Temporal regulation of *ZBTB16* expression by glucocorticoids alters human cortical neurogenesis

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Summary

Glucocorticoids are important for proper organ maturation[1]. Increased exposure to these hormones during pregnancy, as a result of commonly prescribed synthetic glucocorticoids such as dexamethasone in preterm births[2], has been associated with lasting effects on the offspring, including on neurodevelopment and neuropsychiatric disease risk[3]. While the consequences of glucocorticoid excess in term and especially adult brain have been extensively studied, mainly in rodents[4], studies on their effects during early human cortical development are absent. Here we use human cerebral organoids and mice to study cell-type specific effects of glucocorticoids on neurogenic processes. We show that glucocorticoid administration during neurogenesis alters the cellular architecture of the developing cortex by increasing a specific type of gyrencephalic species-enriched basal progenitors that co-express *PAX6* and *EOMES*. This effect is mediated via the glucocorticoid-responsive transcription factor *ZBTB16* as shown with overexpression, genetic knock-down and reporter assays experiments in organoids and embryonic mouse models and leads to increased production of deep-layer neurons. A phenome-wide mendelian randomization analysis of a genetic intronic enhancer variant that moderates glucocorticoid-induced *ZBTB16* levels, as shown with enhancer assays and enhancer-editing in organoids, reveals potential causal relationships with increased educational attainment as well as neuroimaging phenotypes in adults. In this study we provide a cellular and molecular pathway for the effects of glucocorticoids on human neurogenesis that potentially explains postnatal phenotypes and may be used to refine treatment guidelines.

Main

Prenatal development relates to postnatal health. The ‘developmental origin of health and disease (DOHaD) hypothesis’[5] proposes that environmental exposures during critical prenatal periods have lasting effects on cells

and tissues impacting human health throughout life. This is also true for the central nervous system (CNS) with a number of environmental factors shown to impact its cellular architecture and function[6].

An important factor shown to affect CNS development during pregnancy are glucocorticoids (GCs)[7]. These steroid hormones are endogenously present prenatally with a physiological rise close to term, important for the maturation of fetal organs[1]. Exposure to levels of prenatal GCs outside of the physiological range has been related to lasting postnatal effects[3]. An important exposure that results in GC excess is the therapeutic use of synthetic GCs (sGCs) during pregnancy. While the placenta constitutes a protective barrier for endogenous maternal GCs, i.e. cortisol with only as little as 10% of the circulating hormones reaching the fetal compartments, sGCs readily cross the placenta[8] leading to higher exposure of the fetus. sGCs, either betamethasone or dexamethasone, are most commonly prescribed starting at 24 and until 33 gestational weeks (GWs) in pregnancies with high risk for preterm delivery, to facilitate fetal lung maturation[2]. More than 1 in 10 babies are born prematurely every year, a number which amounts to ~15 million preterm births (< 37 GWs) per year[9] of which ~ 615,000 are born extremely preterm (< 28 GWs)[10], highlighting the clinical and societal importance of prenatal sGCs use. Dexamethasone is also administered throughout pregnancy, starting as early as GW6, to female fetuses at risk of virilization due to congenital adrenal hyperplasia[8]. Given the clear beneficial anti-inflammatory effects of sGCs, they are also used during the COVID-19 pandemic in patients requiring oxygen therapy and mechanical ventilation[11]. Pregnant women were not excluded from this treatment course, with the guidelines specifically promoting the use of dexamethasone in women that fulfill the afore-mentioned criteria[12].

The overall molecular and cellular effects of GCs on the term and adult brain are well-characterized in rodents[4]. Interestingly, their effects on early stages of brain development and especially during the neurogenic period, which extends till GW28[13] in humans and thus in the timeframe of sGCs administration for extremely preterm births, have rarely been studied and are absent for human brain or complex models of the human developing cortex. To gain a better molecular and cellular understanding of GC effects on neurogenic processes of the developing human neocortex, we used human cerebral organoids (hCOs) to investigate GC impact on neurogenesis and on the molecular and transcriptional landscape as well as potential mediation of postnatal phenotypes and validated important phenotypes using *in vivo* mouse models.

Glucocorticoids increase the number of basal progenitors

To study GC effects on neurogenic trajectories in the developing neocortex we treated hCOs[14] for 7 days with 100nM of dexamethasone (dex), a dose and time consistent with therapeutic guidelines followed in clinical settings[2] (see Methods for detailed explanation). This treatment was initiated at day 43 (Fig.1a) in hCOS derived from 2 independent iPSC (induced pluripotent stem cell) lines. Days 40-50 were chosen as a time-range when hCOs are actively performing neurogenic processes with all the progenitor cell types present while birth of deep layer neurons is peaking and of upper layer neurons has started[15]. First, we analyzed the specific effects of dex on different progenitor cell types defined by the expression of PAX6 (Paired Box 6) and EOMES (Eomesodermin - also known as T-box brain protein 2 or TBR2). PAX6 is highly expressed in radial glia (RG) cells, EOMES, but not PAX6, in intermediate basal progenitors (IPs), while both can be expressed in certain basal progenitors (BPs). Dex consistently led to a significant increase of PAX6+EOMES+ BPs (Fig.1b,c) in hCOs derived from both iPSC lines compared to veh-treated hCOs. The increased PAX6+EOMES+ BPs were localized in the basal side of the germinal zones, in the subventricular-like zone (SVZ) (Fig.1d and Extended data Fig.1). Moreover, we confirmed the effects of dex on the increase of double-positive BPs by analyzing the number of PAX6+EOMES-, PAX6-EOMES+ and PAX6+EOMES+ progenitor subtypes in Line 2- hCOs using flow cytometry analysis (FCa). We observed a significant increase (+18%) in PAX6+EOMES+ BPs when hCOs were treated with dex compared to veh (Extended

data Fig. 2a-c). Co-administration of the glucocorticoid receptor (GR) antagonist RU486 supported that dex effects are mainly mediated by the GR and not the mineralocorticoid receptor (Extended data Fig. 2a,d,e).

Focusing on neurons, dex led to an increase of immature neuronal somata (doublecortin, DCX+) in the ventricular-like-zone (VZ, Fig.1e,f) and to a decrease of the DCX zone thickness in the cortical-like plate (CP, Fig.1e,g), potentially pointing to later-born neurons still migrating to their final destination. This putatively prolongated neurogenesis can be related to the increased numbers of PAX6+EOMES+ BPs. These BPs have high proliferative capacity, undergoing not only neurogenic but also self-renewing proliferative divisions, which comes in contrast to PAX6-EOMES+ IPs that primarily undergo one neurogenic division producing two neurons[16–21]. Interestingly, PAX6+EOMES+ BPs are abundant in the inner and outer SVZ of mammals with a gyrified brain, such as ferrets, primates and humans, and are one of the mechanisms responsible for the increased neurogenic potential of these species[17]. In lissencephalic species, like rodents, this cell type is rare with the vast majority of BPs being IPs[16–21]. Overall, GCs seem to increase neurogenic processes that are enriched in gyrified species, highlighting how an environmental factor can affect neurogenesis and subsequently the cellular architecture of the brain.

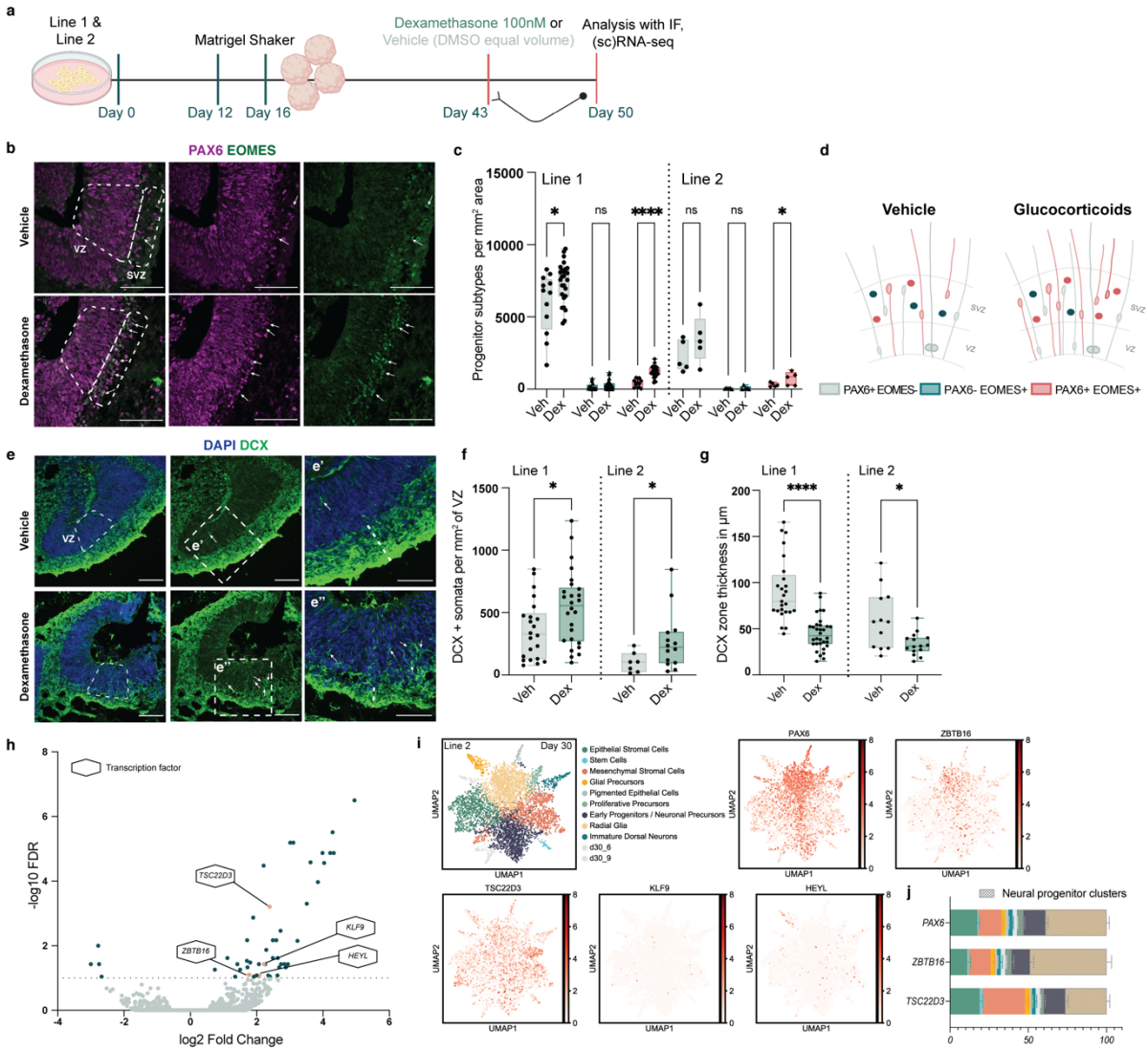

Figure 1l Glucocorticoids increase basal progenitors that co-express PAX6 and EOMES. a, Treatment and analysis workflow in human cerebral organoids (hCOs) derived from two iPSC lines. **b,** Representative images of day 50 hCOs at veh and dex conditions stained for PAX6 and EOMES. Arrows indicate cells that co-express PAX6 and EOMES; Scale bars, 100µm. **c,** Quantification of the progenitor subtypes in each treatment condition normalized by mm² of quantified total area in hCOs produced from two iPSC lines. **d**, Schematic representation of the effects of dex on progenitors, highlighting the increased numbers of basal

progenitors co-expressing PAX6 and EOMES. **e,** Representative images of day 50 hCOs at vehicle and dex conditions stained for DCX and DAPI. Arrows indicate DCX positive somata in the VZ; Scale bars, 100µm. **e' and e'',** Zoom-ins of the areas shown in vehicle and dex ventricles respectively in figure e. **f,** Quantification of DCX somata found in the VZ normalized per mm^2 of area. **g,** Quantification of DCX zone thickness in µm. **h,** Volcano plot of DE gene expression analysis in bulk RNA sequencing. Grey dots, genes with non-significant expression changes at an FDR cutoff of 10%; Blue dots, genes with significant expression changes; Orange dots denote TFs labeled with their gene name. **i,** UMAP plots of cell clusters and TFs in day 30 Line-2 hCOs from Cruceanu et al., 2022. **j,** Percent of the fraction of total of cells positive for each TF in each cluster. Color scheme follows the one in Fig.1i. FDR, false discovery rate with Benjamini-Hochberg correction. DMSO, dimethyl sulfoxide; IF, Immunofluorescence; Seq, sequencing; Veh, vehicle; Dex, dexamethasone; VZ, ventricular-like zone; SVZ, subventricular-like zone; BPs, basal progenitors; TFs, transcription factors. Significance was tested with two tailed Mann-Whitney comparison between treatment and vehicle. P-values: **** <=0.0001, * <=0.05, ns >0.05

Transcriptional response to glucocorticoids during neurogenesis

We next aimed to identify genes and pathways responsible for the effects of GCs on neurogenesis. For this we used bulk RNA sequencing (RNA-seq) of Line 2- day 45 hCOs following the exact treatment paradigm as above (100nM of dex or vehicle for 7 days, treatment start at day 38). At a 10% FDR cutoff, fifty genes were differentially expressed (DE) (Extended data Table 1). Given the essential role and developmental specificity of transcription factors (TFs) in determining neurodevelopmental processes[22], we decided to focus on this class of proteins. Out of the 50 DE genes only 4 were TFs, *TSC22D3* (TSC22 Domain family member 3), *KLF9* (Kruppel-like factor 9), *ZBTB16* (Zinc finger and BTB domain-containing protein 16) and *HEYL* (HEY-like protein) (Fig.1h). To narrow in on progenitor-specific responses, we took advantage of a single-cell (sc)RNA-seq cerebral organoid dataset that we previously published[15] (Line 2, 30 days). From the four TFs, *KLF9* and *HEYL* were very lowly expressed in hCOs, thus excluded as candidates (Fig.1i). *TSC22D3* had a ubiquitous expression pattern with the minority of TSC22D3+ cells located in neural progenitor clusters (42.5% of TSC22D3+ cells located in "Proliferative Precursors", "Neural Precursors", "Radial Glia" clusters, Extended data Table 2). In contrast, *ZBTB16* was enriched in the neural progenitor clusters (64.7% of ZBTB16+ cells), following the expression pattern of *PAX6* (Fig.1i,j). *ZBTB16* (also known as *PLZF*- promyelocytic leukemia zinc finger protein) has been associated with the regulation of the balance between self-renewal and differentiation of stem cells in multiple organ systems including the brain[23], so we decided to focus on this TF. It belongs to the Krüppel-like zinc finger family of TFs and is very well conserved across species. It has nine zinc finger motifs in the C terminus that comprise the DNA binding domain of the protein, a protein-protein interaction domain at the N terminus and a less well characterized middle RD2 (repressor domain 2) domain and can act as a transcriptional activator or repressor[23].

Glucocorticoids alter the very dynamic neurodevelopmental expression pattern of *ZBTB16*

ZBTB16 is dynamically expressed during rodent and human neurodevelopment with high brain expression early in gestation and a subsequent downregulation to low levels. In rodents, *Zbtb16* is expressed until E10.5 (embryonic day 10.5) in the forebrain[24] when it is downregulated to non-detectable levels during neurogenesis (Extended data Fig. 3a,b). In contrast, in human fetal cortex *ZBTB16* is expressed during the initial stages of neurogenesis (Extended data Fig. 3c), indicating expression of this TF during the neurogenic period in humans but not in rodents. We analyzed the *ZBTB16* expression pattern in our model of the developing human neocortex, the hCOs. *ZBTB16* was dynamically expressed in hCOs with high RNA (Fig.2a) and protein (Fig.2b) levels at the early stages of organoid development, until approximately day 40, with a subsequent decrease when mature neurons emerge (MAP2+ cells, Microtubule-associated protein 2) (day 50, Fig.2a). We found *ZBTB16* enriched in the apical and basal side of the VZ, mainly expressed by progenitor cells (SOX2+ cells, SRY-Box Transcription Factor 2) and not expressed by mature neurons (MAP2+) (Fig. 2c), resembling the RNA expression pattern we observed with the

scRNA-seq data (Fig.1i). Thus, *ZBTB16* exhibits a very dynamic expression pattern in hCOs with high protein expression during the initial period of neurogenesis, consistent with data reported from human fetal cortex (Extended data Fig.3c).

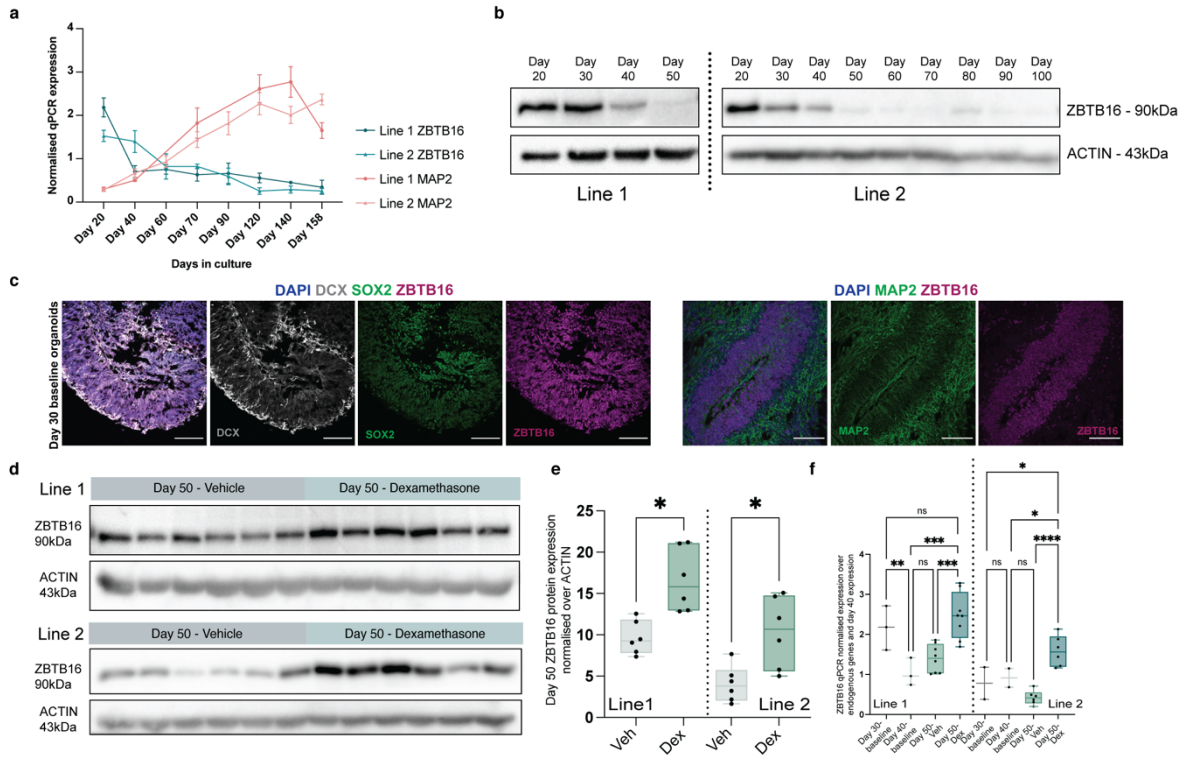

Figure 2| Glucocorticoids alter the expression profile of *ZBTB16*. a, qPCR results (mean +/- SEM) for ZBTB16 and MAP2 in hCOs of different developmental stages. **b,** Western blots of ZBTB16 and ACTIN proteins in hCOs of different developmental stages. **c,** Representative images of day 30 baseline hCOs stained for the immature neuron marker DCX, the progenitor marker SOX2, the mature neuronal marker MAP2, ZBTB16 and the nuclear marker DAPI Scale bars, 100µm. **d,** Western blots of ZBTB16 and ACTIN proteins in hCOs treated with 100nM of dex at day 43 and analysed 7 days later at day 50. **e**, Quantification of the effect of 100nM 7 days dex treatment on ZBTB16 protein expression in day 50 hCOs normalized over ACTIN. **f,** qPCR plot for ZBTB16 mRNA expression in hCOs and expression at days 30-50, with vehicle and dex condition at day 50. qPCR, quantitative polymerase chain reaction; hCOs, human cerebral organoids; Veh, vehicle; Dex, dexamethasone. For **e** significance was tested with two tailed Mann-Whitney comparison between treatment and vehicle. For **f** significance was tested with one-way ANOVA with Benjamini, Krieger and Yekutieli multiple testing correction (p= 0.0003, F= 10.39, DF= 3). Mann-Whitney p-values for e or post-hoc p-values for f: **** <=0.0001, *** <=0.001, ** <=0.01, * <=0.05, ns >0.05

Next, we examined the levels of ZBTB16 expression after dex administration in hCOs (100nM dex, for 7 days starting at day 43- Extended data Fig.4a). Treatment with dex resulted in increased *ZBTB16* expression at the RNA (Extended data Fig.4b) and protein level (Fig.2d,e) in the progenitor cells that line the VZ (Extended data Fig.4c,d). In fact, dex alters the tightly-regulated developmental expression pattern of this TF by reversing its levels to those of day 30 and younger hCOs (Fig.2f). Together, these results suggest that dex maintains high *ZBTB16* expression during later stages of neurogenesis, at developmental time-windows with physiologically lower levels of this TF.

ZBTB16 mimics the effects of glucocorticoids on basal progenitors

To test whether the effect of dex on BPs is mediated via *ZBTB16*, we overexpressed ZBTB16 and GFP from a bicistronic plasmid or GFP from a monocistronic control plasmid, in hCOs starting at day 43 when ZBTB16 expression is already declining (Fig.2a,b). Subsequent analyses were performed 7 days after the electroporation,

at day 50 (Fig.3a). In order to explore effects on progenitor subtypes of the VZ and the SVZ and on neurons of the CP, we divided the electroporated area in three bins of equal height and analysed the effects of ZBTB16 overexpression on GFP+ electroporated cells.

ZBTB16 overexpression led to increased numbers of Ki-67+ cells (Fig. 3b,c), indicating an increase in proliferation potential. In analogy to the experiments with dex, we next co-analyzed PAX6 and EOMES expression. Indeed, ZBTB16 overexpression led to a similar phenotype to that of dex, with an overall 25.7% increase in PAX6+EOMES+ BPs (Fig. 3d,e) in bins B and C (Fig.3f, 23.8% increase in bin B and 43.1% increase in bin C), which reflect the basal parts of the VZ, the SVZ and the CP. Interestingly, there was a substantial increase of these BPs in bin C (43.1%) which associates to the CP of the organoids, pointing to a potential identity change of this bin to resemble an area with cells typically found in the inner and outer SVZ of gyrencephalic species. In addition, we also found more layer V neurons with ZBTB16 overexpression (BCL11B-positive cells, BAF Chromatin Remodeling Complex Subunit- also known as CTIP2, show a 14% increase, Extended data Fig. 5a,b) indicating increased neuronal production. The birth of these neurons, however, is potentially delayed as they are still migrating and have not yet reached their final destination in bin C (Extended data Fig. 5c, 11.2% more BCL11B+GFP+ cells in bin A and 8.1% less BCL11B+GFP+ cells in bin C). Thus, ZBTB16 overexpression increases the amount of double-positive BPs and of later-born neurons, effects that resemble the ones of dex (Fig.1c-g).

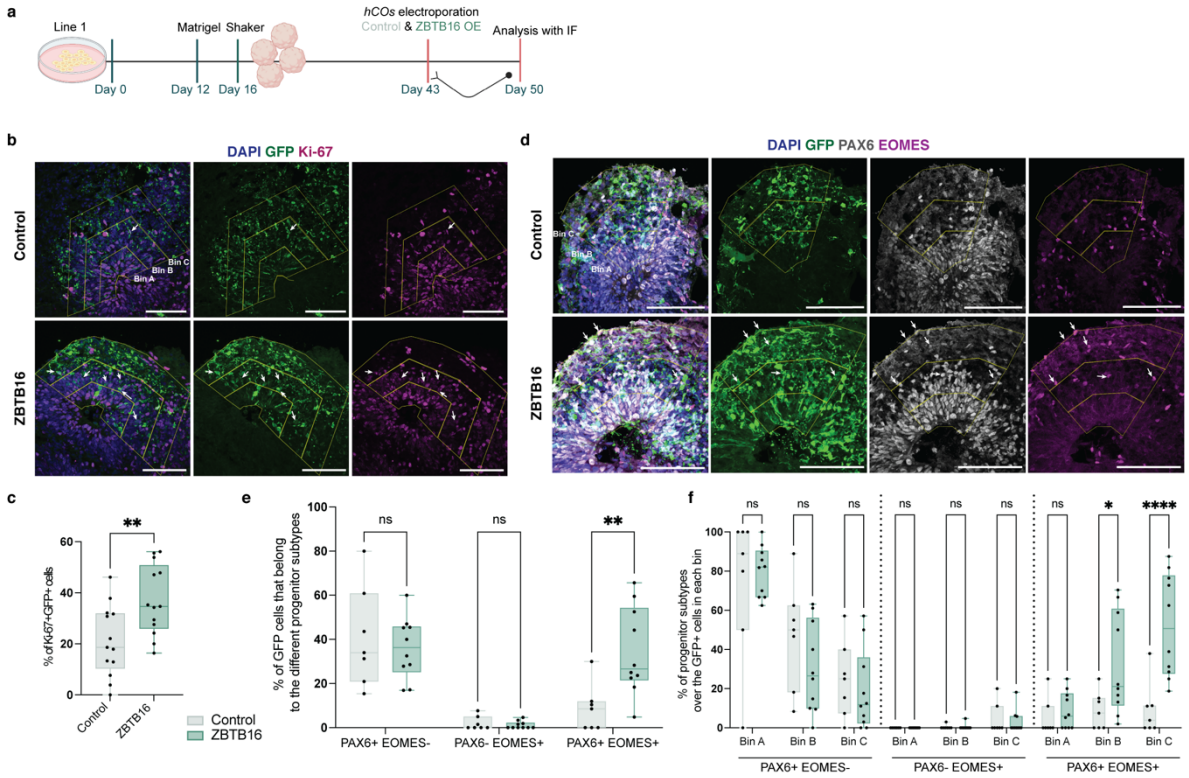

Figure 3| *ZBTB16* increases PAX6+EOMES+ basal progenitors in human cerebral organoids. a, Schematic of Line 1-derived hCOs electroporations and analysis workflow for ZBTB16 OE. **b,** Representative images of day 50 hCOs at control and ZBTB16 OE conditions stained for Ki-67, GFP and DAPI. White arrows indicate GFP cells that express Ki-67; Scale bars, 100µm. **c,** Quantification of the total number of GFP cells that are Ki-67 positive normalized over total GFP cells. **d,** Representative images of day 50 hCOs at control and ZBTB16 OE conditions stained for PAX6, EOMES, GFP and DAPI. Arrows indicate GFP cells that co-express PAX6 and EOMES; Scale bars, 100µm. **e,** Quantification of the GFP cells belonging in each progenitor subtype normalized by total GFP cells. **f,** Quantification of the GFP cells belonging in the different single positive progenitor subtypes in each bin and condition normalized by total GFP cells of each bin. hCOs, human cerebral organoids; OE, overexpression; IF, Immunofluorescence. For **c** significance was tested with two tailed Mann-Whitney comparison between ZBTB16-overexpression

and control plasmid. For **e&f** significance was tested with two-way ANOVA with Benjamini, Krieger and Yekutieli multiple testing correction (e: p.interaction= 0.0069, F= 5.5, DF= 2/ f: p.interaction = 0.0002, F= 4.17, DF= 8). Mann-Whitney p-values for c or post-hoc p-values for e&f: **** <=0.0001, ** <=0.01, * <=0.05, ns >0.05

ZBTB16 is necessary for the glucocorticoid effects on basal progenitors

In view of the similarity of the dex and ZBTB16 overexpression phenotypes on BPs, we sought to determine whether ZBTB16 is in fact necessary for the dex-induced phenotype. To achieve this, we used CRISPR-Cas9 to knock-out (KO) exon 2 of the *ZBTB16* locus in the Line 2- iPSCs. Exon 2 encodes for more than 50% of the protein and includes the initiating ATG, the BTB/POZ domain and the first two zinc fingers of the binding domain[25]. We created heterozygous Line 2- (from now on called ZBTB16$^{+/-}$) iPSCs where one allele of the *ZBTB16* locus is wild-type and one allele has exon 2 excised. A full KO of exon 2 in both alleles was not viable at the iPSC cell stage. We then treated ZBTB16$^{+/+}$- derived (CRISPR control Line 2- iPSCs) and ZBTB16$^{+/-}$-derived day 43 hCOs with 100nM dex for 7 days and analysed ZBTB16 protein expression as well as the relative abundance of PAX6+EOMES-, PAX6-EOMES+ and PAX6+EOMES+ progenitor subtypes with FCa at day 50 (Fig.4a).

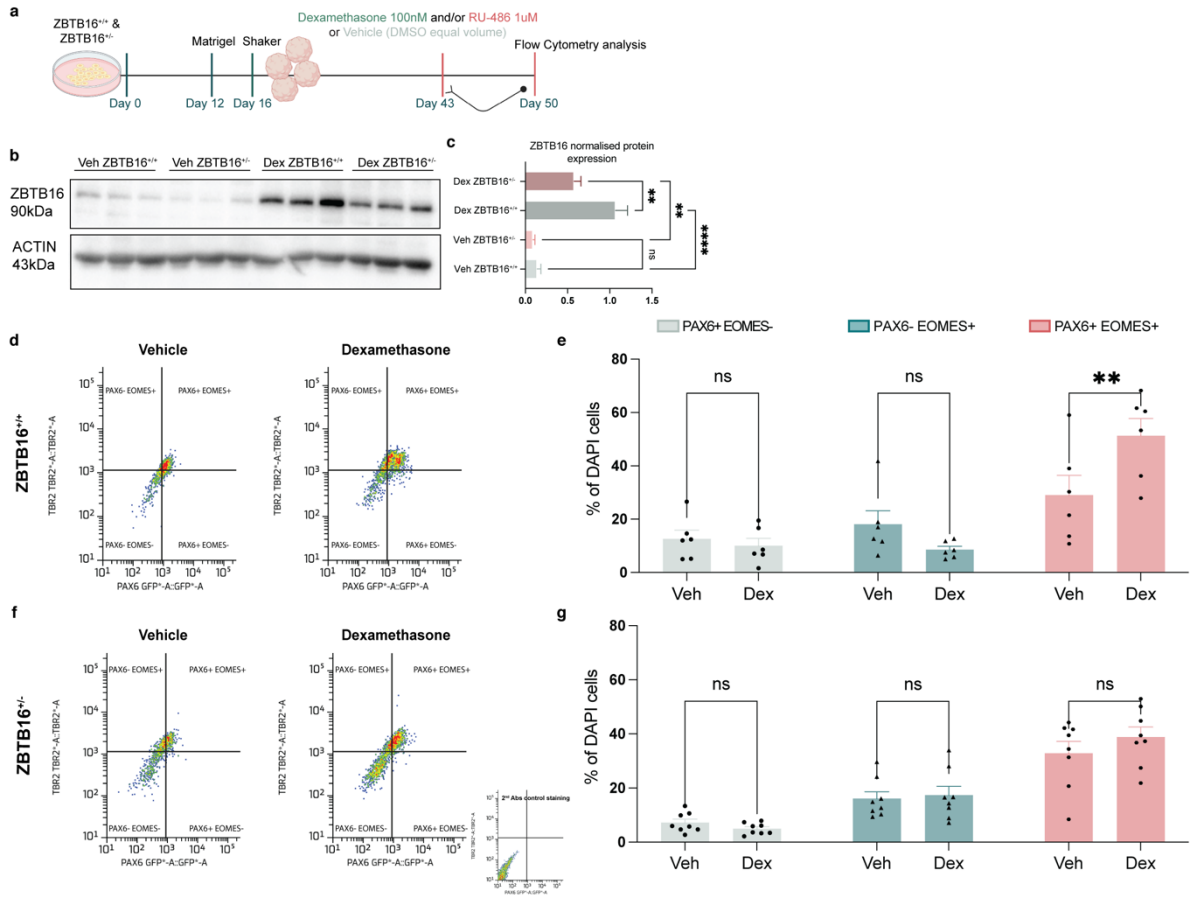

Figure 4| *ZBTB16* mediates the effects of glucocorticoids on PAX6+EOMES+ basal progenitors. a, Treatment paradigm and analysis workflow in hCOs derived from edited Line 2- iPSCs either with ZBTB16$^{+/+}$ or ZBTB16$^{+/-}$ genotypes. **b,** Western blots for ZBTB16 and ACTIN proteins in ZBTB16$^{+/+}$ or ZBTB16$^{+/-}$ derived hCOs at different treatment conditions. **c,** Quantification of the Western blot results for ZBTB16 normalized over ACTIN. **d,** Representative images of FCa of ZBTB16$^{+/+}$-derived hCOs per dex treatment condition. TBR2 is an alternative name for EOMES. **e,** Quantification of the FCa results. Percentages of DAPI cells in each progenitor subtype and treatment condition. **f,** Representative images of FCa analysis of ZBTB16$^{+/-}$-derived hCOs per treatment condition. **g,** Quantification of the FCa results. Percentages of DAPI cells in each progenitor subtype and treatment condition. DMSO, dimethyl sulfoxide; hCOs, human cerebral organoids; Veh, vehicle; Dex, dexamethasone; FCa, flow cytometry analysis. For **c,e&g** significance was tested with two-way ANOVA with Benjamini, Krieger and Yekutieli multiple testing correction

(c: p.interaction = 0.03, F= 6.6, DF= 1/ e: p.interaction = 0.0068, F= 5.9, DF= 2/ g: p.interaction = 0.97, F= 0.38, DF= 2). Post-hoc p-values: ** <=0.01, ns >0.05

ZBTB16$^{+/-}$ hCOS showed significantly less increase of the ZBTB16 protein following dex treatment than the ZBTB16$^{+/+}$ hCOs (46% less increase, Fig.4b,c). FCa of the ZBTB16$^{+/+}$ hCOs validated the increase of PAX6+EOMES+ BPs under dex treatment (Fig.4d,e- 22.2% increase, similar to the increase found with the Line 2 wild-type hCOs (Extended data Fig. 2b,c). However, in the ZBTB16$^{+/-}$ hCOs, the number of double-positive BPs was not significantly increased in the dex condition in respect to veh (Fig.4f,g- 6% non-significant increase). Together, these results indicate that ZBTB16 is necessary for the effects of GCs on PAX6+EOMES+ BPs and suggests that it could potentially play a key role in the maintenance of the PAX6+EOMES+ BP population in gyrified species under baseline conditions.

Heterochronic *ZBTB16* expression in mouse fetal brain is sufficient to induce basal progenitors typically enriched in gyrified species

While the principles of neurogenesis are similar among all mammalian species, differences exist in respect to their temporal progression as well as to the different progenitor subtypes populating the SVZ which play a key role in the overall neurogenic potential. In lissencephalic species BPs double-positive for PAX6 and EOMES are very rare[16], the neurogenic period is much shorter[26] (9 days in mice compared to 110 days in humans) and *ZBTB16* is not physiologically expressed at any point during the neurogenic period (Extended data Fig.3a,b). In order to analyse if altered expression of *ZBTB16* during neurogenesis would lead to increased numbers of PAX6+EOMES+ BPs also in a lissencephalic species, we performed *in utero* electroporations at E13.5 in mouse brains with the same plasmids as for the hCOs and analysed the effects of human *ZBTB16* overexpression at E16.5 (Fig.5a). Considering the more complex cortical cellular architecture of the mouse brain as compared to hCOs, we divided the electroporated area in five bins of equal height where bin A includes the VZ and the SVZ, bin B the SVZ and intermediate zone (IZ), bin C the IZ and bins D and E the CP.

In mice, ZBTB16 overexpression significantly changed the distribution of the GFP+ electroporated cells. ZBTB16+GFP+ cells accumulated in the SVZ with less cells reaching the outer-most part of the cortical plate (Fig.5b,c), indicating possible identity changes and/or altered timing of differentiation. Similar to hCOs, we found more Pax6+Eomes+ BPs (Fig.5d,e, 8.8% overall increase) in bin A and bin B (Fig. 5f, 16.5% increase in bin A and 11.8% increase in bin B). Interestingly, in mice, Pax6-Eomes+ IPs, which are the vast majority of endogenous BPs of lissencephalic species[16], were significantly decreased after ZBTB16 overexpression (Fig.5e,f, 10.6% decrease in bin A and 15.1% decrease in bin B). Our results thus suggest that ZBTB16 overexpression in lissencephalic neurogenesis leads to increased gyrified species-enriched BPs at the expense of the endogenous neurogenic progenitors.

As discussed, the double-positive BPs have a higher proliferative potential which contributes to the higher abundance of neurons in gyrified species[16,17]. This seems to also translate to lissencephalic species where physiologically these BPs are very rare. Here, we also found that ZBTB16 overexpression led to increased numbers of deep layer V neurons expressing Bcl11b (33.3% increase, Fig.5g,h) and layer VI neurons expressing Tbr1 (T-box brain transcription factor 1, 4.3% increase, Extended data Fig.6a,b) but not of upper layer IV neurons that express Satb2 (SATB Homeobox 2, Extended data Fig.6d,e), supporting the increased neurogenic potential of these gyrencephalic BPs also in other species. When examining the distribution of neurons, we found an accumulation in the SVZ- and IZ- areas with less neurons having reached the CP after ZBTB16 overexpression (Fig.5i & Extended data Fig.6c,f). This finding again lends support to the fact that the higher proliferative capacity of the PAX6+EOMES+ BPs associates with a potentially longer neurogenic period similar to what we observed

following dex treatment and ZBTB16 overexpression in hCOs. In fact, when analyzing the distribution of neurons across the five bins not 3 but 6 days post electroporation (i.e. at day E19.5/birth) there were no significant differences for the distribution of cells with ZBTB16 overexpression (Extended data Fig.6g-k), indicating that migratory processes are probably not affected.

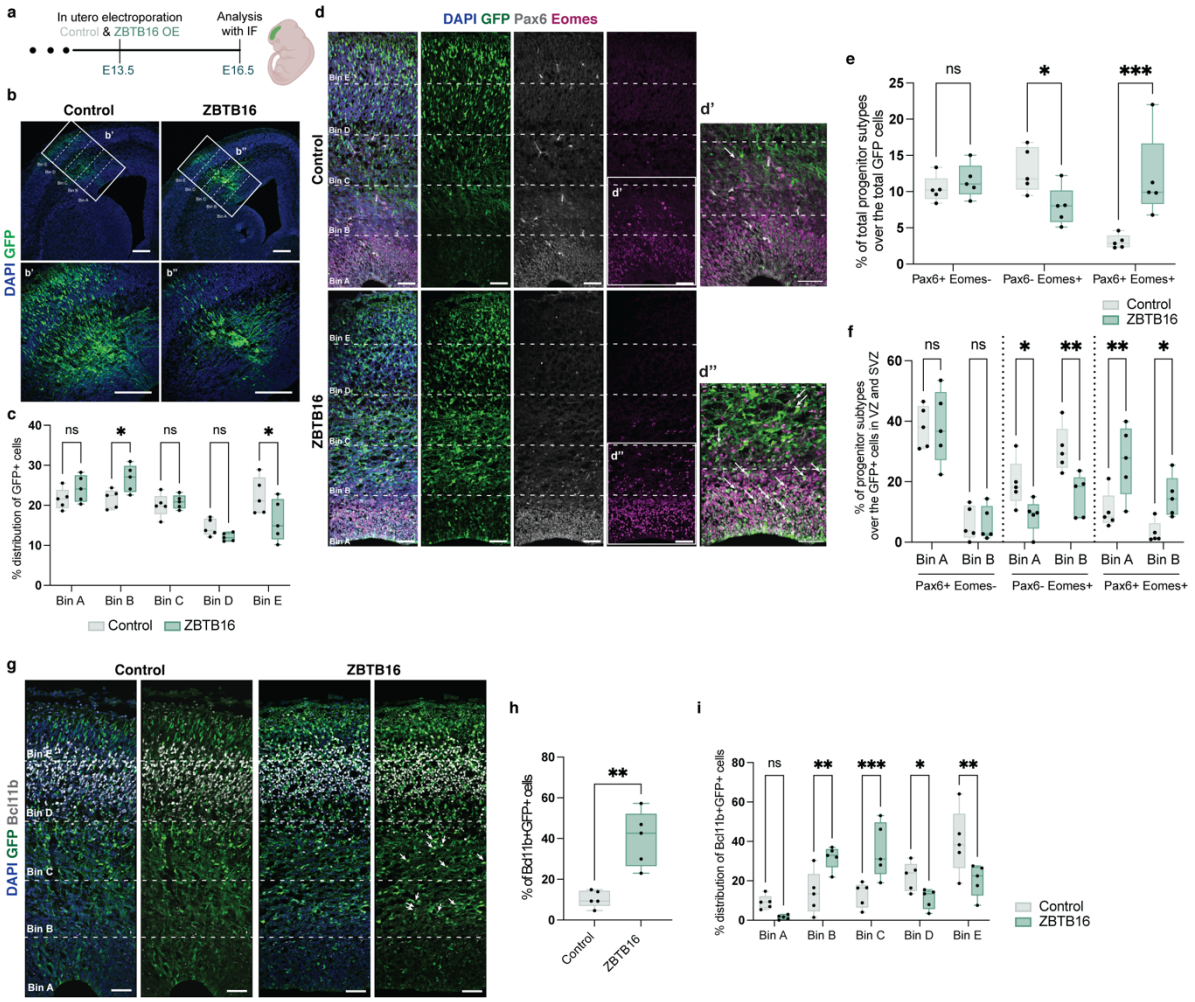

Figure 5l *ZBTB16* increases PAX6+EOMES+ basal progenitors and deep layer neurons in a lissencephalic species. a, Workflow of the *in utero* electroporations of *ZBTB16* and analysis in fetal mice. **b,** Representative images of E16.5 fetal mouse brains at control and ZBTB16 OE conditions stained for GFP and DAPI. Box indicates the electroporation areas and **b', b''** are zoom-ins; Scale bars, 100µm. **c,** Quantification of the distribution of GFP cells in each bin normalized by the total number of GFP cells. **d,** Representative images of E16.5 fetal mouse brains at control and ZBTB16 OE conditions stained for Pax6, Eomes, GFP and DAPI. **d' and d''** are zoom-ins. Arrows indicate GFP cells that co-express Pax6 and Eomes; Scale bars, 50µm. **e,** Quantification of the GFP cells belonging in the different progenitor subtypes normalized by total GFP cells. **f,** Quantification of the GFP cells belonging in the different positive progenitor subtypes in each bin normalized by total GFP cells of each bin. **g,** Representative images of E16.5 fetal mouse brains at control and ZBTB16 OE conditions stained for the layer V neuronal marker Bcl11b, GFP and DAPI. Arrows indicate GFP cells that express Bcl11b; Scale bars, 50µm. **h,** Quantification of the GFP cells that are Bcl11b+ normalized by total GFP cells. **i,** Quantification of the distribution of Bcl11bB+GFP+ cells in each bin normalized by total number of Bcl11b+GFP+ cells. OE, overexpression; IF, Immunofluorescence. For **h** significance was tested with two tailed Mann-Whitney comparison between ZBTB16-overexpression and control plasmid. For **c,e,f&i** significance was tested with two-way ANOVA with Benjamini, Krieger and Yekutieli multiple testing correction (c: p.interaction = 0.003, F= 4.7, DF= 4/ e: p.interaction = 0.0003, F= 11.84, DF= 2/ f: p.interaction < 0.0001, F= 6.58, DF= 5/ i: p.interaction < 0.0001, F= 10.66, DF= 4). Mann-Whitney p-values for h or post-hoc p-values for c,e,f&i: *** <=0.001, ** <=0.01, * <=0.05, ns >0.05

ZBTB16 directly induces *PAX6* expression

Considering that dex via *ZBTB16* seems to sustain *PAX6* expression in EOMES+ cells, even in a lissencephalic species where physiologically they are mutually exclusive[27–29], and that *ZBTB16* is a TF, we analysed the activatory capacity of ZBTB16 on *PAX6* human promoters. *PAX6* has three promoter regions that regulate tissue-specific expression and that are highly conserved between humans and rodents: the P0, P1 and Pa promoters [30,31] (Extended data Fig. 7a). Using luciferase assays, we found that ZBTB16 activates the P1 promoter of *PAX6* but not the P0 and Pa promoters (Extended data Fig.7b). This suggests that ZBTB16 could regulate *PAX6* expression via the P1 promoter which is active during neocortical development, in comparison to the P0 and Pa promoters which are minimally active[30]. This could be a potential mechanism responsible for sustaining *Pax6* expression in Eomes+ cells even in mice by ZBTB16 overexpression circumventing the negative feedback loop that physiologically ensures that *Pax6* and *Eomes* are not co-expressed in rodents[27–29].

Glucocorticoids interact with the *ZBTB16* genetic and epigenetic landscape

In order to analyse the mechanisms via which the physiological temporal expression pattern of ZBTB16 is affected by GCs, we looked at the gene regulatory landscape of the human *ZBTB16* locus in response to GCs. ENCODE data indicate the existence of intronic GR-response elements (GREs) at the human *ZBTB16* locus (Fig.6a). We have previously shown that activation of GR leads to DNA methylation changes in GREs of target genes which associates with changes in gene transcription[32,33]. To identify the molecular mechanism by which dex via GR induced *ZBTB16* expression, we treated day 30 hCOs with 100nM dex for 7 days and used HAM-TBS[34] (Highly Accurate Method for Targeted Bisulfite Sequencing) to measure DNA methylation of all GREs, as identified by public GR-ChIP sequencing datasets (Extended data Table 3) and additional non GR-related GREs (Fig.6a). Out of 55 CpGs covered in this assay, 36 were located within GREs and 19 in enhancer elements lacking GR binding sites. While only 1 of the CpGs outside these GR-binding regions showed significant DNA methylation level changes following dex stimulation (Extended data Table 4), this was true for 18 of the 36 CpGs around GREs (Fig.6b and Extended data Table 4). All significantly altered GRE-CpGs are located in enhancer regions that loop to the transcriptional start site of *ZBTB16* ("GeneHancer" track in Fig.6a and Bothe *et al.*, Life Science Alliance, 2021[35]). Our data thus support a model in which GR binds to these enhancer elements as evidenced by altered DNA methylation and increases ZBTB16 transcription.

Knowing that environmental factors, including GCs, can interact with the genetic landscape to modulate their effects on expression[36], we next analysed the genetic landscape of *ZBTB16*. We catalogued the SNPs (single nucleotide polymorphisms) in the *ZBTB16* locus previously associated with neurobehavioral and brain structural outcomes (GWAS Catalogue & Extended data Table 5) and identified rs648044 as the only variant associated with both (Extended data Table 6). rs648044 has been associated with educational attainment in two GWAS (genome-wide association study) for this trait (Lee *et al.*[37]: N= 1,131,881, FDR = $9*10^{-9}$ & Okbay *et al.*[38]: N= 3,037,499, FDR = $2*10^{-8}$) and with generalized cortical thickness[39] (N= 35,657 individuals, FDR = $6*10^{-9}$). In the latter GWAS on cortical morphology, gene-level analysis also identified the whole *ZBTB16* locus to be significantly associated with generalized cortical surface area (FDR = $7.2*10^{-14}$) and thickness (FDR = $1.9*10^{-8}$), thus suggesting relevance of *ZBTB16* for adult cortical morphology. In addition, rs648044 is located within the responsive GR-enhancer area identified with the DNA methylation analysis (Fig.6a), pointing to a possible role of this SNP in GC-regulation of *ZBTB16* transcription.

We first analysed whether this SNP moderates dex-induced activity of the surrounding 200 bp (base pair) enhancer element using a STARR-qPCR (Self Transcribing Active Regulatory Region- qPCR) approach. We identified that the sequence surrounding rs648044 indeed possesses enhancer activity that is increased with GR activation via dex (Extended data Fig.8a). The extent of the dex-induced activity increase was rs648044 allele-dependent, with

the allele associated with higher educational attainment (A-allele), conferring a significantly stronger increase following dex (Extended data Fig.8b). Next, we used CRISPR-Cas9 to KO 400 nucleotides surrounding rs648044 in Line 2- iPSCs in order to identify whether this enhancer affects *ZBTB16* transcription in hCOs. The rs648044 genotype of Line 2- iPSCs is heterozygous (G/A), with the rarer A allele (allele frequency = 0.35) creating a degenerated partial GR binding site (described half site[40]: AGXACAG, rs648044 creates: AGCAGAG, Extended data Fig.8c). A KO of the A allele resulted in rs648044$^{G/-}$ cells that only carry the G allele (Extended data Fig.8c). Using the edited cell line (rs648044$^{G/-}$) and the control cell line carrying both alleles (rs648044$^{G/A}$), we found that the absence of the A allele confers significantly less induction of *ZBTB16* following 100nM dex in day 30 hCOs treated for 7 days (Extended data Fig.8d), suggesting that the enhancer containing rs648044 modulates dex-mediated transcriptional effects on *ZBTB16* in hCOs.

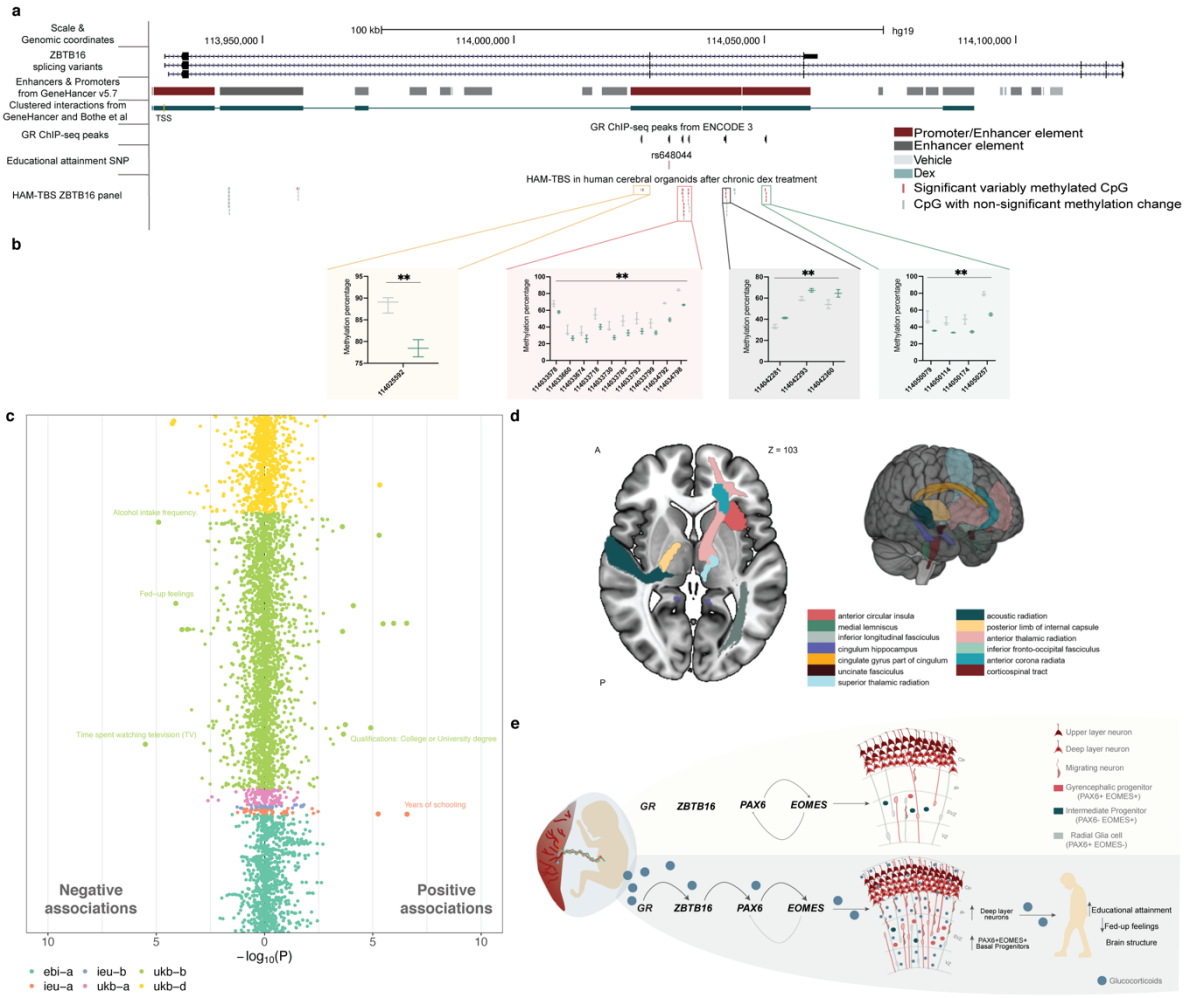

Figure 6l Glucocorticoids interact with the genetic and epigenetic landscape of ZBTB16 to impact postnatal neurobehavioral and structural phenotypes. a, Graphical representation of the ZBTB16 locus including the position of the amplicon for HAM-TBS. **b,** HAM-TBS results for CpGs with significantly altered DNA methylation levels following exposure to 7 days of 100nM dex vs. vehicle. **c,** Plot depicting Phe-WAS-MRa associations as -log of the p-value for rs648044 with various phenotypes from the UK Biobank. Single phenotypes are depicted as individual points. Associations are presented based on negative (negative MRa estimate.i.e. lower quantitative measures with A-allele effects) and positive (positive MRa estimate, i.e. higher quantitative measures with A-allele effects) effects. Color coding reflects the different sources of GWAS depicted in the legend. Traits that remain significant following Benjamini-Hochberg correction are shown with larger dots and are listed in Extended data Table 8. All neurobehavioral traits are labeled. **d,** Illustration of significant MRa associations between brain imaging phenotypes and rs648044 mapped onto the brain atlases. All effects are listed also in Extended data Table 9. **e,** Summary of the effects of glucocorticoids on ZBTB16 expression, cortical cellular architecture during development and postnatal phenotypes. GR,

glucocorticoid receptor; ChIP, chromatin immunoprecipitation; Seq, sequencing; SNP, single nucleotide polymorphism; HAM-TBS, highly accurate method for targeted bisulfite sequencing; Dex, dexamethasone; MRa, mendelian randomization analysis. For **b** significance was tested with two-way ANOVA with Benjamini, Krieger and Yekutieli multiple testing correction (GH11J114152 enhancer: p.treatment < 0.0001, F= 7.33, DF= 31/ GH11J114174 enhancer: p.treatment< 0.0001, F= 37.32, DF= 3). Post-hoc p-values: ** <=0.01

Glucocorticoids x rs648044 effects on *ZBTB16* relate to beneficial postnatal outcomes

Given the functional effects of rs648044 on GC-induced ZBTB16 expression in the developing cortex, we used phenome-wide mendelian randomization analysis (MRa-PheWAS) to identify causal effects of *ZBTB16* levels on 7,323 phenotypes from the UK Biobank and the NHGRI-EBI GWAS Catalog (Extended data Table 7) that include, among many others, neurobehavioral traits and adult neuroimaging data. We used rs648044 as exposure and the magnitude of the allele-specific expression changes following dex in the STARR-qPCR experiment as instrument to test for these associations. MRa-PheWAS provided strong evidence for associations with multiple outcomes as indicated by the QQ-plot (Extended data Fig.8f). MRa on various phenotypes, including endophenotypes and diseases (N= 4,360), showed significant associations of GC-altered *ZBTB16* expression with 22 phenotypes after multiple testing correction. These included positive associations with years of schooling and whether individuals had obtained a college or university degree (Fig.6c and Extended data Table 8), which are direct measures of educational attainment. This supports the idea that the functional effects of rs648044 on GC-induced *ZBTB16* transcription in the developing cortex, are putatively causally-related to educational attainment postnatally. In addition, GC-induced *ZBTB16* transcription was negatively associated with “fed-up feelings” a phenotype related to neuroticism[41], “alcohol intake” and “time spent watching television” (Fig.6c), suggesting associations of higher *ZBTB16* levels with beneficial postnatal outcomes and decreased associations with negative outcomes (Fig.6c). Given the previously published relationships of both educational attainment[42–45] as well as rs648044[37–39] with cortical volumes and white matter measures, we also ran an MRa-PheWAS on all neuroimaging phenotypes (N = 3,143) in the UK Biobank using the same instrument. We observed 21 significant associations after multiple testing correction (Fig.6d, Extended data Fig.8g and Extended data Table 9). Most evidence indicated that GCs x rs648044-mediated increases in *ZBTB16* expression were significantly associated with altered white matter measures and with higher anterior circular insula thickness (Fig. 6d). Thus, higher GC-induced *ZBTB16* expression in rs648044 A allele carriers is associated with higher educational attainment and increased cortical thickness as well as altered white matter measures postnatally. This suggests that the genetic association of this variant with adult neurobehavioral and brain structural measures could be mediated in parts by its effects on GC-induced *ZBTB16* levels in early brain development and in consequence their effects on neurogenesis.

Discussion

With this work we sought to understand the effects of GC excess during human cortical neurogenesis at the cellular and molecular level. We found that GCs increase a particular population of neural progenitor cells that co-express PAX6 and EOMES. These progenitor cells were found at the basal side of the germinal zones and are known to be enriched in species with gyrified brains related to a higher proliferative capacity, thus contributing to increased neuronal production[16,17,46]. We show that these effects of GCs are mediated by an alteration of the developmental expression profile of a TF called *ZBTB16*. *ZBTB16* activates a forebrain active promoter of *PAX6*, potentially explaining how GCs increase the numbers of PAX6+EOMES+ BPs. The increase in these specific BPs is followed by increased numbers of deep layer neurons and may relate to beneficial postnatal outcomes at the neurobehavioral level, like increased education attainment, and altered brain structure (Fig.6e).

Precise temporal and spatial regulation of gene expression by TFs is key for the proper unfolding of neurogenic processes[22]. Thus, we focused on GC-effects on TFs as the potential molecular mechanism responsible for their

effects on neurogenesis. We showed that GCs alter the tightly regulated developmental expression profile of a TF called *ZBTB16* in hCOs which in turn mediates the GC-effects on neurogenesis. While ZBTB16 shows a dynamic expression pattern in both gyrencephalic and lissencephalic species, the expression among species differs according to developmental time-windows. In mice, *Zbtb16* appears at E7.5 in the neuroectoderm, increases until E10.5 and is subsequently downregulated and finally only expressed in specific areas of the hindbrain and the septum but not the forebrain[24], so that in rodents *Zbtb16* is not expressed during cortical neurogenesis. This is contrary to the expression pattern observed in human fetal brain and hCOs (Extended data Fig.3), where *ZBTB16* is expressed during the initial stages of neurogenesis. This points to possible divergent actions of this TF in gyrencephalic and lissencephalic species neurodevelopment and highlights this protein as important for gyrified-species enriched neurogenic processes at baseline as well as in response to GCs.

Indeed, overexpressing ZBTB16 in the mouse fetal brain during the neurogenic period (E13-E16, when *Zbtb16* is not anymore expressed physiologically) leads to increased Pax6+Eomes+ BPs. In lissencephalic cortical development *Pax6* and *Eomes* create a positive feedforward cascade that self-regulates with direct negative feedback effects[27–29], ensuring mutually exclusive expression of these proteins. This seems to be overridden by asynchronous overexpression of *ZBTB16*. We also show that *ZBTB16* directly activates the *PAX6* promoter P1[30], that is functional in the forebrain, and sustains PAX6 expression potentially leading to the increased PAX6+EOMES+ BPs both in hCOs and in mice. Thus, via the action of *ZBTB16,* GCs have the ability to extend or open a sensitive developmental time-window for the production of gyrencephalic-enriched BPs in a lissencephalic species resulting in enhanced neurogenic potential with higher production of deep layer neurons.

Given the high prevalence of premature births, GC excess during human neurodevelopment through administration of sGCs is a very common phenomenon[9]. In fact, yearly in about ~615,000 extreme preterm pregnancies sGC treatments would take place in a period of active neurogenesis, before GW28[10,13]. While endogenous GCs are important for the maturation and function of fetal organs[1], prenatal excess of GCs has been extensively associated with long-term metabolic, endocrine and cardiovascular problems[6] and risk for neurodevelopmental[47] and mental[48] disorders in the offspring. Evidence from a recent meta-analysis of studies including more than 1,25 million children re-affirms the association of exposure to sGCs with negative effects on cognitive and neuropsychiatric outcomes when administered to children with late-preterm or term birth. However, the authors also report a significantly lower risk for neurodevelopmental impairments in children with extremely preterm birth (<28 GWs), that were treated with sGCs between GW22 to GW27[3]. This meta-analysis could be pointing to potential differential effects of GCs on neurodevelopment depending on the developmental time-window they were administered in.

One process that is different among extremely preterm and term or adult brains and might contribute to these dichotomous effects is human cortical neurogenesis, which peaks ~GW20 and is reduced but present until GW28 in the SVZ of the brain[13]. This means that extremely preterm born children are still treated within the time window of active cortical neurogenesis. Such differential effects are supported by a study in mice in which dex administration during neurogenesis was associated with anxiolytic and anti-depressive like behavior in the adult offspring[49] while increased, prolonged exposure to GCs following completion of cortical neurogenesis has repeatedly been associated with increased anxiety and depressive-like behaviors and decreased cognitive ability in these animals[50].

With this work we highlight a potential molecular and cellular pathway for the lasting effects of prenatal sGC exposure during neurogenesis and pinpoint *ZBTB16* as an important hub gene mediating GCs effects on cortical neurogenesis and potentially postnatal phenotypes. The latter is supported by results from an MRa-PheWAS where we find potentially causal associations of GC-altered *ZBTB16* levels in neurodevelopment with higher educational attainment, lower neuroticism measures and altered cortical and white matter measures.

Overall, our work provides a molecular and cellular mechanism of how GCs administered early in development, during the neurogenic period, affect the cytoarchitecture of the developing cortex which in turn associates with

postnatal outcomes at the brain structural but also neurobehavioral level. This work highlights how an environmental factor can affect brain development and postnatal health and underscores the importance of taking the developmental time-window into account when studying prenatal exposures. In addition, the new knowledge provided potentially explains the associations of early sGC use with beneficial behavioral and neurodevelopmental measures found in the literature and, thus, may help refine sGC treatment guidelines according to the stage of pregnancy they are administered in.

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Data availability

Scripts and data for the MRa-PheWAS analyses are openly available via https://osf.io/4ud6q/ for full transparency.

Data for the bulk RNA-seq are deposited as BioProject under accession code PRJNA865917.

Acknowledgments

LD acknowledges support by the Joachim Herz Foundation. We thank Mira Erhart, dr. Michael Czisch and dr. Benoit Boula for informative discussions on the brain structural measures. We thank Nathalie Gerstner and dr. Janine Knauer-Arloth for discussion on data analysis.

Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

ACK conceived the idea, designed and carried out experiments, performed analyses, provided critical intellectual input, generated and revised the paper draft; CC designed and performed omics experiments, provided critical intellectual input and revised the paper draft; CK designed and performed electroporation experiments, provided critical intellectual input and revised the paper draft; LD performed analysis and revised the paper draft; MHL performed cell biology experiments and revised the paper draft; NK & DP performed the phenome wide mendelian randomization analysis and revised the paper draft; SR performed analysis and revised the paper draft; VS, BW, MK & SS performed experiments; MRH supported project organization and experimental procedures; ML designed and performed CRISPR-Cas9 experiments, provided critical intellectual input and revised the paper draft; SC provided critical intellectual input and revised the paper draft; EBB conceived the idea, obtained funding, supervised the study, designed experiments and analysis pipelines, provided critical intellectual input and contributed to paper draft writing and revisions.