tree with spines as well as a thin process with varicosities resembling an axon.

DISCUSSION

Here, we show that focal Tsc1 knockout in the postnatal SVZ results in mTOR pathway activation and migratory alterations characterized by the heterotopia along the migratory path, olfactory bulb disorganization and neuronal misrouting to cortical and subcortical structures. In addition, newborn Tsc1null neurons displayed a reduced migration rate and an enhanced dendritic tree.

To explore whether postnatal neurogenesis contributed to some of the lesions observed in TSC patients, we used two strategies. First, we used inducible transgenic mice, in which tamoxifen injections led to Tsc1 loss in nestin-expressing cells, including SVZ cells. This resulted in the formation of SEGA-like lesions as reported recently (41). In addition, we found small heterotopias (i.e. clusters of misplaced cells)
TSC patients, in particular the heterotopias commonly called olfactory hamartomas (32,33). These heterotopias had either a mixed neuroglial or neuronal phenotype. The neuronal heterotopias contained cytomegalic, spiny neurons with enhanced mTOR activity and the presence of axons. The majority of these neurons were GFP\(^+\), suggesting that they were born during the neonatal period (P1–10). The mixed neuroglial heterotopia contained a mixture of early born GFP\(^+\)/RFP\(^+\) cells and GFP\(^+\)/RFP\(^-\) cells born ~3–7 days prior to preparing brain sections (P28). These data suggest that the continual generation of cells from Tsc1\(^{null}\) SVZ neural progenitor cells leads to progressive accumulation of cells in the lesions and their subsequent increase in size. It remains unclear why some cells remains stalled along the migratory path. A premature differentiation as seen by the presence of cells with astrocytic and neuronal morphology could result in cell immobility. Alternatively, the presence of immature-looking cells with a radial-like morphology suggests that neural progenitor cells (i.e., radial glial cells) did not fully differentiate and remain stalled in the SVZ. Addressing this issue is outside the scope of this study. During migration, Tsc1\(^{null}\) neuroblasts displayed a slowed migration speed and a more complex morphology than Tsc1\(^{homo}\) neuroblasts. Once in the olfactory bulb, Tsc1\(^{null}\) neurons formed micronodules, suggesting that their positional cues were altered. In addition, they displayed a hypertrophic dendritic tree consistent with a role of the mTOR pathway on dendritogenesis (52).

Another major finding is the presence of GFP\(^+\) neurons in the cortex and nucleus accumbens around the anterior commissure. Cortical neurons were spiny and displayed morphologies reminiscent of pyramidal neurons. They also exhibited a thin process resembling an axon. Although only 10–15 were found per slice, they clustered in groups of 2–6 and could have a major impact on local circuit function. The function of these ectopic neurons will clearly warrant further investigation.

Our findings have major implications and raise important new questions. It is highly conceivable that Tsc1\(^{null}\) cells born along the SVZ around the time of birth in humans (neonatal period in mice) can generate olfactory lesions. In addition, because neural stem cells and neurogenesis persist in juvenile and adult humans (19,20,28), the continual generation of Tsc1\(^{null}\) cells from the SVZ in TSC patients may feed and increase in size the lesions that are formed neona tally or embryonically. Finding micronodules in the olfactory bulb raises the possibility that such abnormal structures may also be generated during embryonic life and would not be visible through conventional imaging. This finding together with the presence of ectopic neurons in the cortex and subcortical regions calls for closer examination of TSC tissue outside the tubers. Finally, TSC shares histological and clinical features with other disorders of cortical malformations such as focal cortical dysplasia and ganglioma that are associated with defects in neurogenesis (22). Collectively, we thus propose that neurogenesis along the SVZ progressively contributes to alterations in the forebrain during the neonatal and juvenile period resulting in progressive circuit disruption and perhaps neuropsychiatric instability in patients with TSC and other cortical malformations.
transgenic mice: R26R mice obtained by crossing the following two lines of Tsc1 null mice (Jackson Laboratories) to generate Eisch, UT Southwestern) that we had crossed with nestin-CreERT2 mice (kind gift from Dr Amelia MA, USA). For additional experiments, and Women’s Hospital, Harvard Medical School, Cambridge, MA, USA). Two lines of mice were generated by David J. Kwiatkowski (Brigham and Women’s Hospital, Harvard Medical School, Cambridge, MA, USA). For additional experiments, Tsc1 fl/fl mice were crossed with nestin-CreERT2 mice (Jackson Laboratories, RFP for tdTomato). These two lines were performed on littermate animals.

**MATERIALS AND METHODS**

**Animals**

Research protocols were approved by the Yale University Institutional Animal Care and Use Committee. Experiments were performed on littermate Tsc1^fl/wt^/R26R and Tsc1^fl/mut^/R26R mice obtained by crossing the following two lines of transgenic mice: Tsc1^fl/wt^ (Jackson Laboratories) and Tsc1^wt/mut^ (NCI) that we had crossed with R26R-Stop-RFP mice (Jackson Laboratories, RFP for tdTomato). These two lines of mice were generated by David J. Kwiatkowski (Brigham and Women’s Hospital, Harvard Medical School, Cambridge, MA, USA). For additional experiments, Tsc1^fl^/nestinCreERT2 mice (kind gift from Dr Amelia Eisch, UT Southwestern) that we had crossed with R26R-Stop-YFP mice (Jackson Laboratories) to generate Tsc1^fl^/nestinCreERT2 mice. Mice were prescreened for successful electroporation prior to sacrificing by viewing with an epifluorescence microscope or a Kodak 4000 imager.

**Genotyping**

Tail or toe samples were prepared using the standard protocols. We used previously published primers (53,54). The primer combination allows for the detection of the wt Tsc1 allele at 295 bp, the fl allele at 480 bp and the mutant allele at 370 bp. The fl allele contains the LoxP sites surrounding the sequence to be excised upon Cre recombination. The mutant allele lacks the sequence flanked by LoxP sites, thus making the hamartin protein non-functional.

**Neonatal electroporation**

Electroporation is as we described previously (45,47). Plasmids (2–3 µg/µl) were diluted in phosphate buffered saline containing 0.1% fast green as a tracer; 0.5–1 µl of plasmid solution was injected into the lateral ventricles of cold-anesthetized neonatal pups using a pulled glass pipette (diameter <50 µm). Five square pulses of 50 ms duration with 950 ms intervals at 100 V were applied using a pulse ECM830 BTX generator and tweezer-type electrodes (model 520, BTX) placed on the heads of P0–1 pups.

**Genomic DNA isolation**

Fresh, unfixed tissue was subjected to a proteinase K dilution and genomic DNA isolated using a DNeasy kit (DNA extraction). Quantification of DNA purity and concentration was performed, and ~0.19 µg of DNA was added to PCRs. 10 × PCR buffer (Invitrogen) was added to 10 mM dNTP mix, 50 mM MgSO4, autoclaved DNase-free deionized H2O and Platinum Taq. The reaction volume was 25 µl after adding 1 µl of each forward and reverse primer with 3 µl of the appropriate sample. PCR was performed in a BioRad MyCycler (32–36 cycles). For recombination detection, 5 µl of product was subjected to a second round of PCR. Amplicons were visualized by running samples diluted in 10 × blue juice DNA loading buffer loaded onto a 2% agarose gel run at 100 V for 30 min along with a 100-bp DNA ladder (Invitrogen).

**Microdissection, RNA extraction and RT–PCR**

Trizol reagent (750 µl) plus 200 µl of chloroform were added to each olfactory bulb sample and passed through a 22-gauge 1.5-inch needle and then vortexed. Following centrifugation for 15 min at 4°C and 12,000 g, the top aqueous phase was transferred to a fresh reaction tube. After adding 1000 µl of ethanol, the sample was vortexed for 1 min and centrifuged at 8000 g for 15 s. Pellets were rinsed three times with 75% ethanol. Following centrifugation, the RNA was eluted with RNase-free deionized H2O prior to determining its concentration and purity on a spectrophotometer. The samples with contamination were subjected to an additional ethanol/sodium acetate precipitation.

For reverse transcription polymerase chain reaction (RT-PCR), 2.12 µg of RNA was mixed with deoxynucleotide triphosphates (dNTPs), random primers (Invitrogen) and RNase/DNase-free deionized H2O prior to determining its concentration and purity on a spectrophotometer. The samples with contamination were subjected to an additional ethanol/sodium acetate precipitation.

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Slice preparation and immunostaining

P28 mice were deeply anesthetized with pentobarbital (50 mg/kg). The brain was then quickly removed and placed in 4% paraformaldehyde overnight at 4°C, then washed in 1× PBS. The region of electroporation was imaged using a Kodak 4000 imager. The next day, 100-μm-thick slices were prepared using a vibratome (Leica VTS 1000). Immunostaining was performed in free-floating 100-μm-thick slices as described previously (55). Free-floating sections were blocked in PBS containing 0.1% Triton X-100, 0.1% Tween-20 and 2% bovine serum albumin and incubated in primary antibodies (see below) overnight at 4°C. After several washes in PBS containing 0.1% Tween-20, slices were incubated with the appropriate secondary antibody [Alexa Fluor series at 1:1000 (Invitrogen) or Cyanine series at 1:500 (Jackson ImmunoResearch)] for 1 h at room temperature. Primary antibodies were rabbit anti-pS6 (1:1000; Cell Signaling; S235/236, 2F9, catalog #4856), mouse anti-NeuN (1:500; Millipore), GFAP (1:100, DAKO) and DCX (1:500, Santa Cruz Biotechnology). Each staining was replicated in slices from three different mice. Z-section images were acquired on a confocal microscope (Olympus FluoView 1000) with a 20× dry objective (N.A. 0.75). Low-magnification images were acquired with a 10× dry objective or a dissecting scope (SZX16 with a SDF PLAPO 1× PF objective). Images were analyzed using Imaris 4.0 (Bitplane AG) and reconstructed using ImageJ 1.39t software (Wayne Rasband, NIH) or Photoshop CS3.

Cell size and pS6 immunostaining analyses

To measure cell size, Z-stack images of mRFP+ cells in coronal sections were acquired with a 20× dry objective (Numerical Aperture, 0.75) using high–low settings to minimize saturation. In ImageJ, the frehand tracing tool was used to outline cell somas. Cell size was represented relative to the indicated controls. To measure nuclear size, Z-stack images of coronal sections stained for NeuN and DAPI were acquired with a 40× dry objective (Numerical Aperture, 1.20). Likewise, the frehand tracing tool was used to outline neuronal nuclei.

To measure mTOR activity, immunostaining was performed using an antibody to phosphorylated serine 235/236 of pS6. Serial Z-stacks were acquired under the same settings for the

Figure 8. Newborn Tsc1null neurons are rerouted to cortical and subcortical areas. (A) Coronal sections with green rectangles indicating the approximate locations of images shown in (B)–(I). (B) The confocal image of GFP+ neurons (green) in the ACB and around the aco counterstained with DAPI (blue). (C) The zoom of the image in the white square in (B). (D) The confocal image of GFP+ and RFP+ neurons and astrocytes (bushy cells) in cortical layer II. (E) GFP+ (black) neurons in cortical layer II. (F) The confocal image and projections of a GFP+ cortical layer II cell that immunostained positive for NeuN (red). (G) The confocal image of GFP+ neuron in the deep layer of the cortex. (H) Zoom of the neuron in the white square in (G). (I) The confocal image of GFP+ neuron in the deep layer of the cortex. Inset: GFP fluorescence overlaid with DIC to illustrate the location of the corpus callosum. CC, corpus callosum; CP, caudate putamen; ACB, accumbens nucleus; aco, anterior commissure. Scale bars: 140 μm (B) 70 μm (C), 80 μm (D and G), 40 μm (E, F and I) and 30 μm (H).
ipsilateral and contralateral coronal sections of an olfactory bulb. Regions of interest (ROIs) were generated using an elliptical selection tool, and average intensities for each ROI were determined. For both cell size and pS6 staining measurement, three sections per mouse were analyzed.

**Acute slice migration assays**

P21 mice were deeply anesthetized with 20 mg/kg Nembutal and brains were dissected into ice-cold, oxygenated (95% O2, 5% CO2) high-glucose Dulbecco’s modified eagle medium (DMEM). The 300-μm-thick sagittal slices were obtained using a Leica vibratome. After incubating for 1 h in room temperature DMEM, slices were transferred to a heated (35°C) perfusion chamber for 1 h. Individual fluorescently labeled cells in the RMS or RMS-OB were visualized using a confocal Olympus FluoView 1000 system. Confocal Z-stack images (4 μm spaced sections over 60–100 μm) were acquired with a 20× immersion objective (XUMPLFL, N.A. 1.20) every 5 min for at least 90 min. Movies were analyzed in ImageJ software (NIH). Image stacks were realigned and RFP-fluorescent cells were tracked using ImageJ plug-ins [Stackreg (56) and MTrackJ written by Dr E. Meijering, Bio-medical Imaging Group Rotterdam]. Individual fluorescent cells were tracked using the MTrackJ plug-in. Cell migration speeds were calculated with a macro program as described previously (47,48).

**Olfactory bulb organization**

Sagittal slices were optically sectioned in the Z-plane at 4 μm intervals at a 4× magnification. Sections were stacked and converted to an 8-bit digital image followed by binary watershedding in ImageJ. Images were loaded and processed in geological image analysis software (GIAS v1.12, Beggan and Hamilton, 2010). Default settings were applied, and the nearest neighbor distance Ra was calculated. Organization is then measured as the theoretical Ra (based on a Poisson distribution) minus experimental Ra.

**Morphometric analysis**

Complete images of RFP+ neurons were acquired in coronal sections using a Fluoview 1000 confocal microscope and 20× objective with a 2× digital zoom to focus on basal neurites. Neurites were traced with simple neurite tracer software (FIJI, GNU GPL v3). Scholl analyses were carried out using dendrite length as a measure of morphological complexity. Confocal Z-stacks from three different square fields of view were taken from each olfactory bulb section, and this was done for three different OB sections in a randomly selected series from each animal. At least three animals were analyzed per condition.

**Statistics**

Analysis of cell size and pS6 was performed on an average of 20–25 cells per slice, respectively, from 3 to 4 animals in each condition. The laminar distribution of cells was performed on 1–2 slices for 3–4 animals of each genotype. Data were presented in Origin 8.0. Statistical significance was determined using the unpaired Student’s t-test or the Fisher’s exact test when mentioned with P < 0.05 for significance (KyperPlot 2.0). Data are presented as the mean ± SEM.

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**Conflict of Interest statement**

None declared.

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