Signal requirement for cortical potential of transplanted human neuroepithelial stem cells

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The cerebral cortex develops from dorsal forebrain neuroepithelial progenitor cells. Following the initial expansion of the progenitor cell pool, these cells generate neurons of all the cortical layers and then astrocytes and oligodendrocytes. Yet, the regulatory pathways that control the expansion and maintenance of the progenitor cell pool are currently unknown. Here we define six basic pathway components that regulate proliferation of cortically specified human neuroepithelial stem cells (cNESCs) in vitro without the loss of cerebral cortex developmental potential. We show that activation of FGF and inhibition of BMP and ACTIVIN A signalling are required for long-term cNESC proliferation. We also demonstrate that cNESCs preserve dorsal telencephalon-specific potential when GSK3, AKT and nuclear CATENIN-β1 activity are low. Remarkably, regulation of these six pathway components supports the clonal expansion of cNESCs. Moreover, cNESCs differentiate into lower- and upper-layer cortical neurons in vitro and in vivo. The identification of mechanisms that drive the neuroepithelial stem cell self-renewal and differentiation and preserve this potential in vitro is key to developing regenerative and cell-based therapeutic approaches to treat neurological conditions.
The cerebral cortex develops from the forebrain vesicle of the mammalian neuroepithelium. Forebrain specification of the anterior neuroepithelium happens shortly after the neural commitment of the epiblast. After this initial specification, dorsal forebrain neuroepithelial cells divide symmetrically to expand the progenitor cell pool. Upon neural tube closure, cells divide both symmetrically to expand the progenitor cell pool and asymmetrically to produce differentiated cells. During early forebrain development, differentiating neural progenitors initially give rise to glutamatergic neurons of the six cortical layers, each with specific connectivity to other brain regions. At later stages, progenitor cells stop producing neurons and start generating astrocytes and oligodendrocytes.

While the dynamics of neural cell fate production from dorsal forebrain neuroepithelial cells are well established, the molecular mechanisms that instruct their expansion and maintenance remain poorly understood. It is known that preservation of multipotent neural progenitor cells in the forebrain requires fibroblast growth factor (FGF) receptor activity. However, neuroepithelial cells isolated from the embryonic rodent telencephalon show limited proliferation in FGF alone. Complementation of FGF with undefined components of brain-vessel endothelial cell conditioned media can extend the proliferation capacity of mouse neuroepithelial progenitors and preserve their early cortical potential transiently, indicating that the cells can self-renew if the precise signals are present.

The investigation of neural progenitor cell potency, cortical development or pathogenesis in humans is restricted by ethical and technical boundaries. This limitation has been overcome by the in vitro use of human pluripotent stem cells; that can be used to model the early developmental potential of the cells required for the self-renewal of the cells. Transplantation of neural progenitors was not accomplished or the differentiation potential of the proliferating progenitors was not cortical.

Here we identify six signalling pathway components that instruct permanent self-renewal and fate preservation of dorsal forebrain neuroepithelial stem cells. In addition to FGF, self-renewal requires the simultaneous inhibition of BMP and ACTIVIN A signalling, reduced GSK3 and AKT activity and low levels of β-catenin (CTNNB1) nuclear activity. We achieve this complex regulation with six factors (6F condition). This results in permanent culture and expansion of cortical specified neuroepithelial cells (cNESCs), which express the dorsal telencephalic markers FOXG1, PAX6, EMX2, LHX2 and OTX1/2, and give rise to glutamatergic projection neurons of lower and upper cortical layers, as well as oligodendrocytes and astrocytes. The 6F condition is sufficient for single cell-derived cNESC colony formation and preserves the embryonic dorsal forebrain-specific differentiation potential of the cells. We also demonstrate that developmental signalling pathways implicated in neural tissue development, such as EGF, SHH and NOTCH signalling, are not required for the self-renewal of the cNESCs. Transplantation of cNESCs into the mouse brain demonstrates that these cells can differentiate into active glutamatergic projection neurons in the adolescent cortex and receive synaptic inputs. Our results show that cells with dorsal forebrain specification can be maintained by selective pathway regulation for long-term in vitro and preserve their early developmental potential.

**Results**

**Generation of cortical neuroepithelial cells.** We established dorsal forebrain-specific neuroepithelial stem cells from human pluripotent stem cells (hPSCs) using chemical inhibitors of TGFβ (TGFβRI) and BMP (BMPRI) signalling (Fig. 1a and Supplementary Fig. 1a–c, Supplementary Table 1). Similar to other neural induction protocols, efficient neural conversion of hPSCs was seen by day 8 in the majority of cells (>95%; Supplementary Fig. 1a), as indicated by the upregulation of neuroepithelial marker, SOX1 (Supplementary Fig. 1b,c), downregulation of pluripotency marker, OCT4 (Supplementary Fig. 1a), and maintenance of neuronal stem cell marker, SOX2. We confirmed neural specification by examining the expression of OCT4 and NESTIN, a neural progenitor cell marker in day 11 differentiated NESCs compared to undifferentiated hPSCs. We observed decreased OCT4 and increased NESTIN mRNA expression in induced NESCs (Supplementary Fig. 1d), which was similar to mRNA expression levels in human foetal forebrain-derived neural stem cells [CB660](21). When we examined forebrain specification in these cells, we found that induced NESCs showed increased expression of EMX2, PAX6, FOXG1 and OTX2, region-specific transcription factor mRNAs that together mark the dorsal forebrain vesicle (Supplementary Fig. 1d), and no change in the expression of ventral forebrain markers LHX6 and NKX2.1, ventral midbrain markers FOXA2 and EN1, and hindbrain marker HOXB1 (Supplementary Fig. 1d). Immunocytochemistry confirmed the expression of SOX1, SOX2, FOXG1, NESTIN and PAX6 markers (Fig. 1b–e). These results indicate efficient induction of dorsal forebrain/cortex specified NESCs from hPSCs within 8–11 days, which we termed cNESCs.

**Regulation of TGFβR and GSK3 regulates maintenance of cNESCs.** Several studies have shown that dorsal forebrain specification is lost during long-term culture of neural stem cells in FGF and EGF. We tested whether additional extracellular signals are required to maintain FGF-dependent self-renewal of cNESCs. We investigated modulation of the WNT, BMP/TGFβ, EGF, SHH, NOTCH pathways, which have all been implicated in the regulation of cortical excitatory neuron specification.

WNT signals are important positive regulators of forebrain fate and induce cell proliferation. WNT signal is mediated via the inhibition of GSK3, which can upregulate CTNNB1 and BMP/TGFβ/SMAD signalling and alter neuroepithelial cell specification. Therefore, we tested if FGF in combination with inhibitors of GSK3 (GSK3i), BMPRI and/or TGFβRI, could maintain the SOX1-positive cNESC population. After neural induction, cNESCs-derived from 3 different human Embryonic Stem Cell (hESC) lines (H1, H9 and CA129) were propagated for five passages in neural maintenance media with FGF and various combination of inhibitors (Fig. 1b). Both FGF alone and FGF and EGF were insufficient to maintain a high ratio of SOX1 positive cells. Addition of (4) BMPRI, (5) TGFβRI, (6) GSK3i, (7) GSK3i and TGFβRI and (8) BMPRI and TGFβRI with FGF reduced the ratio of SOX1 positive cells, while addition of (9) FGF, GSK3i and BMPRI showed no change compared to cNESCs in (2) FGF alone or (3) FGF and EGF. Strikingly, we found that neural cells derived from all three hESC lines cultured in FGF and all three inhibitors (termed 4F for 4 factor condition) contained the highest proportion of SOX1-positive cNESCs (mean value 86%; Fig. 1f). Conditions 2, 4, 5, 6 and 8 did not support the propagation of at least one of the three different hESC lines and led to significant cell death, suggesting cell line-dependent variation.

In agreement with other studies, we found that cNESCs showed limited proliferation in FGF alone. To determine whether 4F could improve the long-term proliferation of SOX1-positive cells we compared the growth of cNESCs in: (a) FGF alone, (b) EGF and FGF, (c) 4F using chemical inhibitors (FGF, LDN193189 (BMPRI), SB431542 (TGFβRI), CHIR99021 (GSK3i), abbreviated as FLSC) or d) 4F using protein antagonists (FGF,
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NOGGIN (BMPi), FOLLISTATIN (ACTIVINi), and CHIR99021 (GSK3i), abbreviated as FNFC. Confirming previous findings, after six passages, cNESCs in FGF alone and FGF and EGF had a lower proliferation rate than in previous passages. In contrast, cNESCs in both FLSC and FNFC showed sustained proliferation and supported the formation of neuroepithelial colonies (Fig. 1g, h). To confirm that the expansion of cNESCs in 4F was not associated with any specific chromosomal abnormalities we examined the karyotype of cNESCs maintained for over 100 days in vitro (more than 50 population doublings) in two independent cell lines and found no chromosomal aberrations (Supplementary Fig. 1e).

Fig. 1 Induction and maintenance of SOX1 positive dorsal forebrain neuroepithelial cells. a Neural induction scheme of hPSCs in the presence of TGFbR and BMPR inhibitors. After cortical neural induction NES cells express NEST1 in SOX2 positive cells b SOX1 in TUBB3 negative cells c, FOXG1 d, and PAX6 e in SOX2 positive cells. The experiment was repeated with 5 biologically independent cell lines. Scalebar: 25 μm f SOX1 positive cell ratio in passage 5 cultures of hES (H1, H9, CA1) derived cNESCs treated with various combination of factors (n = 3 independent cell lines, 10 datapoints per each cell line per group, red bars are mean ± SEM, one-way ANOVA, Tukey’s test). g Normalised model of cell number changes of cNESCs (Data are presented as mean ± SEM, 2-way ANOVA, Dunnett’s test) over 10 passages (30 days). h Phase contrast image of H1 derived cNESCs on day 4 (top) and on day 14 of culture (bottom) forming rosette structures. The experiment was repeated with 4 biologically independent cell lines. Scalebar: 50 μm. i Colony formation assay of cNESCs cultured in 4F prior to seeding at 200 cells/cm² density. j Schematic presentation of developmental signalling pathway components targeted in our assay, protein ligands are in bold and chemical inhibitors are in italic, 4F components are in red. k Quantification of cell number changes after 96-h treatment of cNESCs with indicated ligands or chemical inhibitors compared to 4F condition (n = 3 independent experiments, data are presented as mean ± SEM, 1 way ANOVA, Tukey’s test, white bars are p < 0.01, grey bars are not significantly different from control, red line indicates starting cell number). Source data are provided as a Source Data file.
Next, we tested if 4F was able to support the proliferation of a single cNESC in the absence of additional signals that could be produced by adjacent cells. We plated cNESCs previously maintained in 4F at clonal cell density in either 4F or FGF alone, without supporting cells or conditioned media. cNESCs in FGF alone rarely formed colonies (1 in 3000 cells, n = 3) both at low and high oxygen levels (3 and 20%; Fig. 1i and Supplementary Fig. 1f). In contrast, single cNESCs in 4F supported single cell derived colony formation at both low and high oxygen levels at an efficiency of around 1%, demonstrating that four pathways can maintain the neuroepithelial specification of a single cNESC (Fig. 1i and Supplementary Fig. 1f).

4F did not include developmental factors previously shown to support neural progenitor cell maintenance in vitro, such as EGF, NOTCH ligands or SHH. Therefore, we tested their role in cNESC proliferation as we had observed a decrease in the proliferation of cNESCs in FGF or FGF with EGF (Fig. 1j, k; bars 14, 15). Although cNESCs can activate all three receptors (Supplementary Fig. 1g–j), the inhibition of these developmental signalling pathways had no effect on the proliferation of cNESCs in the presence of the 4F (Fig. 1k).

Our signalling screen showed that a change in the activity of any factor in the 4F condition affects cell proliferation even when the other three components are present (Fig. 1k); activation of ACTIVIN A signalling, removal of GSK3 inhibitor or inhibition of the FGF receptor by PD173074 all caused a reduction in cell proliferation (Fig. 1k). Our results suggest that FGF signalling is mediated by both the MAPK (bar 7) and PI3K (bar 8) axis, as inhibition of either pathway significantly reduced cNESC numbers (Fig. 1k). Moreover, AKT (bar 9) inhibition did not interfere with the cell proliferation in 4F, suggesting that high activity of AKT is not required for PI3K signalling in cNESC maintenance (Fig. 1k).

In summary, we determined that FGF signalling through the MAPK and PI3K pathways and inhibition of GSK3 and SMAD signalling are required for cNESCs maintenance, while additional developmental signals like NOTCH, EGF and SHH are not required.

**Multitopic cNESCs differentiate to glutamatergic neurons.**

Next, we tested whether the differentiation potential of the cNESCs was maintained after prolonged culture. We differentiated cNESCs maintained in vitro for 75 days. After 30 days of differentiation, cultures contained β3-tubulin (TUBB3)-positive neurons (approx. 60% of cells) that also expressed MAP2 and NEUN (approx. 40% of cells) (Fig. 2a, b), and Synaptophysin (SYP) positive presynaptic complexes were detectable on MAP2-positive dendrites (Fig. 2b) indicating that cNESC derived neurons started to mature. After neuronal differentiation cNESCs could also differentiate into GFAP-positive astrocytes, and O4-positive oligodendrocytes (Fig. 2a, c). Thus, confirming that cNESCs maintain multipotency during long-term culture.

To further characterise the types of cortical neurons produced, we analysed their neurotransmitter expression. The majority of cells were glutamatergic projection neurons (vesicular glutamate transporter type 1 (VGLUT1)-positive) (Fig. 2d). GAD67 positive and vGAT positive interneurons comprised around 10 and 2%, respectively, of the total number of neurons (Fig. 2e, f).

Next, we determined whether cNESC-derived neurons were electrophysiologically active mature cells. After 45 days of in vitro...
With increased depolarizing voltage steps, we were able to detect both fast voltage-gated Na⁺ currents and subsequent voltage-activated K⁺ currents (Fig. 2k), confirming the presence of necessary ion channel components for action potentials. Moreover, addition of glutamate receptor agonist N-methyl-D-aspartic acid (NMDA, 1 mM) and consequent activation of postsynaptic receptors resulted in the induction of excitatory membrane currents (Fig. 2l). Together,
these results demonstrate the functional maturation of neurons derived from cNESC.

GSK3 and CTNNB1 regulation maintains proliferative cNESC. Our data showed GSK3 inhibition improved cNESC proliferation in FGF alone or in the 4F (Fig. 1g), however early anterior neuroepithelium specification requires low CTNNB1 activity. Therefore, we tested whether forebrain specification is maintained in the presence of GSK3 inhibition. Gradually increasing GSK3 inhibition (from 0 to 3 μM GSK3i/CHIR) in cNESC caused an increase in the proliferation rate of cNESC and an immediate decrease in the number of FOXG1-positive cNESC (Fig. 3a, b and Supplementary Fig. 2a). Increasing levels of GSK3 inhibition were associated with an increase in the mRNA levels, and a decrease in AXIN2 expression of canonical WNT/CTNNB1 target genes AXIN2 and LEF1, and a decrease in EMTX2, FOXG1 and OTX2 mRNA levels, whereas PAX6 mRNA level did not change (Fig. 3c). Long-term maintenance of cNESC in the 4F media reduced the potential of the cells to differentiate to TBR1 and BCL11B (Ctip2) positive deep layer neuronal subtypes compared to direct differentiation of cNESC without culture in 4F (Supplementary Fig. 2b). Together, these results suggested that low levels of both GSK3 and CTNNB1 activity are needed to maintain dorsal forebrain specification in cNESC.

We aimed to establish the conditions that support cNESC proliferation while maintaining dorsal forebrain specification by regulating the WNT/CTNNB1 activity by lowering the GSK3 activity in combination with low CTNNB1 transcriptional activity. We tested two different chemical inhibitors of CTNNB1 signalling, XAV939 and KY02111 (Fig. 3d). XAV939 inhibits Tankyrase activity, which leads to an increase in AXIN2 levels and reduces CTNNB1 level in the nucleus. KY02111 was shown to inhibit CTNNB1 function downstream of GSK3 during cardiomyocyte specification by an unknown mechanism. High GSK3 inhibition (3 μM) rapidly increased mRNA expression of LEF1 in cNESC (Supplementary Fig. 2c). In comparison to GSK3 inhibition alone, GSK3 inhibition together with Tankyrase inhibition by XAV939 reduced the transcription of LEF1 to control levels while the addition of KYO2111 had no effect. Therefore, we tested whether dual inhibition of GSK3 and CTNNB1 transcriptional activity could maintain FOXG1 and PAX6 expression in SOX2 positive cNESC after neural induction. Six days after replating the cells with or without high GSK3 inhibition (3 μM) reduced the number of both FOXG1 and PAX6 positive cNESC, and this effect was compensated by the presence of 1μM XAV939 (Fig. 3e). Decreasing the concentration of GSK3 inhibitor to 2 or 1 μM in the presence of 1μM XAV939 preserved the expression of FOXG1, PAX6 and SOX2 in most of the cells. Based on the high proliferation rate and sustained marker expression of cNESC at 2μM CHIR and 1μM XAV (C2X1), we decided to use this condition for further experiments.

It has previously been reported that neural progenitor proliferation and survival in frog and mouse embryos are regulated by sub-cellular localization of FOXG1 and FOXO proteins via AKT-mediated phosphorylation. We found that FGF signalling activates both MAPK and PI3K/AKT pathways in
cNESCs. This was demonstrated by the phosphorylation of ERK and FOXO1/P70S6K and by the reduction in FOXO1 and P70S6K phosphorylation following inhibition of AKT kinase activity (Supplementary Fig. 2d). As we found that AKT inhibition does not interfere with cNESC maintenance (Fig. 1k), we inhibited AKT to enhance FOXG1 function. cNESCs cultured in 6F with Tankyrase and AKT inhibitor (termed 6F for 6 factor condition) showed sustained expression of FOXG1 in cNESCs. Next, we aimed to determine whether cNESCs cultured in 6F would maintain the expression of cortical markers FOXG1,
OTX1/2, PAX6 during extended culture. cNESC s cultured in 6F for 75 days (15 passages or 50 population doublings) maintained expression of FOXG1 in all five hPSC lines tested (Supplementary Table 2). No decrease in the ratio of FOXG1/SOX2 positive cells (Fig. 3f, g) was observed. Moreover, the expression of high levels of OTX1/2 (caudo-lateral cortex) or PAX6 (dorso-frontal cortex) was maintained in the SOX2 positive cNESC s from all hPSC lines (5/5; Supplementary Fig. 2e, f). The cNESC s proliferated consistently in the 6F condition and maintained a normal karyotype after more than 180 days (approximately 120 population doublings) in culture (Supplementary Fig. 2g). In addition, we found that most of the cells in the culture were actively proliferating. There was a low ratio of P21/Cyclin-dependent kinase inhibitor 1 positive quiescent cells among the total cells in the culture (Supplementary Fig. 2h, i).

Next, we tested whether 6F could support the formation of single cell derived colonies. Three percent of cNESC s cultured in 6F at a low density (200 cells/cm²) formed colonies (Fig. 3b, i and Supplementary Fig. 2j, k). Removal of either AKT or BMP and TGFβ inhibitors resulted in colony formation, albeit at a reduced level (Fig. 3h). Similarly, to the 4F condition, colony formation was also seen with inhibition of NOTCH, EGFR or SMO receptors (Fig. 3i, j).

We then tested if this sustained cortical specification also preserved the potential of the cNESC s to differentiate into TBR1 positive neurons. We found that both bulk cNESC s and single cNESC derived clones cultured in 6F maintained the expression of dorsal forebrain progenitor cell markers and retained the capacity to generate TBR1 positive neurons (Fig. 3k, l and Supplementary Fig. 2k, l). However, when single cell derived clones were cultured in 6F media lacking the Tankyrase inhibitor (XAV939), an increase in CTNNB1 activity led to increased expression of AXIN2, LEF1, PAX6 and NGN1 genes and reduced expression of FOXG1, OTX2, DLL3, DLL1 and ASCL1 mRNA (Supplementary Fig. 2m) compared to cNESC s in complete 6F. These results suggest that cNESC s require a balanced regulation of GSK3, CTNNB1 and AKT activity to maintain a dorsal telencephalic fate and that the regulation of these pathways supports the formation of single cell derived colonies with early developmental potential.

cNESC s maintain embryonic cortical gene expression. The 6F condition preserved key marker expression and differentiation potential of early cortical neuroepithelial progenitors. To confirm the forebrain-specific gene signature of cNESC s, we compared the transcriptomes of 6F cNESC s (Group 1; H1JA-derived clones and CA1J and H1JA-derived bulk cultures, Fig. 3k, Supplementary Fig. 2l, k); hPSC differentiated to dorsal forebrain neuroepithelium for 8–10d without culture in 6F condition (Group 2; H1, H7, H9 and CA1J); mid-hindbrain specified NESC s (mhbNESC s) (Group 3; derived in FGF and EGF from hPSCs - AF22, 23[18] from human embryos - SAI1,336; and hPSCs (Group 4; H1, H7, H9, and CA1), (Fig. 5a). 6F cNESC s showed different gene expression signatures from mhbNESC s and hPSCs (Fig. 5a) and were most similar to dorsal forebrain specified cultures (Group 2), mhbNESC s (Group 3) clustered differently from both hPSC s (Group 4) and all dorsal forebrain specified samples (Group 1) (Fig. 5a).

We then analyzed gene clusters that were specifically expressed in cNESC s. Our analysis revealed that 6F cNESC s (Group 1) expressed genes related to neural differentiation, neurogenesis, axon development, cell-cell signalling and cell adhesion. We detected increased expression of genes related to forebrain development, ciliary morphogenesis and negative regulation of both neurogenesis and gliogenesis (Supplementary Cluster E, Supplementary Fig. 4a). Since we were able to detect the enrichment of forebrain development-specific genes in our transcriptome analysis of 6F cNESC s samples, we specifically examined genes related to forebrain and mid-hindbrain specification and development in 6F cNESC s and mhbNESC s (Group 3, Supplementary Fig. 4b-d). Both cNESC s and mhbNESC s had similar expression levels of neural progenitor genes SOX1, SOX2, NES, NOTCH1, PAX6 (Fig. 5b). Strikingly, 6F cNESC clones showed upregulation of forebrain-specific genes, including OTX2, FOXG1, FEZF2, SP8, EMX2, SIX3 while mhbNESC s showed higher expression of DPPA4, PAX3, EN2, GBX2, IRX2, HOXB2, GLI2 and HHIP, thus demonstrating distinct gene expression patterns that reflect their respective anatomical regions (Fig. 5b, Supplementary Fig. 4b-d).

To further characterize the identity of 6F cNESC s, we performed long-read RNA sequencing of passage 20+, 6F cNESC s (H1JA and CA1J). To specifically understand changes in gene expression that were associated with the AKT and Tankyrase inhibition, we cultured cNESC s in 6F and then removed these two factors (6F-2F) for 10 days prior to sequencing. We found sustained expression of cortical neuroepithelial markers SOX2, FOXG1, OTX2, EMX2, LHX2 in both the 6F and 6F-2F conditions (Fig. 5c). However, cNESC s in the 6F-2F condition had reduced expression of 254 genes such as OLIG1, OLIG2, NKX6-2, FGFR3 expressed in the ventral forebrain and later in oligodendrocyte lineage and elevated expression of 70 genes such as neural crest lineage genes like CDH11, MXSI, SNAI2 indicating a small, but rapid change of the transcriptome after removal of the AKT and Tankyrase inhibitors.

cNESC s preserve cortical differentiation programme. During development, the differentiation of glutamatergic projection neurons follows an “inside first, outside last” pattern, which results in the formation of the six layers of the neocortex. To determine whether 6F cNESC s could recapitulate this pattern, we examined the potential and timing of cNESC s differentiation to lower- and upper- layer neurons. Strikingly, we saw the layer 6, TBR1 positive neurons appear first (Fig. 4a, b), followed by layer 5 specific BCL11B positive neurons (Fig. 4a, b) and finally the appearance of upper layer specific SATB2 positive neurons (Fig. 4a, b). The generation of neurons was followed by the emergence of GFAP positive astrocytes (Fig. 4b). This showed that 6F cNESC s preserve the “inside first, outside last” embryonic pattern of differentiation (Fig. 4b, right panel).

To determine whether individual cNESC s can retain multilayer cortical differentiation potential in 6F, we performed long-term culture of subcultured cNESC colonies. We plated passage 10 cNESC s (Shef6) at clonal density in a 10 cm dish in 6F medium and subcultured 48 colonies after 10 days in 96 well plate (Supplementary Fig. 3a). 45 out of 48 colonies could be plated and expanded in the 96 well plate. To determine whether the Shef6 cNESC clones maintained dorsal forebrain specification in 6F we examined the FOXG1, PAX6 and SOX2 expression. All cNESC clones (45/45) expressed SOX2, FOXG1 and PAX6 markers (Supplementary Fig. 3b), demonstrating the maintenance of dorsal forebrain specification in the 6F condition. To assess whether 6F cNESC s clones maintained multilayer specific differentiation potential, we differentiated cells by withdrawing 6F and looked for the presence of TBR1 positive or BCL11B positive deep layer cells, and SATB2 positive upper layer cells. We found that all clones (45/45) had the potential to differentiate into TBR1 positive layer 6 and BCL11B positive layer 5 cells in 30 days (Supplementary Fig. 3c). After sixty days of differentiation, we were also able to identify SATB2 positive upper layer cells in all differentiating clones (Supplementary Fig. 3c), demonstrating the maintenance of multilineage potential of cNESC s in the 6F condition.
To determine how the gene expression of the cNESCs in the 6F and 6F-2F conditions compared to bona fide embryonic cortical neuroepithelial progenitors, we used the single cell transcriptomic data from day 11.5 mouse embryonic cortices or gestational week 5 to 20 human cortices. Both datasets contained progenitor cells that expressed SOX2, FOXG1, EMX2, PAX6 and LHX2 genes. This gene expression signature was used to identify dorsal forebrain/cortically specified cortical neuroepithelial progenitors (Supplementary Fig. 5a, b).

To determine the gene signature of cNESCs we examined the differentially expressed genes in 6F and 6F-2F cNESCs when compared to isogenic hESCs. To confirm dorsal forebrain cortical neuroepithelial identity, we compared cNESCs gene expression to cortex-derived cells (i) from the Yuzwa et al. mouse embryonic brain dataset, and then specifically to Foxg1/Sox2 expressing (ii) Pax6/Lmx2/Emx2 (iii) expressing subpopulations. The genes enriched in Foxg1/Sox2 (i) and Pax6/Lmx2/Emx2 (ii) were also highly expressed in 6F and 6F-2F cNESCs (Fig. 5d, Supplementary Fig. 6a). In addition, cNESCs showed high expression of dorsal forebrain genes EMX1, FEZF2, SOX2, HES1, HES5, MYCN. These were associated with transcriptional regulation, neural stem cell maintenance, negative regulation of neuronal differentiation and forebrain radial glia cell differentiation (Clusters 1 and 2, Supplementary Fig. 7a). When we compared these clusters of genes in the mouse cNESCs we found no significant difference in the global expression between the 6F and 6F-2F conditions, which confirmed our previous findings in panel A (Fig. 5c, Supplementary Fig. 8a). We found an upregulation of genes in Cluster 4 in the 6F-2F condition, including genes expressed in the neural crest lineage such as Id3, Notch4, Cdh11, but altogether these genes were not associated with any gene ontology (GO) term or KEGG biological pathway (showed a Supplementary Fig. 7a).

We then compared 6F and 6F-2F cNESCs to human embryonic and foetal cortices. Similar to the analysis performed for mouse cortical neuroepithelial progenitors, we examined the differentially expressed genes in 6F and 6F-2F cNESCs when compared to isogenic hESCs. To confirm dorsal forebrain cortical neuroepithelial identity, we compared cNESCs gene expression to cultured NESCs, gestational week 5, 6, 8, 16, 19, 20 human cortical progenitors (i), and then specifically to FOXG1/SOX2 expressing (ii) or PAX6/LMX2/EMX2 (iii) expressing subpopulations (Fig. 6). The genes enriched in FOXG1/SOX2 (ii) and PAX6/LMX2/EMX2 (iii) were also highly expressed in 6F and 6F-2F cNESCs (Fig. 5a, Supplementary Fig. 6b). These highly expressed genes included, FOXD1, SOX1, EMX2, FEZF2, EMX1, and LHX2, and were not expressed at earlier (gestational week 5 or 6 or later gestational stages (gestational week 16, 19 or 20). When we compared the expression of these genes in the 6F and 6F-2F cNESCs, we could find no significant difference (Clusters 4 and 5, Fig. 6, Supplementary Fig. 8b). cNESCs in the 4F condition significantly elevated the expression of genes such as ZIC1, HHIP, ITGB1, NOTCH2, NOTCH4, MSX1 CDH11, GATA2 expressed in the neural crest lineage and associated to biological processes related to neural crest cellular function (Cluster 7 and 8, Fig. 6, Supplementary Figs. 7b, 8b). We found genes upregulated in the 6F condition, but we could not find enriched association to any known biological function (Cluster 9, Fig. 6, Supplementary Figs. 6b, 7b, 8b).

Altogether, our transcriptome analysis verified that 6F cNESCs show a specific dorsal forebrain identity that is different from...
mid-hindbrain specified NESCs in vitro, and a gene expression signature that is consistent with embryonic cortical progenitors in the mouse and human tissue. Moreover, we find the removal of AKT and Tankyrase inhibitors is associated with changes in gene expression related to ventral forebrain and neural crest cell lineages.

**Transplanted cNESC**s execute a cortical programme in vivo. To determine whether cNESC would follow their early developmental differentiation programme in vivo, we transplanted undifferentiated, proliferating 6F cNESC into the cerebral cortex of 9-week-old mice (Fig. 7a). Seven weeks post-transplantation, we saw formation of rosette-like polarised structures (indicated by arrowheads) that contained Ki67 positive cycling progenitors (Fig. 7b) and NESTIN positive cells (Fig. 7c). By thirteen weeks cNESC differentiated into a large number of hNCAM positive neurons (Fig. 7d). When we analysed these cells for cortical layer specific markers, we found TBR1 and BCL11B positive deep layer cells and CUX1 positive upper layer cells in distinct, non-overlapping populations of cells (Fig. 7e–g, Supplementary Fig. 9a–c). Both deep and upper layer specific human cells gave rise to layer specific, hNCAM positive neurons (Fig. 7h, i, Supplementary Fig. 9d–f). As many cortical neurons project to the contralateral hemisphere via the corpus callosum, we looked for the expression of hNCAM in the hemisphere contralateral to the transplant. We noticed the presence of several hNCAM processes and the absence of Human Nuclear Antigen (HuNu) positive nuclei in the contralateral corpus callosum, suggesting that cNESC-derived cortical neurons can project long distances (Supplementary Fig. 9g). These results show that, not only
Fig. 7 Transplanted cNESCs can complete embryonic cortical differentiation potential in the postnatal brain. a Schematic of 6-week-old mouse coronal brain section indicating the injection site (red arrow) and analysed area (blue rectangle). b After 7 weeks post transplantation polarized rosette structures (arrowheads) of transplanted cNESCs (HIJA, CAI1) contain proliferating Ki67 positive human cells. The experiment was repeated with 2 biologically independent cell lines. Scalebar: 50 μm. c NES1 positive human progenitors were found in the grafts at 7 weeks. Arrowheads indicate rosette structures. Scalebar: 50 μm. d Human NCAM expressing neurons are abundant in grafts of cNESCs seven weeks after transplantation. Scalebar: 1 mm.

e-j Transplanted human cNESCs differentiated in situ to TBR1, e, h, BCL11B, f, l and CUX1, g, j positive HuNu and hNCAM expressing cells by 13 weeks after transplantation. The experiment was repeated with 2 biologically independent cell lines (HIJA, CAI1). Scalebars: 10 μm. k Immunofluorescent detection of differentiated human cells in the NSG mouse cortex 12 weeks after transplantation. Cytoplasmic marker STEM121 (blue) identifies human neurons (HIJA-APCE) that express MAP2 (red) and EGFP (green) indicated with arrowheads and surrounded by mouse cells that are negative for all three markers indicated by asterisks. Several human MAP2 STEM121 double positive neurons were EGFP dim or negative and can be identified only with immunolabelling (see Supplementary Fig. 9H). Nuclei were labelled with DAPI (purple). The experiment was repeated with 3 animals. Scalebar: 10 μm. l Voltage gated sodium and potassium currents can be recorded from EGFP positive human neurons (HIJA-APCE) in the cortical slices of NSG mice 12 weeks after transplantation. Voltage values are indicated under the traces. m Human EGFP positive neurons (HIJA-APCE) surrounded by mainly host rodent cells can fire action potentials after current injection in 80% of patched neurons (4/5) 12 weeks after transplantation. Current steps are indicated next to the traces. n Spontaneous EPSCs could be detected in 40% (2/5 neurons) of EGFP positive human neurons (HIJA-APCE) surrounded by mainly host rodent cells 12 weeks after transplantation. o Post hoc immunofluorescent labelling of recorded cells 12 weeks after transplantation. EGFP positive cells (HIJA-APCE) were filled with ALEXA 594 dye from the recording internal solution. ALEXA 594 (blue) and EGFP (green) positive cells were also labelled for STEM121 (red) confirming the human origin. Surrounding cells are triple negative host cells. Nuclei were labelled with DAPI (purple). The experiment was repeated with 3 animals. Scalebar: 10 μm.

cNESCs maintain their potential to generate both deep and upper layer neurons in vitro but also in vivo.

We then determined whether these neurons were able mature in the host tissue and synapse with other neurons. We labelled cNESCs with EGFP knocked-in to the human AAVS1 locus and transplanted the cells to adolescent mice. 12 weeks after injection we found EGFP positive cells in the grey matter of the mouse cortex that expressed the human specific marker STEM121 and MAP2 (Fig. 7k, Supplementary Fig. 9h). Patch-clamp recording of the transplanted EGFP positive neurons showed that these human neurons express voltage gated sodium and potassium channels and can generate action potentials (Fig. 7l, m). To determine whether the human EGFP positive neurons could connect to surrounding neurons, we recorded synaptic EPSCs from EGFP positive cells, and confirmed their identity by post hoc labelling for EGFP, STEM121 and AF594 from the patching internal solution (Fig. 7n, o). Altogether, these results suggest that transplanted human cNESCs can differentiate in the adolescent cortical tissue to mature neocortical neurons that can establish synaptic connections.

Discussion

Cortical or dorsal forebrain neuroepithelial cells are the founding cells of the cerebral cortex. Before the onset of neurogenesis these cells divide symmetrically to expand the progenitor pool, but the regulation of their self-renewal is not clear. We investigated the necessary developmental signalling pathways for the maintenance of multipotent cortically-specified neuroepithelial stem cells (cNESCs) in vitro. By testing combinations of activators and inhibitors, we found that a combination of six factors (activation of FGFR together with inhibition of BMPR, TGFβR, GSK3, AKT and TNKS; termed 6F) can support the permanent self-renewal of human cNESCs. We showed that: (1) cNESCs can proliferate without changing their cortical marker expression; (2) cNESCs preserve the potential to differentiate to cortical neural cells following the embryonic developmental order; (3) single cNESCs can form colonies and preserve the developmental potential of the bulk population; (4) cNESCs can implement their developmental potential in the cortical tissue.

We found that FGFR activity is needed for human cNESC proliferation as it was shown in mouse forebrain neuroepithelium39, and this is mediated by both MAPK and PI3K pathway, however AKT inhibition allows colony formation and proliferation. AKT has been shown to regulate the nuclear export of FOXG1 in cortical progenitors35, so reduced level of AKT and additional proteins will help the retention of FOXG1 in the nucleus and maintain cortical specification.

Our screen showed that a key component of the self-renewal of cNESCs is the regulation of GSK3 and CTNNB1 activity. Inhibition of GSK3 promotes proliferation and colony formation of human cNESCs, but the level of transcriptional CTNNB1 activity regulates the expression of essential dorsal forebrain specification genes FOXG1, EMX2, PAX6, and OTX1/2. Combined reduction of GSK3 and CTNNB1 transcriptional activity supports cNESC proliferation, colony formation and the maintenance of dorsal forebrain specification and differentiation potential. Removal of the CTNNB1 regulation (XAV939) causes increased nuclear activity that changes the transcriptome of the cNESCs. The continuous elevated CTNNB1 levels reduce FOXG1 expression and change the differentiation potential in the cNESCs as they no longer can form deep layer neurons positive for TBR1 and BCL11B. Studies on GSK3 function in the developing neural tissue showed the requirement of reduced kinase activity to inhibit neural crest formation and neuronal differentiation39,40. Several signalling pathways can alter the activity of GSK3, which acts as a hub protein41,42 regulating the activity of several target proteins involved in cell cycle regulation, mRNA transcription and protein translation. GSK3 can interact with ERK to down-regulate SMAD mediated ACTIVIN A and BMP signalling26,27. We found that reduction of GSK3 activity in cNESCs requires the inhibition of BMP and ACTIVIN A for the self-renewal of cNESCs. BMP inhibition in cNESCs maintains the expression of SOX1, an early marker of neuroepithelium43, while inhibition of ACTIVIN A signalling supports cell proliferation and allows colony-formation of cNESCs. Conversely it has been shown that β-catenin (CTNNB1) transcriptional activity is low during the specification and maintenance of forebrain identity of the neuroepithelium31 and this can be mediated by several inhibitory proteins (TLE2, TCF711, TCF3, HESX1, CTNNBIP1)44–47. In vitro chemical inhibition of Tankyrase by XAV939 can mimic the function of modulatory proteins in stem cells and balance CTNNB1 transcriptional activity48.

NOTCH signalling has been shown to be important in brain development, but dispensable during the neuroepithelial expansion phase specifically in the forebrain19. The anterior neuroepithelium develops without RBPI expression50 and cleaved NOTCH intracellular domain is low in the apical progenitor population and accumulates in the nucleus of progenitors of the
forebrain from the start of neurogenesis at embryonic day 10.5. The switch to NOTCH dependent state of progenitors at the start of neurogenesis is reflected rapid differentiation to neurons of RBPJ null embryonic forebrain cells. Our results define the necessary signals for the early cortical neuroepithelial progenitor self-renewal independently of NOTCH signalling and complements previous observations about this transient state in rodent cell cultures. Human early embryonic neuroepithelial cells show heterogeneity at the transcriptome level and display differences to rodent cells. The FOXG1, PAX6, SOX2 and TJP1 positive neuroepithelial cells are present from Carnegie stage 16 telencephalon before the differentiation of DXC positive deep layer cortical neurons. Transcriptomic analysis indicated dynamic changes of FGF, WNT, NOTCH and mTOR signalling in the neuroepithelial cells during gestational weeks 6 to 9. Our study verified the requirement of FGFR with low GSK3, CTNNB1 and AKT signalling is sufficient for FOXG1, PAX6, SOX2 positive cNESCs in vitro maintenance, even when NOTCH signalling is inhibited. Hedgehog signalling specifies and supports the maintenance of ventral forebrain progenitors, but it is not required for the dorsal forebrain progenitors similar to our observations with the inhibition of SMO activity in cNESCs. The convergence of various signalling pathways to support progenitor cell maintenance in the cortex was shown by the capacity of Hedgehog signals to compensate the accelerated neurogenesis and progenitor pool depletion in the RBPJ null embryos.

We also found that cNESCs do not require EGFR signalling in the 6F condition. EGFR induces elongated bipolar morphology in mouse cortical radial glia cells, allowing the cells to act as scaffold for migratory cells. Long-term exposure of human forebrain neuroepithelial cells to FGFR and EGFR causes their transition to radial glia-like cells and differentiation into astrocytes. EGFR-mediated signalling has been shown to be important for the gliogenic differentiation of neural progenitors, however multi-potent neural progenitors in the cortex can function normally without EGFR activity during development. Our data and previous observations suggest that early cNESCs does not require EGFR activity but when stimulated it causes radial glia-like cell proliferation and the loss of neurogenic cortical differentiation potential.

One of the key properties of self-renewing stem cells is the sustained differentiation potential over rounds of cell divisions. cNESCs retain early cortical potency long-term and preserve the developmental order of corticogenesis. Once the cNESCs are allowed to differentiate by the removal of the 6F neurons of the TBR1 and BCL11B positive deep layers are produced first, followed by SATB2 and CUX1 positive upper layer neurons and later astrocytes. The 6F condition supports the efficient formation of single cell derived colonies of SOX2 positive cNESCs that express FOXG1 and PAX6 dorsal forebrain progenitor markers indicating sufficient regulation of key signalling components to inhibit differentiation or regional repatterning. The individual cNESC clones could differentiate into deep (TBR1, CTIP2) and upper (SATB2) cortical layer specific cells demonstrating the ability of the early dorsal forebrain neuroepithelial cells to proliferate extensively without changing their differentiation potential. The identified 6F control of cNESC self-renewal supports the observation of Shin et al. that blood vessel endothelial cells can secrete factors that support the self-renewal of corticaly specified neuroepithelial progenitors however, results on signals that can reduce GSK3, TGFB, BMPR activity was not reported.

The lack of regenerative potential in the adult and ageing cerebral cortex is hypothesised to be due to the absence of stem cells with embryonic cortical potential and the inability of the tissue to regulate the generation or integration of new projection neurons. The transplantation of undifferentiated cNESCs into the young mouse brain demonstrated that cells with early embryonic developmental potential activate their differentiation programme in the cortical tissue environment and generate deep and upper layer projection neurons. The newly formed neurons could mature to electrophysiologically active cells in the host tissue and form functional synapses that receive inputs from other neurons similar to transplanted mouse embryonic neurons in the adult neocortex. We observed that neuronal differentiation of cNESCs labelled with EGFP delivered by various methods correlated with transgene repression and silencing over time, therefore we cannot rule out that we could not identify a good fraction of mature and synthetically connected human neurons in the live brain slices. The in situ differentiation of transplanted human cNESCs and the survival of cortical neurons for at least 90 days in the adult brain provides an opportunity to investigate the regulatory mechanisms of cell lineage specification of early developmental stage cells and to investigate the regeneration or functional recovery of the adult cortex.

In summary, we present evidence that the six signalling pathway components control the self-renewal of early cortical neuroepithelial stem cells in vitro. The cNESCs preserve their early developmental potential and neuroepithelial cell state until the 6 signalling modulators are under control in vitro. Then, the transplanted human cNESCs differentiate in situ to projection neurons of the cerebral cortex in the young adult brain.

Methods

All human stem cell work complied with the human stem cell guidance and was approved by the Stem Cell Oversight Committee of Canada and the Human Tissue Authority of United Kingdom, all animal work complied with regulations in Canada and United Kingdom and was approved by Mount Sinai Hospital Research Ethical board (Canada) and the Home office (UK).

Human pluripotent stem cell culture and neural induction. Human pluripotent stem cells (hPSCs) E1 (WAe001-A, WICell), H9 (WAe009-A,WICell), CA1 (Mount Sinai Hospital, Canada), SHEF6 (R-05-031,UK Stem Cell Bank), CTRL-2429 (University of Cambridge), 1.53E (Mount Sinai Hospital, Canada) were routinely cultured on mitomycin C treated mouse embryonic fibroblast feeder cells in αSR media with 10 ng/ml FGF2. αSR media contains 80% DMEM-F12 (GIBCO), 20% Knockout serum replacement ( Gibco), 0.1 mM 2-mercaptoethanol, 2 mM Glutamax, 0.1 mM Non-essential amino acids. Prior to neural differentiation hPSCs were transferred to geltrex (GIBCO) coated surface in the absence of feeders and were cultured in N2ESR2 (stem cell technologies) media. On the day of neural induction, hPSCs were replated on geltrex coated surface in N2B27 media supplemented with 10 μM SB431542 and 100 mM LDN193189 and 10 μM Y27632. Details of cell culture media components are listed in Supplementary Table 1.

Neuroepithelial stem cell maintenance and differentiation. Neural cultures were detached from Geltrix first on day 8–10 with Accutase or PBS-EpTA, and replated in neural maintenance media (NES) with 10 μM Y27632 at 3 x 10⁵ cells per cm² on laminin (Sigma) coated surface. NES media contains 50% DMEM-F12, 50% Neurobasal, 0.1 mM 2-mercaptoethanol, 2 mM Glutamax, 1x N2 supplement, 0.05x B27 minus vitamin A supplement (GIBCO). 4F media is made by supplementing NES media with 10 ng/ml FGF2, 3 μM CHIR99021, 1 μM SB431542 or 50 ng/ml ISt (Peprotech), 100 nM LDN193189 or 50 ng/ml NOGGIN. 6F media contains components of the 4F media with 100 nM R02288 (Selleckchem), 100 nM AKTIVIII and 75 nM IRK2206 (Selleckchem), and 1-2 μM XAV939 (Selleckchem). XAV9939 concentrations need to be adjusted to each cell line, 1 μM for H1, CA1, H9, CTRL hPSC-derived cNESCs, 2 μM for SHE6 derived cNESCs. For oligodendrocyte differentiation cNESCs were cultured in 4F and cells were collected in clumps using EDTA-PBS. Floating cell clumps were cultured in 4F media for 2-3 days and the aggregates were transferred to Neural Differentiation 1 Media. Aggregates were cultured for 30 days in Neural Differentiation 1 Media with media changes every 4 days. Floating aggregates were plated onto Poly-D-lysine and laminin coated glass coverslips and cultured for 14 more days in Neural Differentiation 1 Media. Cultures were fixed with 4% Parafomaldehyde and stained with anti-O4 mouse IgM antibody. Details of cell culture media components are listed in Supplementary Table 1.

Proliferation assay. Proliferation of hNESCs cells was assayed by plating 1 x 10⁴ cells per each laminin coated well of a 96 well plate in NES media. Cells were
allowed to attach and spread for 2 h and one volume of seeding media was complemented with equal volume of test media with 2× concentration test molecules. Test media were used in dilution series and had the following highest final concentrations: ACTIVIN A (100 ng/ml, Peprotech), BMP4 (20 ng/ml, Peprotech), FST (50 ng/ml, Peprotech), NOGGIN (50 ng/ml, Peprotech), CHIR99021 (3 μM, Selleckchem), Cyclopamine-KAUDA (200 nM, Millipore), DAPT (5 μM), IGF (100 ng/ml, Peprotech), FGF2 (10 ng/ml, Peprotech), LDN193189 (100 nM, Selleckchem), PD0325901 (1 μM, Selleckchem), PD153035 (2.5 μM), PD173074 (0.3 μM), PI-103 (0.5 μM), Wortmannin (0.1 μM), Purmorphamine (1 μM), SAG (100 nM), SB202190 (20 μM), SB203580 (20 μM), SB505124 (1 μM), SB3431542 (10 μM), LY364947 (5 μM), TGFβ (100 ng/ml), Y27632 (10 μM), JNK-IN-8 (3 μM). Cells were incubated with the indicated chemokines overnight (96 h). Total cell numbers were determined by Alamar blue cell viability assay (Life Sciences) based on cell number titration. Each value is the average of 6 technical replicates, average values were normalized to control condition (4F) and represented as relative values in Supplementary Tables 1, 3.

Clonogenicity assay and single cell clone isolation. H1 (passage 12) and S6 (passage 9) cNESCs were cultured in 4F or 6F medium as described in section ‘Neuroepithelial stem cell maintenance and differentiation’. Three to four days after plating the cells were rinsed with PBS once and detached with 5-min Accutase digestion at 37°C. The cells were collected with 10-fold volume DMEM-F12 with 0.1% BSA and centrifuged at 300 x g for 3.5 min. The pellet cells were resuspended in 1 ml fresh 6F media and triturated by pipetting with 1000 μl pipet for 20 times with 5-min intervals without generating bubbles, then washed with additional 5-fold volume DMEM-F12 with 0.1% BSA at 300 x g for 3.5 min. For clonogenicity assay the pellet cells were resuspended in 1 ml fresh NES media with FGF2 at 20 ng/ml concentration and counted in a haemocytometer. Two hundred cells per cm² culture surface area were plated in poly-D-lysine and laminin coated plastic culture dishes. Two hours after the plating cells attached and spread well, and media was changed to test conditions. Fresh FGF2 was added daily at 20 ng/ml concentration without media change for the first 4 days until clones reached a colony size of 4 or more cells. Media was completely changed after 6 days. Cultures were fixed at indicated timepoints with 4% PFA solution for 5 min at room temperature. After rinsing with PBS, the cell colonies were labelled with Cysyl violet solution for 5 min at room temperature and rinsed with water. The plates were scanned and colonies were counted with Velocity software. Occasionally colonies split to form 2–3 small colonies close to each other and separate from neighbouring colonies. The split colony clusters were counted as one.

For single cell cloning S6 cNESCs were plated in 6F media at 100 cells per cm² culture surface area in a 10 cm dish coated with poly-D-lysine and laminin. FGF2 was added daily at 20 ng/ml concentration without media change for the first 4 days. Media was completely changed after 6 days. Colonies were manually picked at day 10 to 96 wells. Clonal S6 cNESCs were maintained in 96 wells and passaged occasionally colonies split to form 2–3 small colonies close to each other and separate from neighbouring colonies. The split colony clusters were counted as one. In vitro electrophysiology. Recording of action potentials and ionic currents of the iPS-derived neurons were carried out using standard whole-cell current- and voltage-clamp techniques (Richmond and Jorgensen, 1999). Cells were patched with fire-polished 3–7 MΩ resistant borosilicate pipettes (World Precision Instruments, USA) and membrane currents and voltages were recorded in the whole-cell configuration by a Digidata 1440A and a MacLab 7000 A amplifier, using the Clampex 10 software and processed with Clampfit 10 (Axon Instruments, Molecular Devices, USA). Data were digitized at 10–20 kHz and filtered at 2.6 kHz. To record spontaneous action potentials, the input current was held at 0 pA, and hyperpolarizing and depolarizing step or ramp currents were injected to elicit action potentials. To record voltage-gated K+ and Na+ currents, cells were held at −70 mV, and voltage steps from −90 mV to +60 mV were delivered at 10-mV increments. The pipette solution contained (in mM): K-gluconate 115; KCl 25; CaCl2 0.1; MgCl2 5; BaCl2 10; HEPES 10; Na2ATP 5; Na2GTP 0.5; cAMP 0.5; cGMP 0.5; pH 7.2 with KOH; −320 mOsM. The external solution consists of (in mM): NaCl 120; NaHCO3 25; CaCl2 2; KCl 5; CaCl2 2; MgCl2 10; HEPES 10, pH 7.2–7.3 with NaOH; −320–325 mOsM. Leak currents were not subtracted. All chemicals were from Sigma. Experiments were performed at RT (20–22°C).

Acute brain slices preparation. Acute brain slices (200 μm thick) were prepared from the forebrain (coronal cut) from the transplanted NSG mice in ice-cold (−3°C) oxygenated (95% O2–5% CO2) sucrose-based brain isolation solution (containing 120 sucrose, 26 NaHCO3, 1 NaH2PO4, 2.5 KCl, 0.5 CaCl2, 4 MgCl2, 10 D-glucose, pH 7.4, 1 kynurenic acid, in mM) and kept at RT for a 1 h recovery period. After the recovery period the slices were transferred to artificial cerebrospinal fluid solution (aCSF) containing (in mM): 120 NaCl, 26 NaHCO3, 1 NaH2PO4, 2.5 KCl, 2 MgCl2, 2 CaCl2, 10 D-glucose, pH 7.4, kynurenic acid (1 mM) was added to block glutamate receptors, which might be activated during the dissection procedure.
Q-RT-PCR. Total RNA was isolated from cultured cells with Qiagen RNeasy kit according to manufacturer’s instructions. One ug of total RNA was reverse transcribed to cDNA with Qiagen reverse transcription kit. Quantitative RT-PCR detection of specific cDNA sequences was done with SYBR Green mix on a BIORAD thermocycler. Primer pairs for each cDNA target were tested for linear amplification and single product melting peak. Ct values are the average of 3 technical replicates, cDNA abundance is normalised to GAPDH housekeeping gene reads. Details of primer sequences are listed in Supplementary Table 3.

Microarray analysis. Total RNA was assessed for quality and quantity on a Bioanalyzer and global gene expression profiling performed with the Affymetrix microarray. Purified and labelled RNA was analysed on Affymetrix Human Transcriptome Array 2.0 (Affymetrix) according to the manufacturer’s instructions, at Microarray Facility of The Centre for Applied Genomics at Sickkids, Toronto, Ontario.

RNA method normalization, background subtraction, and summarization was performed using Oligo (v13.4.2)40 R package from bioconductor. Normalized gene expression intensities were log2-scaled for all subsequent analyses. Unless otherwise stated, all data presented are representative of at least two independent experiments. A linear model approach and the empirical Bayes statistics were implemented for differential gene expression analysis, as described in limma (v3.26.9)41 R package user guide. P-values were adjusted using the Benjamini–Hochberg method and significance cut-off was set at 0.01. Euclidean distance matrix and complete linkage were used for hierarchical clustering of the samples using log2-transformed gene expression values. Euclidean distance matrix and Ward’s minimum variance method of clustering were used to obtain the gene clusters using Z-score normalized gene expression values. All statistical analyses, Gene Ontology term and KEGG pathway analyses, and data visualization were done in R using basic functions and the following packages: clusterProfiler (v2.4.3)42, ggplot2 (v3.0.1), https://cran.r-project.org/package=ggplot2, pathway (v1.10.1)43 and stats (v3.2.2, http://www.rdocumentation.org/badges/version/stats). The microarray data generated in this study have been deposited in the GEO database under accession code GSE185258.

Library preparation and long-read RNA-sequencing. Cell lysates were homo- geneized with QIAshredder columns (QIAGEN #79654) and total RNA was extracted with RNAeasy mini columns (QIAGEN #74104). RNA was DNase treated using TURBO DNase (ThermoFisher Scientific #AM2238). RNA quality was verified on a 2100 Bioanalyzer (Agilent Technologies) and polyadenylated RNA was enriched using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490L). Libraries were generated according to 100 ng of poly(A) + RNA and Oxford Nanopore Technologies’ Direct cDNA Sequencing Kit (ONT SQK-DCS109), with each sample barcoded with the Native Barcode Expansion kits (ONT EXP-NBD104 and ONT EXP-NBD114), according to the manufacturer’s specifications. Barcoded samples were pooled, loaded on a MinION sequencer on R9 Flow Cells (ONT FLO-MIN106D), and sequenced using the MinION software v19. The long-read RNA-sequencing data generated in this study have been deposited in the GEO database under accession code GSE184081.

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Microarray and long-read RNA sequencing data are available under accession numbers GSE185258 and GSE184081, respectively. Publicly available datasets used in this study can be accessed under accession numbers GSE107122 and GSE81475. The KEGG71,72 databases were used for gene ontology enrichment analysis. The data related to the manuscript are provided as Source Data files. Source data are provided with this article.

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**Author contributions**

B.V.V. designed and carried out the experiments, analysed and interpreted the data and wrote the manuscript, M.F. carried out transplantation experiments and analysed the data, H.Y. and E.L. carried-out experiments, S.G. did the in vitro electrophysiological recordings and interpreted the data, M.Z. interpreted the data, H.P. and R.T.K. carried out transplantation experiments and acute brain slice electrophysiological recordings and analysed the data, S.M.H. and G.K. analysed the microarray and long-read RNA-sequencing data, A.N. interpreted the data and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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