The galactocerebrosidase enzyme contributes to maintain a functional neurogenic niche during early post-natal CNS development

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We report a novel role for the lysosomal galactosylceramidase (GALC), which is defective in globoid cell leukodystrophy (GLD), in maintaining a functional post-natal subventricular zone (SVZ) neurogenic niche. We show that proliferation/self-renewal of neural stem cells (NSCs) and survival of their neuronal and oligodendroglial progeny are impaired in GALC-deficient mice. Using drugs to modulate inflammation and gene transfer to rescue GALC expression and activity, we show that lipid accumulation resulting from GALC deficiency acts as a cell-autonomous pathogenic stimulus in enzyme-deficient NSCs and progeny before up-regulation of inflammatory markers, which later sustain a non-cell-autonomous dysfunction. Importantly, we provide evidence that supply of functional GALC provided by neonatal intracerebral transplantation of NSCs ameliorates the functional impairment in endogenous SVZ cells. Insights into the mechanism/s underlying GALC-mediated regulation of early post-natal neurogenic niches improve our understanding of the multi-component pathology of GLD. The occurrence of a restricted period of SVZ neurogenesis in infancy supports the implications of our study for the development of therapeutic strategies to treat this severe pediatric neurodegenerative disorder.

INTRODUCTION

Genetic defects in the expression and activity of the lysosomal galactosylceramidase (GALC), a key enzyme in the breakdown of myelin-enriched galactolipids, cause globoid cell leukodystrophy (GLD) or Krabbe disease, a rare autosomal recessive lysosomal storage disorder (LSD). This is typically an infantile disease with a fatal course. Clinical manifestations are neurologic with prominent white-matter signs. The pathology consists of rapid and nearly complete disappearance of myelin and myelin-forming cells in the central nervous system (CNS), peripheral nervous system (PNS), reactive astrogliosis, neuroinflammation and infiltration of PAS-positive macrophages (globoid cells). The accumulation of galactosylsphingosine (psychosine), a toxic metabolite, is crucial in the pathogenesis (1).

Hematopoietic cell transplantation (HCT) from healthy donors is beneficial if performed in asymptomatic GLD patients (2) but does not provide definitive cure. HCT delivers functional enzyme to the CNS and PNS by replacement of endogenous macrophage/microglia with donor-derived cells. However, full brain microglia reconstitution from donor-derived circulating

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progenitors requires up to 6 months in mice (3). This slow turnover might preclude obtaining sufficient levels of functional enzyme to counteract the rapid disease progression. Several studies in experimental GLD models established the efficacy of HCT (4) and intracerebral gene therapy (5,6) in providing a permanent source of functional enzyme in CNS tissues and described synergic effects of combined therapies (7–9). However, none of these treatments was able to arrest the disease progression completely. One interpretation of these results was that levels of corrective enzyme were insufficient or were achieved too late in post-natal life, when neuropathology is established and irreversible.

In post-natal and adult mammals, including humans, primitive neural stem cells (NSCs) sustain neurogenesis within restricted areas of the brain (niches). The subventricular zone (SVZ) lining the forebrain lateral ventricles is the largest mammalian stem cell reservoir. In rodents, the complex adult SVZ cytoarchitecture results from relevant modifications of the structure and cell composition that occur in the first postnatal month (10). A subset of SVZ astrocytes (Type B cells) function as primary precursors. They proliferate slowly and generate rapidly dividing transit-amplifying progenitors (Type C cells), which differentiate into neuroblasts (Type A cells). A dense network of astrocytes enwraps chains of neuroblasts migrating along the rostral extension of the SVZ to the olfactory bulb, where they integrate as mature neurons (11–13). In humans, SVZ neurogenesis takes place at high rates during the late gestation period (14) and persists in infancy (15), with neuroblasts migrating to olfactory and cortical regions (16), similarly to what described in young post-natal rodents (17–19). This process declines sharply before the age of 2 (15,16), when the human SVZ progressively acquires distinctive features that will be maintained throughout adulthood, with a loose glial network reduced to an incomplete ‘ribbon’ separated from the ependyma by a ‘hypocellular gap’ (20,21). Thus, perturbation of SVZ neurogenesis at formative stages of CNS development occurring in neonatal/pediatric neurological diseases might hold important therapeutic implications. Yet, our knowledge concerning the physiology of resident SVZ NSC compartments during early postnatal CNS development is incomplete, particularly as regards their behavior during the onset and progression of a severe pediatric neurodegenerative condition, such as GLD.

Several sphingolipids in the GALK metabolic pathway, such as ceramide, lactosylceramide (LacCer) and sphingosine-1-phosphate (SIP), participate to signaling pathways controlling neuronal survival, migration, differentiation, responsiveness to growth factors, synaptic function and neuron-glia interactions (22). Further levels of complexity arise from the metabolic interconnection of these bioactive sphingolipids (23). A dynamic regulation of sphingolipid metabolism appears essential to maintain proper neurogenesis and NSC function during CNS development and to ensure CNS homeostasis in adult life (24). Impaired neuronal survival as well as defects in neurogenesis and NSC function have been described in animal models of neurodegenerative LSDs, such as mucopolisaccharidosis (MPS) IIIB (25), MPS VII (26) and Niemann Pick type C (27). Modulation of GALK levels affects the components of the hematopoietic niche (28), but little is known regarding the potential role of GALK in regulating neurogenic compartiments.

Clinical observations in GLD patients (29) and recent preclinical studies in the Twitcher mouse, a mutant that lacks a functional GALK protein (30,31), suggest that GALK deficiency leads to impairment of neuronal and glial function before the occurrence of demyelination driven by overt tissue storage and neuroinflammation. We report here for the first time that GALK deficiency is associated to perturbation of the morphological, functional and biochemical properties of the neonatal, early post-natal and adult SVZ neurogenic niche in GLD mice. By modulating the pathophysiology of GLD phenotype, either acting on the brain environment (using anti-inflammatory drugs) or restoring the expression of a functional GALK in SVZ-derived NSCs and progenitors by means of ex-vivo lentiviral-mediated gene transfer or by in vivo metabolic cross-correction mediated by neonatal NSC transplants, we clarified the cell-autonomous and non-cell autonomous mechanisms that differently contribute to perturb the SVZ neurogenic compartment at different stages of disease progression.

RESULTS

Altered organization of the SVZ neurogenic niche in symptomatic Twi mice

We assessed the proliferative state, the morphology and the relative cell-type proportions in the SVZ at the level of the lateral ventricles (lv-SVZ) and in the rostral extension (re-SVZ) of FVB/Twitcher mice [from hereon referred to as Twi (5); see Materials and Methods] at different stages of disease progression. We injected bromodeoxyuridine (BrdU) in asymptomatic [post-natal day 2 (PND2) and PND10; one injection] and symptomatic Twi mice (PND30, PND40; four injections at 2 h interval) and in wild-type (WT) littermates. We then analyzed the brains 2 h after the last injection. We observed a significant decrease in the total number of BrdU+ cells in the lv-SVZ of Twi mice when compared with WT littermates as a function of age (Supplementary material, Fig. S1). Ultrastructural analysis revealed similar numbers of neuroblasts (Type A cells) and astrocytes (Type B cells) (Fig. 1A) but decreased numbers of transit-amplifying progenitors (Type C cells) in the mutant lv-SVZ at PND40 (Fig. 1B). The percentage of proliferating (BrdU+) double-cortin (DCX)-positive neuroblasts was significantly decreased in mutants (Fig. 1C) and qualitative analysis suggested altered morphology and organization of DCX+ cells (Fig. 1D).

Indeed, analysis of lv-SVZ wholemounts, which provide a comprehensive, en-face view of this germinal region (32), showed sparse, deranged and tangled DCX+ neuroblast chains in the lv-SVZ of PND40 Twi mutants (Fig. 1E and F).

Astrocytosis and microglia activation characterize several brain regions of symptomatic Twi mice (Supplementary material, Fig. S2; PND40). While glial fibrillary acidic protein (GFAP)-expressing cells showed little changes in morphology and organization in the mutant lv-SVZ (Fig. 1F and G), this region was infiltrated by activated macrophages and microglial cells (CD68+, Iba1+), which are less represented or display resting morphology in WT littermates (Fig. 1H and Supplementary material, Fig. S2).

The morphological and proliferative impairment of the neuroblast cell population observed in the Twi lv-SVZ...
appeared greatly enhanced in the re-SVZ (Fig. 2A and B). Ultrastructural analysis (Fig. 2C–F) indicated a significant reduction in the total number of Type A cells associated with disruption of chain organization, thus pointing to a reduced migration. However, the absence of Type A cell build-up in the Twi lv-SVZ (Fig. 1A) strongly suggested that this ultrastructural picture resulted from a defective production and altered organization of neuroblasts in the mutant lv-SVZ.
rather than from a relevant primary migration defect, which might, however, be present and contribute to the overall scenario (see below). Few apoptotic cells were observed in the lv-SVZ and re-SVZ of Twi mice, as assessed by both ultrastructural analysis and immunofluorescence (IF) analysis using anti-cleaved caspase-3 (C3) antibody (data not shown). Several astrocytic-like cells with ultrastructural features different from the canonical type B cells (Fig. 2D) as well as numerous CD68- and Iba1-immunoreactive cells (Supplementary material, Fig. S2) were found within or close to the re-SVZ of Twi mice when compared with WT controls at PND40. In order to clarify whether the paucity of apoptotic cells in Twi mice might be consequent to their rapid clearance mediated by the macrophagic/microglia population, we resorted to ex vivo systems. We established primary neuronal and glial mixed cultures by freshly isolating lv-SVZ primary cells from PND40 mice and plating them in the growth factor-free medium containing 2% fetal bovine serum (FBS) (33). Two weeks after plating, we assessed the presence of apoptotic cells in the different cell types by IF analysis using antibodies to neuronal (TUJ1), oligodendroglial (O4) and apoptotic (C3) markers. Interestingly, we found increased numbers of apoptotic neurons (C3+TUJ1+) in Twi-derived primary cultures when compared with their WT counterpart, thus suggesting that apoptosis might play a role in the loss of neurons that are detected in vivo (Supplementary material, Fig. S2). Of note, we detected a remarkable increase in apoptotic oligodendrocytes (C3+O4+) in Twi-derived cultures (Supplementary material, Fig. S2), further indicating the susceptibility of this cell type in Twi mice.

Figure 2. Impairment of the neuroblast cell population in the re-SVZ of adult Twi mice. Representative pictures showing (A) DCX+ cells and (B) PCNA+ cells in coronal sections comprising the re-SVZ of PND40 Twi and WT littermates. Note the altered neuroblast cell morphology and organization as well as decreased cell proliferation. (C–D) Electron micrographs of PND40 WT and Twi re-SVZ region with the corresponding masks (C’–D’) illustrating the distribution of neuroblasts (grey area) and astrocytes (line-surrounded cells). Insets: B, Type B cells (astrocytes); A, Type A cells (neuroblasts). Asterisks indicate astrocytic-like cells showing nuclei with dispersed heterochromatin. (E) Schematic drawing showing the re-SVZ level analysed. Scale bars, 400 µm (A) and 200 µm (B), 5 µm (C–D), 2 µm (inset). (F) Quantification of Type B and Type A cells indicates loss of neuroblasts in the mutant re-SVZ. Data are expressed as the mean ± SEM, three sections/animal, n = 3 mice/genotype. Mann–Whitney test, *P < 0.05.
Overall these results indicate that production, survival and organization of migrating neuronal precursors are impaired in the SVZ of symptomatic GALC-deficient mice. The presence of minor changes in the astrocytic population and the expansion of the macrophagic/microglial population in mutants might be potential contributors in determining/maintaining the neurogenic impairment.

**Functional impairment of SVZ-derived neural stem/progenitor cells in early post-natal asymptomatic Twi mice**

During the first two post-natal weeks of age, migrating neuroblasts form large masses that will later organize in chains. Similarly, radial cells/astrocytes and their processes form a dense network that will next develop into glial tubes (10). Such a tissue composition precluded a reliable quantification of the different SVZ cell types *in vivo*. In order to more easily assess whether abnormalities in cell type composition, organization and function might be present in Twi mice at the early stages of post-natal neurogenesis, we resorted to primary mixed cultures (33) and SVZ tissue explants (34).

Primary neuronal and glial mixed cultures were obtained from PND2 and PND10 mice and assessed for their cell type composition using neuronal (TUJ1), astroglial (GFAP) and oligodendroglial (O4) markers. Since we obtained comparable results in PND2- and PND10-derived cultures (Fig. 3A–C), we focus here on data from PND2 mice. We did not find significant quantitative or qualitative differences in the GFAP+ cell population in Twi-derived cultures compared with the WT counterpart (Fig. 3A and D). In contrast, Twi-derived cultures displayed a significant decrease in TUJ1 and O4 immunoreactivity (Fig. 3B–D), measured either by quantification of immunopositive area (Fig. 3B and C) and by direct cell counts performed at 8, 10 and 15 days after plating (Fig. 3E; TUJ1+). Interestingly, the relative proportion of TUJ1+ cells with round-shaped morphology when compared with bipolar/branched morphology was higher in Twi- with respect to WT-derived cultures (Fig. 3F). We measured similar proliferative ability (Fig. 3G; Ki67) and increased apoptosis (Fig. 3H; C3) in Twi- when compared with WT-derived cultures, with a remarkable increase in apoptotic cells in the oligodendrocyte (4.5 ± 1.2 and 22.7 ± 2.7% of C3+O4+ cells on total O4+ cells in WT- and Twi-derived cultures, respectively; mean ± SEM, n = 3; 10 DIV; Fig. 3I) and neuronal (TUJ1+C3+; Fig. 3J) cell populations. Finally, we observed an overall reduced cell migration from SVZ explants isolated from PND2 Twi mice when compared with age-matched WT mice (Supplementary material, Fig. S3).

Results from these experiments indicated reduced survival and/or migration of SVZ-derived neuronal and oligodendroglial progenitors from the first PNDs of life in Twi mutants. In order to assess functional changes of primary precursors (stem cells), which are less frequent and relatively quiescent *in vivo*, we applied the neurosphere assay (NSA) (35). We plated primary cells isolated from the lv-SVZ of PND2, PND10, PND30 and PND40 Twi mice and WT littermates at clonal density (5 cells/μl) in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). The number of primary spheres (diameter >100 μm) counted 7 days after plating reflects the number of sphere-forming cells present in the primary cell suspension, which are mainly represented by transit-amplifying progenitors and stem cells (35,36). We observed a significant impairment in the clonogenic efficiency of Twi-derived primary cells when compared with the WT counterpart at all ages considered (Fig. 4A). Similar results were obtained using the neural colony-forming cell assay (37), in which cells are seeded in a hydrogel matrix in order to circumvent the potential bias related to cell aggregation and primary neurospheres are scored according to their size (Supplementary material, Fig. S3). Decreased numbers of primary neurospheres were not due to increased cell death of GALC-deficient primary cells, as shown by similar cell counts obtained in PND2-derived WT and Twi cultures at 24 h (84.2 ± 7.6% WT and 90.4 ± 7.6% Twi) and 72 h after plating (25.5 ± 4.8% WT and 25.8 ± 2.5% Twi; expressed as live cells/total number of plated cells × 100). Notably, the overall cell migration from Twi-derived primary spheres (PND2 and PND40) was reduced when compared with that measured in WT-derived counterparts (Fig. 4B), resembling the phenotype observed in SVZ explants (see Supplementary material, Fig. S3). The consistent generation of secondary and tertiary neurospheres (Fig. 4C) indicated the presence of self-renewing stem cells in the primary cell populations. However, GALC-deficient cells were impaired in their neurosphere-forming ability at least up to the second subculturing passage, suggesting a cell autonomous defect in self-renewal capacity.

**Inflammation contributes to SVZ functional impairment during disease progression**

While the role of inflammation in worsening CNS pathology in neurodegenerative LSDs is acknowledged, its occurrence and potential role in the early stages of the disease are poorly understood. Inflammation may be detrimental for neurogenesis and NSC functions (30–32). Thus, we sought to assess its potential contribution to the functional changes of the SVZ compartment of Twi mice along the disease progression.

We showed an age-dependent upregulation of mRNA expression of several cytokines and chemokines in Twi brain tissues, firstly detected in early symptomatic mice (PND20) and reaching >100-fold the physiological levels at the late stages of the disease (Fig. 5A). A moderate but significant up-regulation of Chemokine (C-C motif) ligand 2 (Ccl2), Interleukin 1 beta (Il1b) and Tumor necrosis factor alfa (Tnfa) mRNA expression was present in asymptomatic Twi mice (PND2, PND10), suggesting that these molecules might play a key role in the pathogenic cascade. Daily treatment of Twi mice (starting from PND10) with minocycline (a semi-synthetic tetracycline able to cross the blood brain barrier and to inhibit microglia activation) (38) or indomethacin (a non-steroidal anti-inflammatory drug) resulted in down-regulation of expression of several of these molecules (Fig. 5B and Supplementary material, Fig. S4) and in partial reduction of macrophages (CD68+; Fig. 5C) and globoid cells (lectin 4+; Fig. 5D) in brain tissues of treated Twi mice, likely accounting for the delayed onset of symptoms and significant, albeit modest, prolongation of lifespan (Supplementary material, Fig. S4). Twi
Figure 3. Reduced numbers of neurons and oligodendrocytes in SVZ-derived primary cultures from early post-natal Twi mice. (A–C) Box and whiskers plots showing comparable immunopositive area for GFAP (astrocytes) (A) and decreased immunopositive area for TUJ1 (neurons) (B) and O4 (oligodendrocytes) (C) expressed as percentage on nuclear area (ToPro), in SVZ-derived primary cultures from PND2 and PND10 WT and Twi mice analyzed after 15 DIV. Quantification was performed on n = 3–4 independent cultures, 5–7 cover slips/antigen, 9 fields/cover slip. Mann–Whitney test, **P < 0.001; ***P < 0.01; *P < 0.05. (D) Representative confocal merged images showing TUJ1- (pink), GFAP- (green) and O4-immunoreactive cells (green) in PND2 SVZ-derived primary cultures. Nuclei counterstained with ToPro (blue). (E) Primary cultures from PND2 mice were analyzed at 8, 10 and 15 DIV for the presence of neurons (TUJ1+ cells). The overall number of neurons is lower in mutant cultures at every time point considered. Quantification was performed on n = 3 independent cultures, two to three cover slips/experiment, five fields/cover slip (>2000 cells/experiment were analyzed); data expressed as mean ± SEM; two-way ANOVA and Bonferroni post-test, ***P < 0.001. (F) Representative confocal pictures and quantification (box and whiskers plot) showing increased numbers of round-shaped TUJ1+ cells in PND2 Twi-derived cultures following double-labeling immunofluorescence using anti-TUJ1 and anti-Ki67 antibodies.
and WT mice treated with the anti-inflammatory drugs and saline-treated controls received BrdU injections at the day of euthanasia, according to the protocol described above. Both the total number of proliferating cells in vivo and the number of primary neurospheres in vitro were significantly increased in minocycline- and the number of primary neurospheres in vitro were significantly increased in minocycline- (Fig. 5E and F) and indomethacin-treated Twi mice (NSA; Supplementary material, Fig. S4) compared with untreated (UT) controls. However, Type C (Fig. 5G) and Type A cells (Fig. 5H) in the lv-SVZ and re-SVZ, respectively, were not significantly rescued in minocyclin-treated Twi mice. Importantly, treatment with anti-inflammatory compounds did not affect NSC compartments in WT mice (Fig. 5F and Supplementary material, Fig. S4).

GALC deficiency induces early metabolic dysfunction in CNS tissues and SVZ NSCs

The partial recovery of the stem/progenitor cell function upon anti-inflammatory treatment identified a non-cell autonomous contribution of the inflamed brain microenvironment to the functional impairment at the late stages of the disease. We next sought to verify whether a similar contribution might occur in the neonatal/early PNDs.

Despite most of the pro-inflammatory molecules are still at physiological levels in neonatal Twi mice (Fig. 5), we detected up-regulation of GFAP, CD68, Iba1 and C3 expression in PND2 and PND10 mutant brains (Fig. 6A and B). These data suggested initial occurrence of astroglisis, microglia activation/recruitment and apoptosis, likely as a consequence of early storage (lectin +) cells and psychosine accumulation, which we found significantly increased as early as PND2 in Twi brains (Fig. 6C and D). We observed a moderate increase in GFAP expression in the neonatal Twi lv-SVZ (Supplementary material, Fig. S5), while lectin+, CD68+ and Iba1 + cells were abundant within or close to the neonatal Twi lv-SVZ (Fig. 6E and F) and re-SVZ (Supplementary material, Fig. S5).

These data suggested that a potential altered crosstalk between the macrophagic/microglial population and the resident SVZ cell types in the neonatal Twi CNS might contribute to their functional deregulation. Still, the functional defects and the increased susceptibility to apoptotic cell death observed in ex-vivo SVZ primary cultures coupled to the defective proliferation/self-renewing phenotype observed in serially passed SVZ-derived neurospheres strongly implied a direct cell autonomous role of GALC in regulating the cardinal features of neural stem/progenitor cells. Indeed, we detected a significant accumulation of psychosine (>20-fold the physiological level; Fig. 6G) and lactosylceramide (LacCer, natural endogenous substrate of GALC; 2–3-fold the WT levels, assessed by steady-state metabolic labeling with radioactive sphingosine) in primary neurospheres derived from the lv-SVZ of PND2 Twi mice (Fig. 6H). The significant accumulation of LacCer and psychosine is also directly observed in freshly isolated SVZ tissues and in neurospheres derived from PND40 Twi mice (Supplementary material, Fig. S6).

Taken together, these data strongly suggest that early storage, psychosine accumulation and lipid unbalance, which are the primary consequences of GALC deficiency, are mainly responsible for the functional defects observed in the neonatal and early post-natal SVZ stem/progenitor cell compartment. As a secondary effect, due to their ubiquitous distribution in Twi CNS tissues, they likely trigger the up-regulation of inflammatory molecules that occurs before or in close correspondence to the onset of symptoms (Fig. 5).

Rescue of GALC expression and activity by gene transfer and cross-correction ameliorate the functional defect in Twi NSCs and in their progeny

In order to prove the cell-autonomous role of GALC in regulating the proliferation and self-renewal of NSCs as well as the survival and migration of their neuronal progeny, we first restored the expression of a functional GALC by lentiviral vector (LV)-mediated gene transfer in Twi-derived stem/progenitor cells and checked for functional rescue.

We transduced primary cells isolated from the lv-SVZ of Twi mice (PND2 and PND40) and WT littersmates with bi-directional (bd) LV expressing the murine GALC and the reporter protein GFP (bdLV.GALC), applying previously optimized conditions (5). The control vector (bdLV.CTRL) expressed GFP and the reporter protein ΔNGFR. Untransduced and bdLV-transduced primary cells were then plated in the mitogen-containing medium to assess for neurosphere formation and in differentiating conditions to obtain primary mixed cultures. Both bdLVs efficiently transduce primary cells, with a vector copy number (VCN; average number of vector copies integrated in the host genome) ranging from 2 to 12 and the presence of 70–100% of GFP cells (assessed in primary neurospheres), although with different intensity of GFP signal [evaluated by direct fluorescence and fluorescence-activated cell sorting (FACS) analysis; Fig. 7A]. Importantly, spheres derived from bdLV.GALC-transduced Twi primary cells expressed supraphysiological GALC activity (Fig. 7B and C). This correlated with normalization of psychosine and LacCer levels (Fig. 7C and D and Supplementary material, Fig. S6), indicating the function of the transgenic enzyme. GALC overexpression in WT cells did not result in evident sphingolipid unbalance (Fig. 7D). Also, no differences were found in the ganglioside content between Twi and WT cells, irrespective of the treatment considered (Supplementary material, Fig. S6).
material, Fig. S6). The number of spheres counted 7 days after plating revealed normal sphere-forming ability in bdLV.GALC-transduced Twi primary cells, undistinguishable from those of bdLV.CTRL- and bdLV.GALC-transduced WT cells (Fig. 7E). Migration was ameliorated in Twi-derived primary spheres transduced with bdLV.GALC (Fig. 7F). Also, the proportion and the morphology of TUJ1+ cells in primary cultures derived from bdLV.GALC-transduced Twi primary cells were significantly ameliorated (Fig. 7G). In all the experimental conditions, bdLV.CTRL-transduced and untransduced cells (both Twi and WT) showed comparable functional features.

The therapeutic benefit provided by novel gene and cell therapy approaches that have been tested to treat LSDs mainly rely on metabolic cross-correction of enzyme-deficient cells (39). Our previous studies indicate that this mechanism allows restoring physiological GALC levels in Twi NSCs and progeny when compared with supra-physiological levels obtained following direct LV-mediated gene transfer (40). Thus, we finally sought to assess whether the expression and activity of a functional GALC obtained through in vivo cross-correction was sufficient for functional rescue of SVZ cells. We transplanted serially passaged bdLV.GALC-transduced WT NSCs (GALC activity: 5-fold the physiological levels; see Materials and Methods) in the forebrain lateral ventricles of PND2 Twi mice, according to a described protocol (40). UT Twi and WT littermates as well as Twi mice transplanted with UT Twi NSCs (GALC-deficient) served as controls. At PND10 and PND40, we isolated primary cells from the lv-SVZ of Twi mice transplanted with GALC-overexpressing or GALC-deficient NSCs (trTwi\textsuperscript{GALC} and trTwi\textsuperscript{def} mice). Cells were plated to assess for neurosphere formation and to obtain primary mixed cultures. SVZ tissue and primary cells freshly isolated from trTwi\textsuperscript{GALC} mice

Figure 4. GALC deficiency affects the SVZ NSC compartment. (A) Clonogenic efficiency of primary cells isolated from the lv-SVZ of PND2, PND10, PND30 and PND40 Twi mice is reduced when compared with WT littermates, as also shown in representative pictures of primary neurospheres. Quantification was performed on n = 8–23 mice/genotype in three to six independent cultures; data expressed as mean ± SEM, two-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001. (B) Reduced migration of neural progenitors from primary neurospheres derived from the early post-natal (PND2; representative bright-field pictures) and adult lv-SVZ of Twi mouse. Quantification of the migration area was performed on 20–45 neurophores/genotype/age; one-way ANOVA, *P < 0.05, ***P < 0.001. (C) Reduced clonogenic ability of precursors derived from the lv-SVZ of PND2 and PND40 Twi mice is maintained through serial subculturing passages in vitro. n = 3 independent cultures; Mann–Whitney test, *P < 0.05, **P < 0.01. Scale bars: 200 µm (A) 500 µm (B).
expressed 30–50% of WT GALC activity (Fig. 8A). Importantly, primary cells derived from PND10 and PND40 trTwiGALC mice showed normal sphere-forming capacity (Fig. 8B). Neurospheres derived from GFP+ (GALC-overexpressing) donor cells accounted for ≈3% in the primary neurosphere population (272 GFP+ neurospheres/9289 total neurospheres in five independent experiments) and were not considered in the calculation of clonogenic efficiency, although they might contribute to the GALC activity measured in the primary neurosphere population (Fig. 8A).
Figure 6. Early psychosine accumulation, astrocytosis and microglia activation in tissues and NSCs from Twi neonates. (A) Representative confocal pictures showing increased expression of GFAP, Iba1 and CD68 in PND2 Twi brains when compared with WT. Asterisks indicate cells shown in the insets. (B) Western blot analysis indicates increased astrogliosis (GFAP), microglia recruitment/activation (Iba1) and apoptosis (C3) in brains of Twi mice when compared with WT littermates as a function of age and occurring as early as PND2. Analysis performed on $n=3$ mice/genotype/time point. One representative blot is shown. (C) Qualitative and quantitative immunohistochemistry indicates a significant increase in lectin+ cells in the cerebellum of Twi mice when compared with WT littermates as early as PND2. Quantification performed on $n=3$ mice/group, four pictures/brain region; data expressed as mean ± SEM; Two-way ANOVA and Bonferroni post-test; $^* P < 0.05$, $^§ P < 0.05$ (Twi PND40 versus Twi other ages). (D) Increased psychosine levels as a function of age in brains of Twi mice when compared with WT littermates. Quantification was performed on $n=4–6$ mice/group; data expressed as mean ± SEM, two-way ANOVA with Bonferroni post-test; $^* P < 0.05$, $^{***} P < 0.001$, $^# P < 0.01$ (Twi PND20 versus Twi PND2), $^§ P < 0.01$ (Twi PND40 versus Twi PND20). (E–F) Increased expression
improved morphology of TUJ1 rather than a general tropic effect of NSCs underlying Twi (3), further supporting a crucial role of NSC-secreted factors. An important quantitative difference was observed in the oligodendroglial population between treatment groups (Fig. 8D and G; PND10), likely because of the early time point considered (8 DIV when compared with 15 DIV). However, we observed a clear reduction in the total number of apoptotic cells in cultures established from PND10 trTwi GALC mice (C3++; Fig. 8E) resulting from a significant decrease in the number of apoptotic oligodendrocytes and neurons (Fig. 8F and G), which was maintained in primary cultures derived from PND40 trTwi GALC mice (Supplementary material, Fig. S7). No significant decrease in the percentages of apoptotic oligodendrocytes and neurons were observed in cultures derived from trTwi def mice when compared with UT Twi littermates (data not shown). Overall, these data suggest a direct and long-lasting beneficial role exerted by the functional enzyme secreted by engrafted NSCs on endogenous Twi SVZ cells.

**DISCUSSION**

In this study, we describe a novel role of GALC in maintaining a functional SVZ neurogenic niche during the neonatal, early post-natal and adult life by contributing to the control of proliferative and self-renewal of NSCs and to the survival and migration of their neuronal and oligodendroglial progeny. Using gain-of-function strategies in vitro and pharmacological treatment in a GLD murine model, we show that this role is largely cell-autonomous, despite a non-cell autonomous contribution of brain inflammation to the neurogenic impairment occurs during the progression of the disease. Importantly, we provide evidence that the supply of functional GALC provided by neonatal NSC transplants restores function in SVZ NSCs and in their committed neuronal and oligodendroglial progeny. As GLD is typically an infantile disorders, understanding the mechanisms through which enzyme deficiency might alter SVZ neurogenesis at formative stages of CNS development holds important implications for understanding the pathophysiology of this disease and for designing effective therapies.

In rodents, active neurogenesis is a life-long process that characterizes the first post-natal months of life, to cease almost completely by the age of 2 (16). We have previously used primary neuronal/glial cultures and neurosphere cultures to model the cell type composition and NSC function during the critical steps of SVZ early post-natal organization (33). Here, we found a marked loss of neurons and oligodendrocytes as well as a functional impairment of the stem/transit amplifying cell population in cultures derived from early post-natal Twi mice. The remarkable decrease in neuroblast number that we found in the rostral migratory stream was likely the complex outcome of proliferative and chain assembly defects coupled to increased susceptibility to apoptosis occurring the lv-SVZ neuroblast cell population, which were mirrored in vivo in SVZ-derived neurosphere cultures. Interestingly, the trend for decreased numbers of Type C cells in the Twi lv-SVZ suggested that reduced production of neuroblasts from their transient amplifying precursors might contribute to the neurogenic defect. These abnormalities might be the result of a cell-autonomous defect or might be secondary to an altered microenvironment resulting from the tissue response to the progressive lipid storage, or both.

Brain inflammation is considered the most prominent consequence of CNS storage, resulting from activation of the innate immune system, with macrophages and microglia playing a crucial role. Neuroinflammation in GLD patients and animal models (1,42,43) might directly contribute to tissue damage. In addition, it may impact on post-natal neurogenesis, due to an established crosstalk between the immune system and NSC compartments (44). In line with a previous report (42), we show that activation of CNS inflammation in GLD precedes the onset of symptoms, involving elevation of multiple pro-inflammatory cytokines. Treatment of Twi mice started at the asymptomatic stage using two anti-inflammatory drugs with different mechanism of action resulted in down-regulation of several pro-inflammatory molecules and in clinical—pathological amelioration. These results indicate the relevance of the inflammatory compartment as a complementary/additional therapeutic target for GLD and similar LSDs. Importantly, the anti-inflammatory treatment ameliorated the clonogenic activity of SVZ primary cells in vitro, indicating a contribution of the inflammatory environment to the neurogenic impairment observed during the disease progression. Nevertheless, the limited rescued of proliferating/transit amplifying cells in the lv-SVZ and of neuroblast number and organization in the re-SVZ strongly suggested the concomitant presence of a cell-autonomous defect, also implying that functional abnormalities of the NSC compartment might be present in asymptomatic mice.

The neonatal/early PNDs of CNS development in mice roughly correspond to the third trimester of gestation in humans (http://www.translatingtime.net/). This is a period characterized by robust SVZ neurogenesis (14), which persists in the first post-natal months of life, to cease almost completely by the age of 2 (16). We have previously used primary neuronal/glial cultures and neurosphere cultures to model the cell type composition and NSC function during the critical steps of SVZ early post-natal organization (33). Here, we found a marked loss of neurons and oligodendrocytes as well as a functional impairment of the stem/transit amplifying cell population in cultures derived from early post-natal Twi mice. Quantification performed on n = 2 independent cultures, pool of 200–400 primary neurospheres/culture. Student’s t-test, *P < 0.05. (H) Digital autoradiography of a thin layer chromatography following steady state metabolic labeling with radioactive sphingosine shows LacCer accumulation in Twi PND2 primary spheres. The graph reports the quantification of radioactive bands for the different lipid species. Cer, ceramide; LacCer, lactosylceramide; GalCer, galactosylceramide; GlcCer, glucosylceramide; SM, sphingomyelin. Data are expressed as the mean ± SEM, n = 3 independent experiments. Two-way ANOVA, ***P < 0.001. Scale bars: 400 μm (C), 200 μm (A, E).
Figure 7. LV-mediated expression of functional GALC in Twi-derived stem/progenitors rescues clonogenic efficiency and normalizes psychosine and LacCer levels. (A) Primary neurospheres derived from bdLV-transduced Twi primary cells show a VCN ranging from 2 to 17 and the presence of 70–100% of GFP+ cells. The different intensity of GFP signal was assessed by direct fluorescence (representative pictures of primary neurospheres generated by untransduced (UT), bdLV.CTRL- and bdLV.GALC-transduced primary cells are shown) and by FACS analysis (% of GFP+ cells and MFI). Values are the mean ± SD, n=3. Scale bar, 200 μm. Functionality of the transgenic GALC enzyme was assessed by X-Gal colorimetric assay (B) and by direct measurement of enzymatic activity (C) on cells dissociated from Twi and WT primary neurospheres and grown on matrigel-coated cover slips in the mitogen-containing medium for 24 h. (C) GALC activity in bdLV.GALC-transduced Twi cells was ~5-fold higher then in bdLV.CTRL-transduced WT cells and inversely correlated with psychosine levels. (D) Digital autoradiography of a thin layer chromatography following steady state metabolic labeling with radioactive sphingosine shows that accumulation of LacCer species found in bdLV.CTRL-transduced Twi cells is normalized to physiological levels (bdLV.CTRL-transduced WT cells) in bdLV.GALC-transduced Twi cells. The graph reports the quantification of radioactive bands for the different lipid species. Cer, ceramide; LacCer, lactosylceramide; GlcCer, glucosylceramide; GalCer, galactosylceramide; SM, sphingomyelin. Data are expressed as the mean ± SEM, n=3 independent experiments. Two-way ANOVA, ***P < 0.001 versus WT; *P < 0.05 versus Twi bdLV.CTRL. (E–G) Expression of a functional GALC in PND2 primary Twi cells rescues
mice. Recent clinical observations show that the corticospinal tract (CST), whose myelination begins approximately at 30 weeks of gestation and is not completed until 2 years of age, is impaired in GLD-affected neonates (29). These early abnormalities may explain the plateau in motor development seen in HCT-treated infants even in the presence of improved overall brain myelination (2). Abnormalities in CST myelination were reported also in symptomatic Twitcher mice (45), but lack of time-course analysis in that study precluded knowing whether the defect was present at earlier ages. Our data suggest that the loss of oligodendrocyte progenitors might contribute to CST defects in neonatal/early post-natal Twi mice and, possibly, in infants. All our ex vivo data strongly suggest that apoptosis contributes to the loss of neuronal and oligodendrogial cell number in the context of GALC deficiency. The low numbers of apoptotic cells found in vivo, in the SVZ of Twi mice at all ages considered (data not shown) is likely due to the rapid clearance of apoptotic cells sustained by macrophages/microglial cells that we found abundant in the SVZ of Twi mice, displaying activated morphology and phenotype (46).

Early macrophage infiltration/microglia activation in the SVZ and in other brain regions (e.g. cerebellum) likely accounts for the increased chemokine levels detected in brain tissues of PND2 and PND10 Twi mice and supports the idea that these processes might play a role in the pathogenesis. Our data also suggest that a crosstalk between the microglial and the NSC/progenitor cell compartment might occur during early post-natal processes might play a role in the pathogenesis. Our data also suggest that a crosstalk between the microglial and the NSC/progenitor cell compartment might occur during early post-natal CNS development, similar to what happens in the adult rodent CNS (44). In addition to microglia activation, we detect marked upregulation of GFAP expression in several regions of asymptomatic Twi brains. Astrocytes are important in the neuro-inflammatory pathway (47) and GFAP up-regulation is the first histological sign of disease in neuronal ceroid lipofuscinoses, a severe neurodegenerative LSD (48). The moderate astrocytosis that we observed in the neonatal/adult SVZ of Twi mice when compared with other brain regions might be related to functional differences that characterize SVZ astrocytes when compared with prototypic astrocytes in the brain parenchyma (49).

The presence of a 5–6-fold increase in psychosine levels in neonatal Twi brains is in line with previous reports showing that this lysolipid accumulates in fetal and neonatal brain tissues of GLD patients and Twitcher mice, reaching >100-fold the physiological levels at the symptomatic stages of the disease (50,51). Psychosine is mainly synthesized in brain tissues of GLD patients and Twitcher mice, reaching >100-fold the physiological levels at the symptomatic stages of the disease (50,51). Psychosine is mainly synthesized by oligodendrocytes during the active myelination period, but previous data have shown the toxic effect of endogenous psychosine in cultured neurons and astrocytes (31). Our data indicate endogenous psychosine production in NSCs and progenitor cells and support the idea that NSCs tolerate intracellular levels of psychosine that are instead toxic to their committed neuronal and oligodendroglial progenitors (52), thus explaining the observed apoptotic death of neuronal and oligodendroglial cells in Twi-derived primary cultures. Selective psychosine toxicity might be related to the basal levels of GALC activity, which are up to 3-fold higher in committed progenitors and mature cells with respect to NSCs (40). We also hypothesize that the pro-apoptotic activity exerted by psychosine is counteracted by anti-apoptotic and pro-proliferative signals driven by mitogens present in the NSC culture medium. Nevertheless, psychosine accumulation might contribute to the clonogenic impairment observed in Twi primary precursors affecting other cell processes, such as raft-mediated endocytosis and cell signaling, possibly altering responsiveness to growth factors (53).

Glucosylceramide (GlcCer), lactosylceramide (LacCer) and their lyso-derivatives glucosylsphingosine (GlcSph) and psychosine mobilize intracellular calcium (54), which may trigger altered cell adhesion and motility, cytoskeleton rearrangements and process retraction, ultimately resulting in migration defects (55). We speculate that these events are responsible for the enrichment of TUJ1-expressing cells with round-shaped morphology in Twi cultures and contribute to the reduced cell migration observed from Twi SVZ explants and primary spheres. Defective calcium homeostasis as a consequence of GlcCer and GlcSph accumulation has been suggested as a cause for neuropathophysiology in acute neuropathic Gaucher disease (56). In addition, psychosine persistence in lipid rafts might directly impact cell adhesion and anchoring required for normal extension of neuronal processes (57).

In conclusion, our comprehensive analysis indicated that GALC deficiency affects stem/progenitor cell survival, proliferation and self-renewal in the SVZ niche in a cell-autonomous manner. The overall neurogenic defect in mutant Twi mice is due to the contribution of all these mechanisms and to additional inputs of non-cell autonomous origin, whose relative weight dynamically changes as a function of age and disease progression. Little storage of psychosine and LacCer resulting from the genetic GALC deficiency (cell autonomous origin) causes metabolic unbalance, directly inducing apoptosis and functional impairment of NSCs and progenitors in the neonatal/early post-natal SVZ. As a consequence, early astrocytosis, microglia activation and initial upregulation of pro-inflammatory molecules occur. Progressive storage in SVZ and non-SVZ tissues during disease progression further increases inflammation (non-cell autonomous mechanism) and worsen the pathology. This contributes to the neurogenic defect and, more importantly, drives oligodendrogial and neuronal death in CNS tissues, thus resulting in overt tissue damage and neurologic dysfunction. These results further support the idea that despite being a monogenic disease, GLD has a complex multi-component pathology.

Importantly, we show here that restoration of GALC activity in ex vivo-isolated Twi NSCs and progenitor cells obtained by means of LV-mediated gene transfer normalizes intracellular psychosine and LacCer levels, reversing the self-renewal, proliferation and migration defects. However, in a probable therapeutic scenario, delivery of therapeutic vectors will be performed in non-SVZ regions, which might be more accessible while ensuring enhanced transgene dispersal.

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**P-values:** $P < 0.001$, $P < 0.01$, ns, not significant. Scale bars: 300 μm (A), 150 μm (B), 500 μm (F), 100 μm (G).
Alternatively, exogenously transplanted cells might be the source of functional enzyme in CNS tissues (8,40,60). In this perspective, enzymatic rescue of SVZ cells and tissues will rely on the cross-correction mechanism that is the ability of functional lysosomal enzymes to be secreted by naive or gene-corrected cells in the extracellular space (5,58,59).

Figure 8. Rescue of GALK expression and activity in Twi NSCs and in their progeny following NSC transplantation. (A) GALK enzymatic activity on brain tissues (pool of cortical and cerebellar tissues), SVZ tissues, SVZ-derived primary cells and primary neurospheres from NSC-transplanted Twi mice (trTwiGALK), UT Twi and UT littermates analyzed at PND10 and PND40. Tissues from three mice/group were pooled in each experiment (n) to perform enzymatic assay. Data are expressed the mean ± SEM for n = 3. Values marked by the asterisk come from one experiment. (B) Expression of functional GALK in primary cells from trTwiGALK mice ameliorates neurosphere-forming ability (assessed in PND10 and PND40 mice; n = 2–5 independent cultures, 4–8 replicates/experiment). Representative pictures (brightfield+epifluorescence) of primary neurospheres in the different groups are shown. Arrow indicates a GFP + neurosphere, derived from engrafted NSCs. Only GFP-negative spheres were considered to calculate clonogenic efficiency. (C–E) Quantification of the immunopositive area after double-labeling IF analysis using anti-TUJ1 (C), anti-O4 (D) and anti-C3 (E) antibodies in SVZ-derived primary cultures from PND10 UT WT, UT Twi and trTwiGALK mice analyzed after 10 DIV. (F–G) Representative confocal merged pictures and quantification of double-labeled cells following IF analysis show reduced numbers of apoptotic neurons (C3+TUJ1+, arrows; F) and apoptotic oligodendrocytes (C3+O4+; G) in trTwiGALK-derived cultures. TUJ1 and O4, red; C3, green. Quantification was performed on n = 3 independent cultures, two to three cover slips/marker, five to eight fields/cover slip. From (B) to (G): one-way ANOVA and Dunn’s post-test. ***P < 0.001, **P < 0.01, *P < 0.05; ns, not significant. Scale bars: 200 μm (B), 100 μm (F, G).
and to be taken up by adjacent affected cells, mainly through a mannose-6-phosphate receptor-mediated mechanism (61). It has been previously reported that minimal levels of GALC replacement obtained by enzymatic cross-correction are insufficient to reverse psychosine-based lipid raft defects in vitro but are relatively unsuccessful in vivo, in bone marrow-transplanted Twitcher mice (31). The high transduction efficiency of bdLV.GALC allowed us to achieve supraphysiologic GALC levels in gene-corrected Twi cells. We know from our previous studies that GALC-overexpressing NSCs secrete high amounts of GALC that is available for cross-correction of Twi-deficient cells, both in vitro and in vivo, following intracerebral gene delivery (5) or NSC transplantation (40). We report here long-lasting (up to PND40) normalization of clonogenic activity as well as reduced apoptosis in primary precursors and committed neuronal and oligodendroglial progenitors derived from the SVZ of Twi mice transplanted at birth with GALC-overexpressing NSCs. These data strongly suggest that CNS-directed gene/cell therapies performed in early postnatal life rapidly provide levels of enzymatic activity sufficient to restore function in the SVZ niche as well as in other CNS cell types through in vitro cross-correction. Concurrent treatments able to reconstitute GALC activity in non-CNS tissues in the appropriate time-window of opportunity might ultimately result in significant clinical–pathological amelioration.

MATERIALS AND METHODS

Mice

Twitcher mice bear a spontaneous point mutation resulting in a premature stop codon and no residual GALC activity (62). FVB/Twitcher mice were generated in our animal research facility by breeding heterozygous Twitcher (+/−) C57BL6 mice with WT (+/+ ) FVB mice (Jackson Laboratory). FVB/Twitcher (−/−) are referred to as Twi mice in this study. Age-matched FVB/Twi (+/+) littermates were used as WT controls for Twi mice in all the experimental conditions. Twi mice show a slower progressive form of the disease than the canonical Twitcher mice and have more numerous litters. Tremors develop at around PND21, and progress to severe resting tremor, weight loss, paralysis and wasting of hind legs. At 40 days of age, the PNS is severely demyelinated, while CNS tissues show a milder and patchy demyelination. Death occurs within 45–50 days of age. Mouse colony was maintained in the animal facility of the Fondazione San Raffaele del Monte Tabor, Milano, Italy. All procedures were performed according to protocols approved by an internal Animal Care and Use Committee (IACUC #314, #420) and were reported to the Italian Ministry of Health.

Drugs

Minocycline and indomethacin (40 mg/kg and 10 mg/kg, respectively; Sigma-Aldrich) were injected i.p. daily starting at PND10. Mice were analyzed at PND20, PND30 and PND40 or monitored for survival until the human endpoint (less than 50% of the weight of WT littersmates). Bromodeoxyuridine (BrdU; 40 mg/kg; Sigma-Aldrich) was injected i.p (1 injection and 4 injections—one every 2 h—in mice <PND10 and >PND20, respectively). Drugs were dissolved in saline and used immediately. Saline-treated mice were used as controls.

Cell cultures

Primary mixed neuronal/glial culture and neurosphere cultures were established from Twi mice and WT littermates, as previously described (33,40). Briefly, brains were removed and tissue containing the SVZ of the forebrain lateral ventricles was dissected out. Tissues from three to six mice were pooled to obtain the primary cell suspension.

Neurosphere cultures

Primary cells derived from PND2, PND10, PND30 and PND40 mice were plated (2500 cells/cm2; 5000 cells/1 ml dish in 24-well plates) in a chemically defined, serum and growth factor-free medium (control medium) containing basic FGF (FGF2; Peprotech; 10 ng/ml) and EGF (Peprotech; 20 ng/ml) (growth medium). Under these culture conditions, a fraction of the cells proliferate to form floating clonal primary spheres (neurospheres). The number of primary neurospheres was counted after 7 days. Cloning efficiency was expressed as the number of neurospheres counted in each well/number of cells plated × 100.

Primary mixed cultures

Primary cells derived from PND2, PND10 and PND40 mice were plated on Matrigel-coated cover slips (growth factor reduced; BD Biosciences; 80,000 cells/cm2) in control medium in the presence of 2% FBS (Euroclone). After 8, 10 and 15 days in vitro (DIV), cultures were fixed with paraformaldehyde (PFA) 4% and processed for indirect immunofluorescence assay.

Tissue explants containing the lv-SVZ from PND2 mice and SVZ wholemounts from PND40 mice were prepared as previously described (32,34).

NSC transplantation

Transplantation of NSCs in the lateral ventricle of newborn mice (PND2) was performed as described (40). GALC-overexpressing serially passaged NSCs used for intracerebral transplantation experiments were prepared, characterized and used as previously described (40). Briefly, serially subcultured WT NSCs (passages 5–10) were transduced using bdLV.GALC (MOI 100). LV-transduced neurosphere bulk cultures (5–10 subculturing passages post-transduction, corresponding to 20–50 days in culture) and matched serially subcultured Twi NSCs were enzymatically dissociated (Accumax, Sigma-Aldrich; 4 min at 37 °C) and plated in growth medium. Cells were collected 48 h later, centrifuged, mildly mechanically dissociated, counted by Trypan Blue exclusion (viability >90%) and resuspended (2.5 × 105 cell/µl) in PBS+ 0.1% DNase (Sigma-Aldrich). Cells were kept in ice and used within 1 h. Cell viability assessed at the end of the
transplantation procedure was >80%. Animal heads were trans-illuminated in order to identify the lateral ventricles and NSC suspensions were rapidly injected (1 μl/injection site; bilateral) through trans-cutaneous insertion of the tip of a hand-drawn glass capillary without exposing the skull. Coordinates for ventricular injections were assessed in pilot dye injection experiments. The overall survival of injected mice was ~90%.

GALC activity was 37.4 nmol*h/mg in untransduced WT NSCs and 205.88 nmol*h/mg in bdLV.GALC-transduced WT NSCs used for transplantation. Twi NSCs display no residual GALC activity.

Experimental groups
UT Twi (n = 20), UT WT (n = 23), NSC-transplanted Twi (n = 22), in six separate experiments; NSC-transplanted mice and UT controls were euthanized at PND10 (n = 51; four experiments) and PND40 (n = 14; two experiments) and SVZ cells were isolated and plated for neurosphere-forming assay and to generate primary mixed cultures, as described above.

Tissue isolation and processing
A group of mice were killed by CO₂ exposure and decapitated. The two brain hemispheres were separated and either quickly frozen in liquid nitrogen or immediately processed to obtain tissue extracts. Another group of mice was anesthetized with Avertine (2, 2, 2-Tribromoethanol; Sigma-Aldrich) and intracardially perfused via the descending aorta with ice-cold 0.9% NaCl followed by 4% PFA (Sigma-Aldrich) in 0.1 M PBS (Euroclone). Brains were collected, equilibrated for 24 h in 4% PFA and included in 4% agarose. Serial coronal vibratome sections (six series, 40 μm thick) were processed for histology and immunofluorescence analysis.

LV-mediated gene transfer
Bidirectional LVs allowing the coordinate expression of two transgenes driven by the human phosphoglycerate kinase promoter were produced and titrated as described previously (5): the control vector (bdLV.CTRL) encodes for two reporter genes (GFP and the truncated form of the low affinity nerve growth factor receptor, ΔNGFR). The therapeutic vector (bdLV.GALC) was generated by replacing the ΔNGFR cDNA of bdLV.CTRL with the murine GALC cDNA C-terminally tagged with the human influenza hemagglutinin epitope. Two hours after plating, primary cells were incubated overnight with bdLV.GALC or bdLV.CTRL (10⁷ TU/ml, MOI 100). The LV-containing medium was then removed, fresh medium added and cells incubated for additional 7 days in order to obtain primary neurospheres or plated onto matrigel coated cover slips to obtain primary mixed cultures. We assessed the transduction efficiency measuring the VCN by qRT-PCR and GFP fluorescence [percentage of GFP+ cells and mean fluorescence intensity (MFI)] by FACS analysis, using untransduced cells as negative control (5).

Immunohistochemistry and immunofluorescence
BrdU immunohistochemistry
Free-floating vibratome sections were incubated for 10 min in 3% H₂O₂ in methanol; after washing in 0.1 M PBS (3 × 5 min), they were incubated for 20’ at 54°C in a denaturing solution (60% formamide, SSC 2X—0.3 M sodium chloride, 30 mM sodium citrate; all from Sigma-Aldrich). Then, samples were rapidly washed with SSC 2X and incubated with 2N hydrochloric acid for 30’ at 37°C, equilibrated with 0.1 M pH8.5 boric acid for 10’ and then blocked with the blocking solution. Anti-BrdU antibody was added and incubated o/n at 4°C. After 3 × 10 min washings with 0.1 M PBS, slices were incubated with biotinylated secondary antibody in 0.1 M PBS, 1% NGS. After 3 × 10 min washings with 0.1 M PBS and 5 min incubation with 100 mM pH 7.5 Tris–HCl, slices were incubated 1 h with the VECTASTAIN ABC kit (PK-6100Vector Laboratories). After 3 × 10 min washings with 100 mM pH 7.5 Tris–HCl, reaction with the substrate 3–3 diamino-benzidine tetrahydrochloride (DAB, 167 μg/ml in Tris–HCl 100 mM + H₂O₂ 1:3.000) was performed. Slices were dehydrated and mounted with EUKITT. Samples were visualized with a Nikon Eclipse E600 microscope. In case of double labeling with lineage-specific markers, the protocol for immunofluorescence on tissues was followed after the blocking passage.

Image acquisition
Samples (cell cultures and tissues) were visualized with: (i) a Nikon Eclipse E600 epifluorescence microscope; images were acquired using a Nikon DMX 1200 digital camera and ACT-1 acquisition software (Nikon); (ii) a Zeiss Axioplan2 microscope using double laser confocal microscopy with a Zeiss Plan-NeoFluar objective lens (Zeiss, Arise, Italy); images were acquired using a Radiance 2100 camera (Bio-Rad, Segrate, Italy) and LaserSharp 2000 acquisition software (Bio-Rad). Images were imported into Adobe Photoshop CS4 and adjusted for brightness and contrast.

Montages were generated using the Photomerge function of the Adobe Photoshop CS4 software starting from a variable number (4 to 12) of low power images (×10 or ×20 magnification).

For quantification of TUJ1, GFAP, O4, C3 and DAPI immunofluorescence, 4–10 fields/cover slips were photographed at ×20 magnification using a threshold defined on cultures in which primary antibodies were omitted. For quantification of lecin histochemistry, CD68, Iba1 and GFAP immunohistochemistry, pictures of defined brain areas were sequentially acquired at ×10 magnification using a threshold defined on the signal level of WT tissues (for lectins) or of tissues in which the primary antibodies were omitted (CD68, Iba1 and GFAP). Images were analyzed by the ImageJ software to quantify signal intensity corresponding to immunopositive areas (expressed as pixels) for each marker and for nuclei (when appropriate). In order to minimize the bias and variability of the analysis, samples to be compared for signal...
quantification of a given marker were photographed and analyzed simultaneously.

For counting of lineage-positive cells (neurons, astrocytes, oligodendrocytes), 1000–2000 cells/cover slips were analyzed. For the double BrdU+DCX+ cells counting on tissue slices, about 100 BrdU+ cells were analyzed and checked for DCX expression using a ×63 oil objective confocal microscope. For apoptotic cell counting, 200–500 TUJ+ or O4+ cells/cover slips were analyzed and checked for C3 expression using a ×40 or ×63 oil objective confocal microscope.

To ensure blind quantification and counting, all slides were randomized and coded with an identification number. The code was then revealed once results were tallied.

### Electron microscopy

Mice were perfused with 2% glutaraldehyde +1% PFA in 0.1 M PBS. After dissection, brains were post-fixed for 2 h and cut with a vibratome (300 μm thick slices). Vibratome sections were fixed in osmium-ferrocyanide for 1 h, stained with 1% uranyl acetate, dehydrated and embedded in Araldite. Ultra-thin sections were examined under a Philips CM10 transmission electron microscope.

### Migration assay

Single tissue explants and individual primary neurospheres were picked and transferred onto cover slips in a drop of Matrigel/growth factor-containing medium (1:2, vol:vol). After gelification (15 min at 37°C), control medium (no growth factors) was added and explants/spheres were incubated for 24–48 h. Pictures were acquired and analyzed using the ImageJ software. The migratory area was expressed as the percentage of the area surrounding the core of the explant/sphere (corona) over the total area (core + corona).

### Reverse transcription and qRT-PCR

Total RNA from tissues was extracted according to the manufacturer protocol of TRIZOL Reagent (Invitrogen). mRNA reverse transcription was performed according to the manufacturer protocol of QuantiTect reverse transcription kit (Qiagen). qPCR was performed in Optical 96-well Fast Thermal Cycling Plates (Applied Biosystem) on ABI PRISM 7900 Sequence Detector System (Applied Biosystem), using the following thermal cycling conditions, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 30 s. Each sample was run in triplicate in a total volume of 25 μl/reaction, containing 12.5 μl 2× QuantiFast SYBR Green PCR Master Mix, 2 μl of template cDNA and 1 μM QuantiTect Primer Assays (all from Qiagen). Relative expression of mRNA for the target genes was performed by the comparative C_{\text{T}} (ΔΔC_{\text{T}}) method using the β-actin gene as control. The relative mRNA levels were expressed as fold change (Twi over WT or Twi-drug treated over Twi UT/saline-treated).

### Western blot

Cells and tissues were lysed with radioimmunoprecipitation assay buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) added with protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. Proteins were quantified by means of Bradford assay (BioRad) and normalized on an albumin standard curve. Forty micrograms of total protein from brain tissue lysate were re-suspended in sample buffer, heated for 5 min at 95°C and separated via SDS–PAGE under reducing conditions. Western blotting was performed with standard procedure and the PVDF membrane (Millipore) was hybridized using the antibodies indicated in Supplementary material, Table S1.

### Lipidomic analyses

Tissues were weighted and homogenized in ice-cold 70% methanol in water. The homogenate was adjusted to a final concentration of 100 μg/ml. Lipid analyses were performed by mass spectrometry at Zora Biosciences Oy (Espoo, Finland). Under the experimental conditions used, glucosylceramide and galactosylceramide were not resolved in two separate species. Thus, we refer to this species as Glc/GalCer represents the contribution of both species.

### Thin layer chromatography following steady state labeling with [1-^3H]sphingosine

Cells were pulsed for 2 h with 3×10^{-8} M [1-^3H]sphingosine dissolved in cell-conditioned medium (5 ml/dish) and chased for 48 h. Under these conditions, free radioactive sphingosine was barely detectable in the cells and all sphingolipids, including ceramide, sphingomyelin, neutral glycolipids and gangliosides were metabolically radiolabeled. Tritium-labeled phosphatidylethanolamine was also obtained due to the recycling of radioactive ethanolamine formed in the catabolism of [1-^3H]sphingosine. The radioactivity associated with cells was determined by liquid scintillation counting and was similar in all cell preparations. Lipid extraction, phase partitioning, alkaline treatment on the organic phases, monodimensional TLC and radioimaging were performed as described previously (63). Under these experimental conditions, glucosylceramide and galactosylceramide were not resolved in two separate bands. Thus, the band we refer to as GlcCer represents the contribution of both species.

### GALC activity

GALC enzymatic activity on brain, SVZ tissues and cell cultures was measured using a modified X-Gal histochemistry according to a described protocol (64) or on cell lysates, using a standardized biochemical assay (65).

### Psychosine quantification

Tissues and cell pellets were frozen in liquid nitrogen. Dimethylpsychosine was added to brain-homogenates (1 mg of protein) and cell-homogenates (0.2–0.5 mg of protein) as internal standard. After extraction by methanol:chloroform and evaporation to dryness of the organic layer, the sample extract was reconstituted in acidic methanol. After injection and elution over a C8 analytical column, psychosine and its internal standard were detected by positive ESI in the MRM
mode on a TSQ Quantum AM mass spectrometer. Psychosine concentrations were established by the use of calibration standards prepared in the corresponding matrix (brain tissues or cells). Concentrations were expressed per mg protein.

**Statistics**

*In vitro* and *in vivo* cell counts and data obtained following the quantification of immunopositive area by the ImageJ software were analysed with GraphPad Software and expressed as the mean ± SEM. Unpaired Student *t*-test, one or two-way ANOVA followed by Bonferroni post-tests were used when appropriate (statistical significance: *P* < 0.05). One-way ANOVA was used to compare >2 sets of data, and two-way ANOVA was used in case of evaluating the effect of two variables. Non-parametric tests were used in case of non-Gaussian distribution of the data (evaluated by the normality test). The log-rank test was used to compare Kaplan–Meier survival curves.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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