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Cell biology of brain development and evolution

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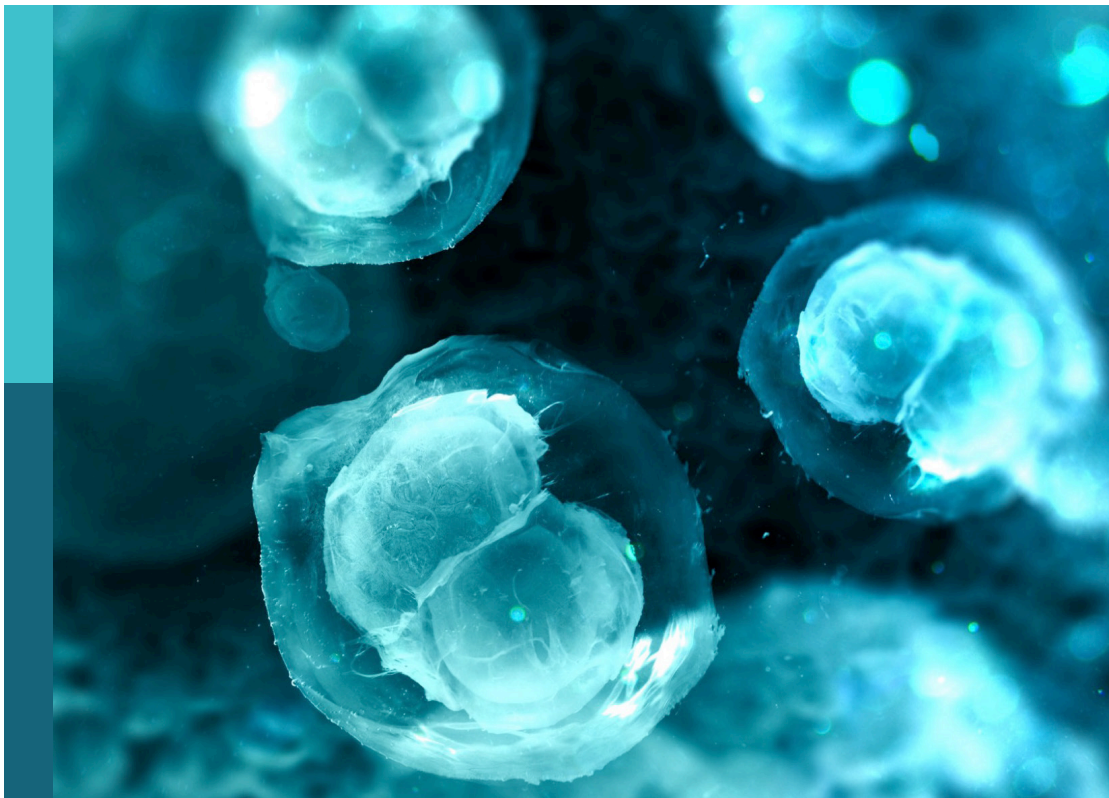

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Cell biology of brain development and evolution

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Editorial: Cell biology of brain development and evolution

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Editorial on the Research Topic
Cell biology of brain development and evolution

Introduction

Since the early days of neurobiological research, embryological, anatomical and histological approaches have led the way in giving us fundamental information about how the nervous system evolves and develops, both in health and disease. Rather than being replaced, such classical approaches have been constantly updated and diversified: from the most basic preparation steps integrating advances in precision sectioning and tissue clearing, to observation and quantitation benefitting from the latest multi-photon, light sheet, correlative, and serial block-face electron microscopy advances, and complemented with automated tissue manipulation and image processing tools. In parallel, transformative advances in molecular biology methods for control and manipulation, all the way up to optogenetics, developmental optogenetics and gene-editing, have been seamlessly integrated to grow a powerful toolkit of innovative models, *in vitro*, *in vivo* and *ex vivo* alike, to probe brain development and evolution (Shinmyo et al.; Vaid and Huttner). Among the latter, the rise of 3D organoid cultures has been providing the invaluable capacity to bridge between 2D cell cultures and native tissues and organs, opening up spatiotemporal neural trajectories to unprecedented experimental tractability (Kaluthantrige Don and Kalebic). "Omics" techniques, including whole genome, epigenome, transcriptome and proteome, are also contributing to give us a more global and detailed view of species-specific differences, as well as of the transient vs. permanent states that each cell can adopt during development (Buisan et al.).

Cell biology of neural stem and progenitor cells

The dawn of this new millennium has seen a major leap in our knowledge on the identity of mammalian neural stem and progenitor cells (NSPCs) and the mechanisms through which they ultimately generate all the neural lineages in the brain. Vaid and Huttner provide in this Research Topic a broad review of the cell biological features of neocortical NSPCs (ncNSPCs) and how they ultimately generate neurons by going from fully epithelial to partially epithelial to fully differentiated nerve cells. The analysis of these components has

permitted to identify and characterise the major ncNSPC types, in both apical and basal zones of the developing cortical wall. In the past, knowledge about the diversity of NSPC types was intrinsically hampered by the lack of diversity of model systems. Therefore, an important focus is given here to the species-specific differences that have progressively been uncovered thanks to comparisons of the traditional cell-based and rodent models to more recent models appropriate for larger and more complex brains, such as those of gyrencephalic species. From their interphase morphology to their cell division behaviour, the mechanistic dissection of these differences will be crucial in the efforts to nail down the nexus between structure and function in the brains of different species.

The coordination between stem cells proliferation and differentiation is achieved by balancing different types of divisions, such as symmetric proliferative vs. asymmetric self-renewing vs. asymmetric neurogenic division. Casas Gimeno and Paridaen review the current status of research about division symmetry in the developing brain, and highlight the intrinsic dichotomy that the term entails: indeed, the term symmetry refers both to the fate symmetry/asymmetry, and to the cell biological basis of it. Apical radial glia (aRGs) offer the paradigm for the establishment of asymmetry, because of their extreme epithelial architecture, with a very small apical plasma membrane, bearing a primary cilium protruding into the ventricle, a tightly regulated angle of mitosis and an extremely elongated basolateral plasma membrane. Interestingly, all these features have been implicated as regulators of asymmetric division and fate, begging the question of how, if all these different sources of asymmetry are potentially at work, the information they carry can be functionally integrated at the single cell level. A possible explanation emerging from recent studies suggests that these mechanisms are strongly cell type-, region- and stage-specific, adding time and space to the complexity of neural progenitor cell biology.

Cellular players

Recent work has uncovered an unexpected diversity of neural stem and progenitor cells in development and evolution. However, as the studies deepen, it is becoming obvious that an understanding of brain in ontogeny and phylogeny requires extending of our gaze beyond the neural lineage to incorporate systems and cell types that are increasingly claiming central stage: the astrocytes and the vascular system.

Falcone elucidates the present status of research on the evolution of astrocytes, reviewing what is known from invertebrates to primates, discussing a clear trend towards increased complexity in the structure and function of astrocytes. Apart from quantitative differences, there are also qualitative difference in primates, with a specific cell type identified as being primate specific. These findings highlight the relevance of looking at all cell types when studying brain evolution and should invite us to consider cellular complexity and diversity when studying human neurodevelopmental disorders as well. In addition, it is for us intriguing to note that the increase in astrocyte's structural complexity mirrors an increase in morphological complexity also of bRGs during evolution, warranting a focused effort to identify the underlying genetic drivers of this increased complexity in both cell types and probe the extent of their overlap.

The vascular system provides tissues with oxygenation and metabolic substrates, yet while the negative consequences of hypoxia during brain development are well established, the long-term consequences of oxygen excess, remain less clear, despite its potential therapeutic use to compensate for insufficient brain growth caused by genetic and environmental factors. Markert and Storch in this Research Topic use their established *in vivo* hyperoxygenation model system to contribute a necessary analysis of the long-term effects of hyperoxygenation throughout mammalian brain neurogenesis. They confirm that more neurons can be generated by hyperoxygenation during mid-neurogenesis, specifically more Layer 5 (L5) neurons and associated synaptic markers. These effects are, however, shown to be non-permanent, and the L5 neuron and synaptic marker numbers come back to wt levels shortly after birth. Interestingly, their results also suggest that microglia, rather than apoptosis, could be responsible for selectively eliminating the excess number of neurons. It will be interesting to further explore the duration of the effects of hyperoxygenation in different models of brain disorders or injury.

Vogenstahl et al. illustrate the role of the vasculature in developmental neurogenesis and the change in paradigm we have been witnessing in the last decades, with the vasculature moved from being recognized for its well-known role of nutrient supply, to a more regulatory and instructive role both in hindbrain and in forebrain. Specifically, two processes have emerged as crucially dependent on vasculature: neurogenesis and neuronal migration. The vasculature provides a niche for basal progenitor division and signaling, with the basal process of RGs also physically associated with vessels. It will be relevant to dissect the molecular mechanisms and outcomes of this intimate interplay to better define the nature of the basal progenitor/vasculature niche, including also a mechanobiological account of the vasculature and bloodstream as pressure carrier.

Modelling different brain architectures

When analyzing differences between the brains of different clades, few differences are as conspicuous as the structure of the outer surface of the cerebral cortex. In gyrencephalic mammals, the appearance of folds in the outer surface of the cortex is thought to have arisen from an interplay between the larger lateral expansion of the basal zones of the developing cortical wall -as opposed to the more constrained apical ventricular zone-, and the spatial limits imposed by the size and shape of the skull. In these Research Topic, Shinmyo et al. and colleagues provide a timely review of efforts to elucidate the functions of these folds as well as the mechanisms responsible for their formation. Focalized attention is given to the use of appropriate model organisms to study different brain architectures. Whereas the mouse is an appropriate model for the study of the mammalian lissencephalic architecture, it falls short when studying the gyrencephalic architecture. The ferret has therefore emerged as a relevant alternative to probe the intricacies of cortical brain folding that could be potentially extrapolated more meaningfully to the human setting. Being a relatively small domesticated mammal, as well as having a long history of use in biomedical research has made ferrets an attractive animal model for studying brain development and evolution. Shinmyo et al. and colleagues also shed light on an often overlooked but important aspect of brain folding, namely the development of the cortical fiber layers.

The iPSCs (r)evolution

iPSCs technology has revolutionized the way we study the human condition at the cellular and molecular level, allowing us to study key aspects of human brain development and neuronal maturation as they unfold *in vitro*.

iPSCs-derived neurons

A glimpse into the human condition is given by the possibility to generate neurons from iPSCs to study the mechanism(s) driving their maturation. The majority of the effort has focused so far on the transcriptional readout in terms of coding transcriptome. In this Research Topic, Kuruş et al. have extended the analysis to the non-coding transcriptome, focusing on long non-coding RNAs, transcripts generally longer than 200 nucleotides that do not generate any corresponding translated proteins and are emerging as crucial regulators of developmental and differentiation dynamics.

Organoids as a potential alternative for basal progenitor research

In recent years, iPSCs- and ESCs-derived brain organoids have progressed to the point of being a viable alternative for the study of many aspects of neural development and evolution. While primary tissue remains the benchmark, its use presents many limitations and constraints, scientific, technical and ethical alike and is obviously not viable for extinct species. For all their advantages, model organisms, including ferret and even primates, by definition cannot give a full picture of the larger and more folded brains of humans and other hominids. Also, in-tissue genetic modification and labelling, e.g., *via* viral infection, electroporation and microinjection, can typically only reach subsets of cells. These limitations have greatly increased the interest in 3D alternatives, such as organoids. In this Research Topic, Kaluthantrige Don and Kalebic discuss advances in a crucial Frontier for brain organoid research, namely the recapitulation of basal radial glia (bRG) biology. In contrast to the apical ventricular zone, the complexities of the basal zones and their progenitors remain very challenging to replicate in organoids, constituting a major obstacle to the study of human brain evolution and modelling of the bRG-dependent human-specific neurodevelopmental disorders. Progress is being made, however, and the *in vitro* generation of proper subventricular zones, fiber layers and the neuronal layers may soon be on the horizon. These exciting challenges notwithstanding, the review also illustrates how organoids have already contributed to relevant discoveries regarding bRGs and its implications for the evolution of animals with higher encephalization.

The genomic basis of recent human history

The availability of *in vitro* models recapitulating features of the developing human brain, the access to ancient genomes and the advent of genome editing motivated researchers to model (at least partially) features of ancient human brain in a dish. Of outstanding importance in fueling this line of interest is the insight and predictive power derived by comparing archaic genomes to the ones of contemporary humans. Buisan et al. provide us with the perspective from paleogenetics and ancient DNA by focusing on introgression deserts, which are parts of the genome showing no or very little traces of archaic DNA. By comparing publicly available RNA sequencing datasets, and by looking specifically at divergence in the transcriptional readout of desert regions, Buisan et al. show that the cerebellum, and other areas outside of the cortex exhibit the highest degrees of transcriptional divergence. In addition to this spatial and area-specific information, of note, time is also a crucial dimension when looking for differences; indeed, as the highest divergence was found in development. This observation is extremely relevant also in a physiopathological context, as many genes responsible for brain disorders (even the ones arising during adulthood) are most highly expressed during development, pointing to development as the most relevant window for human evolution and pathology alike.

Concluding remarks

The temporal dissection summed up above clearly illustrates the potential derived from integrating different approaches and scales of analysis in the conjoined study of brain development and evolution. For it is at the spatiotemporal intersection, and along both the evolutionary and developmental axis, that the insights from the cutting edge experimental approaches and model systems discussed in this special issues, can most powerfully contribute to a contemporary molecular understanding of the human condition.

Author contributions

FM-B, GT, and ET conceptualised and wrote the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptome Dynamics of Human Neuronal Differentiation From iPSC

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The generation and use of induced pluripotent stem cells (iPSCs) in order to obtain all differentiated adult cell morphologies without requiring embryonic stem cells is one of the most important discoveries in molecular biology. Among the uses of iPSCs is the generation of neuron cells and organoids to study the biological cues underlying neuronal and brain development, in addition to neurological diseases. These iPSC-derived neuronal differentiation models allow us to examine the gene regulatory factors involved in such processes. Among these regulatory factors are long non-coding RNAs (lncRNAs), genes that are transcribed from the genome and have key biological functions in establishing phenotypes, but are frequently not included in studies focusing on protein coding genes. Here, we provide a comprehensive analysis and overview of the coding and non-coding transcriptome during multiple stages of the iPSC-derived neuronal differentiation process using RNA-seq. We identify previously unannotated lncRNAs *via* genome-guided *de novo* transcriptome assembly, and the distinct characteristics of the transcriptome during each stage, including differentially expressed and stage specific genes. We further identify key genes of the human neuronal differentiation network, representing novel candidates likely to have critical roles in neurogenesis using coexpression network analysis. Our findings provide a valuable resource for future studies on neuronal differentiation.

Keywords: iPSC-derived neuronal differentiation, transcriptome profiling, lncRNAs, coexpression, WGCNA

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HIGHLIGHTS

- We provide an overview of the past and current advancements in iPSC-derived cell differentiation.
- We summarize the transcriptome during critical stages of iPSC-derived neuron differentiation.
- We identify the distinct characteristics of each stage, including coding and lncRNA genes.

1 INTRODUCTION

Increasing number of studies have highlighted that pluripotent stem cell (ESC/iPSC) technologies provide a notable platform to generate specific types of neuron from healthy and patient-derived iPSCs, *in vitro* models to elucidate the biological cues of neuronal development and the cellular/

molecular basis of neurological disease (Chambers et al., 2009; Karumbayaram et al., 2009; Nizzardo et al., 2010; Lancaster et al., 2013; Chanda et al., 2014; Bardy et al., 2015; Akbari et al., 2019a). To generate neurons from iPSCs, it is crucial to utilize stepwise protocols that mimic the signaling and molecular events which occur throughout brain development *in vivo*. First attempts developed neuronal lineage with differentiation steps upon embryoid bodies formation (Gaspard et al., 2008). A few of the major barriers in this field are the purity, viability, maturity and functionality of iPSC-derived cells. Chambers and others showed that treatment by SMAD inhibitor during differentiation increases the efficiency of neuronal lineage generation in adherent culture conditions (Chambers et al., 2009). Other groups modified this protocol afterwards to further maturation and long-term culture. Following SMAD inhibition, neural precursor cells (NPC) were being enriched and expanded during neurogenesis (Shi et al., 2012). Moreover, increase in our knowledge about the coordination of brain development has permitted to develop specific regions of the brain *in vitro* (Muratore et al., 2014; Tao and Zhang, 2016; Kikuchi et al., 2017). More recently, the utilization of iPSCs and 3D cell culture systems added another dimension to the generation of organ-like structures, termed organoids, to dissect the molecular events during brain development (Lancaster et al., 2013; Paşca et al., 2015; Birey et al., 2017). Therefore, an integrative approach combining molecular biology and bioengineering approaches with computational biology methods has been implemented to overcome these limitations and generate reliable, functional *in vitro* models. These *in vitro* models allow to investigate the transcriptome dynamics and characteristic parameters of generated cells during neuronal specification.

In the last decades, genome-wide studies have revealed that mammalian tissue specific coding and non-coding RNAs (ncRNAs) play critical roles in the regulation of biological/developmental processes, such as lineage commitment, cell fate decision and organogenesis (Cabili et al., 2011; Hu and Shan, 2016; Perry and Ulitsky, 2016; Rosa and Ballarino, 2016; Pal and Rao, 2017). Transcriptome profile of pluripotent stem cell-derived neurons was obtained using RNA-seq data and utilized to improve differentiation of neurons (Wu et al., 2010; Lin et al., 2011; Hjelm et al., 2013; Stein et al., 2014). Analysis of gene expression dynamics in human iPSC-derived neurons provide a solid framework to study early neural developmental process, progenitor differentiation, distinct axonal development (Compagnucci et al., 2015; Grassi et al., 2020; Lindhout et al., 2020). Large-scale transcriptomics studies in bulk or single cell level tried to dissect quantitative changes in neurons gene expression and map the neurons to the temporal and spatial brain development based on transcriptome similarity (van de Leemput et al., 2014; Close et al., 2017; Tanaka et al., 2020). Long non-coding RNAs (lncRNAs) are a sub-type of ncRNAs with a length of more than 200 nucleotides that originate from coding and non-coding locations of the genome (Lee, 2012; Ulitsky and Bartel, 2013). In particular, lncRNAs participate in the patterning of cellular reprograming, maintenance of pluripotency, and specification of stem cells. In this regard, lncRNAs such as rhabdomyosarcoma 2-associated transcript (RMST), in interaction with other genes such as SOX2 (Ng et al., 2012; Ng et al., 2013), mediate neurogenesis, Pax6 upstream antisense RNA (PAUPAR) lncRNA and Pax6 co-regulate gene sets and recruit transcription coactivators that affect the growth of neural progenitor cells (Vance et al., 2014), the PNKY lncRNA maintains the neural stem cell pool (Ramos et al., 2015), and the lncRNA DNMT1-Associated Long Intergenic Non-Coding RNA (DALI) is expressed in the embryonic brain, where it governs the proper differentiation and specification of neurons and maturation of neuroblastoma cells (Chalei et al., 2014).

Previous studies showed a repertoire of 4,000–20,000 lncRNA genes are differentially expressed in different cell types of the human brain (Vance et al., 2014; Ramos et al., 2015). However, the relationship between lncRNAs and neural lineage commitment is yet not described in depth. Therefore, we investigated the transcriptome dynamics of lncRNAs along with the protein coding genes to address the challenge of elucidating the characteristic features of cells during different stages of neural differentiation from iPSCs. We further performed genome-guided *de novo* transcriptome assembly to predict high confidence lncRNA genes not found in previous annotations. Our main goal was to investigate the stage-specific expression and possible function of protein coding genes and lncRNAs over the course of iPSCs-derived neural differentiation, as well as to identify previously unannotated lncRNAs with potentially key roles in the process. Our study proposes potential functions of annotated and novel lncRNAs based on coexpression network and hub gene analysis, and provides a useful resource for further studies that examine the roles of lncRNAs in biological processes, such as mammalian development and neurogenesis.

2 RESULTS AND DISCUSSION

2.1 *In Vitro* Differentiation of iPSCs Into Neurons Using Monolayer Culture Conditions

Human iPSCs cultured in feeder-free monolayer conditions were exposed to the neural induction medium, and subsequently re-plated in neuronal progenitor medium (NPM). Cells no longer exhibited pluripotent stem cell morphology during neural induction and progenitor expansion, and adopted an extended progenitor morphology instead (**Figure 1A**). Immunostaining analysis on day zero revealed that a majority of the cells were positive for the pluripotency marker OCT3/4, but not the neuronal progenitor markers PAX6 and NESTIN. Afterwards, we passaged and differentiated the iPSCs to generate neuronal progenitor cells. To assess neural progenitor (NP) generation, we first stained the cells with NP markers following differentiation. Between days 25–28 of neural progenitor generation, almost all cells were positive for the NP markers PAX6 and NESTIN. In addition, the OCT3/4 gene expression started to decrease, and a majority of the cells were negative for the pluripotency marker. Second, we passaged and cultured the NPCs as single cells for 21 additional days to further differentiate them, and analyzed the expression of markers indicating mature neuron cells on days

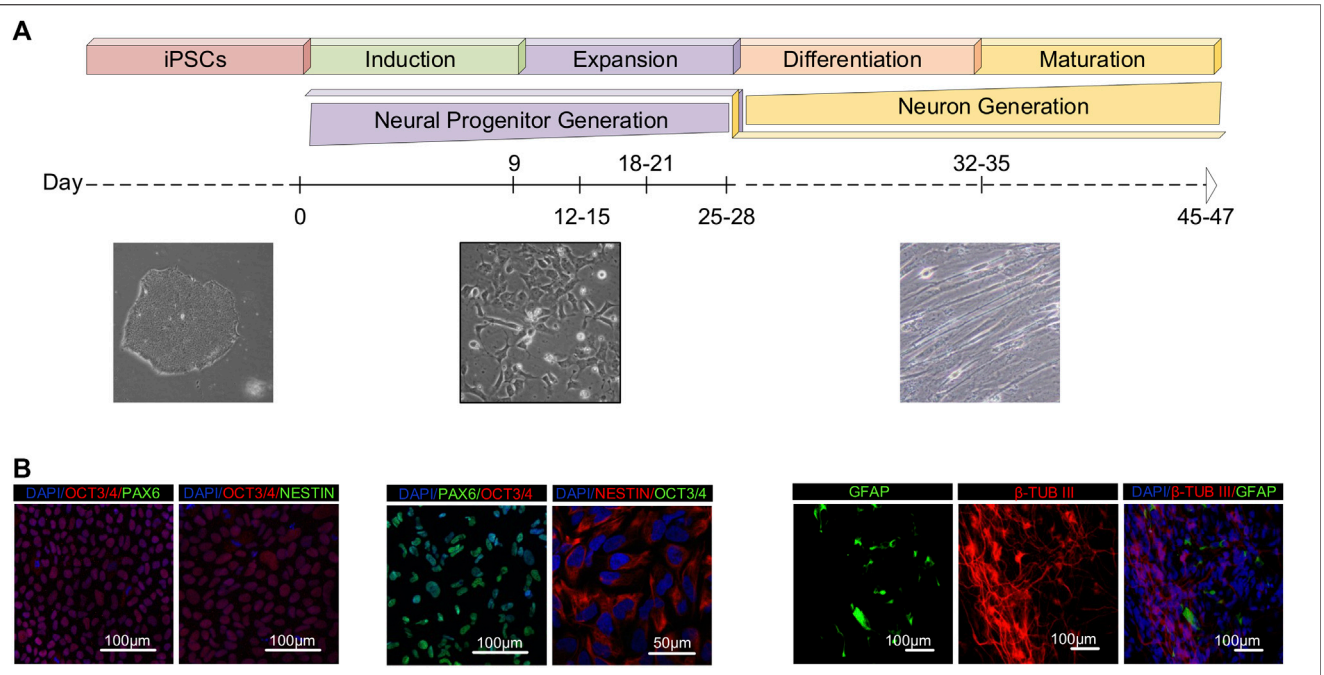

FIGURE 1 | Generation and characterization of human iPSCs-derived neurons. **(A)** A schematic representation of the *in vitro* culture system used for stepwise differentiation of human iPSCs into neurons. Timeline and representative bright-field images of cell morphology during stages of differentiation from day 0 to day 45. **(B)** The samples for RNA-seq analysis were collected on day 0 (pluripotent), days 9–12 (induced progenitor), days 25–28 (expanded progenitor), days 32–35 (differentiated/neuronal precursor) and days 45–47 (mature neuron). Confocal images of the cells showing expression of the pluripotency marker (OCT3/4), neural progenitor markers (PAX6, NESTIN) and neural markers (GFAP, β-TUBIII). Nuclei were visualized with DAPI.

45–47. The maturation step resulted in the generation of class III β-TUBULIN-positive neurons with a very low proportion of GFAP-positive cells (**Figure 1B**). Taken together, this data indicates the iPSCs efficiently differentiated into neural cells.

2.2 Genome Guided *De Novo* Transcriptome Assembly and Transcriptome Profiling of iPSC Derived Neuronal Like Cells

Following the characterization of the cells, we sought to understand the transcriptome profile of the cells using the RNA-seq technique. In addition to quantification of genes described in our annotation file (GENCODE GRCh38 human reference genome, Release 34), we used a robust novel lncRNA identification pipeline to identify whether any counts not aligned against annotated genes could have originated from previously unannotated transcripts (see **Section 4**). We have identified 354 high-confidence previously unannotated lncRNA candidates (herafter referred to as novel lncRNAs) (**Supplementary File S1**). After filtering lowly expressed genes from expression data, we used unsupervised hierarchical clustering of Pearson correlation values of the samples to generate a correlation heatmap (**Figure 2A**). The undifferentiated iPSCs (I1/I2) clustered together, while the neuron progenitor generation stages (P1/P2 and G1/G2) formed a separate cluster from the neuron generation stages (D1/D2 and M1/M2), as we expected during the neural differentiation process. Looking at the distribution of RNA-seq read counts across protein-coding, long non-coding, and other non-coding transcripts, we observed that long non-coding transcripts formed a very small fraction of total read counts in all samples (**Supplementary Figure S1**). We then looked at the distribution of reads across lncRNA transcript classes in more detail, divided into intergenic, antisense, sense overlapping, and sense intronic lncRNAs, further categorized as either annotated or novel (**Figure 2B**). Samples in different stages had varying read count distributions, suggesting a dynamic transcriptome profile, with annotated long intergenic non-coding RNAs (lincRNAs) comprising over 50% of the transcriptome in each stage, and a low percentage (<25%) of reads aligning to novel lncRNAs. We further characterized the distributions of the expressions of protein coding, annotated long non-coding, and novel non-coding genes in the samples (**Supplementary Figure S2**). We observed that lncRNA expressions are lower compared to protein coding gene expressions in all samples, with a few outlier lncRNA expressions being higher than protein coding genes in the same sample. Finally, we analyzed the length, exon distribution, and expression characteristics of protein coding, annotated lncRNA, and novel lncRNA genes expressed in our samples (**Figures 2C–E**), in order to determine whether the novel lncRNA characterization has been compatible with previously annotated lncRNAs. The analysis revealed protein coding genes as longer and with a higher exon count than both lncRNA categories (**Figures 2C,D**), as well as having higher expression (**Figure 2E**). Our results showed that our transcriptome sequencing and analysis have been consistent with expected findings.

We also inspected independent iPSC-derived neuronal differentiation datasets to observe whether reads in the

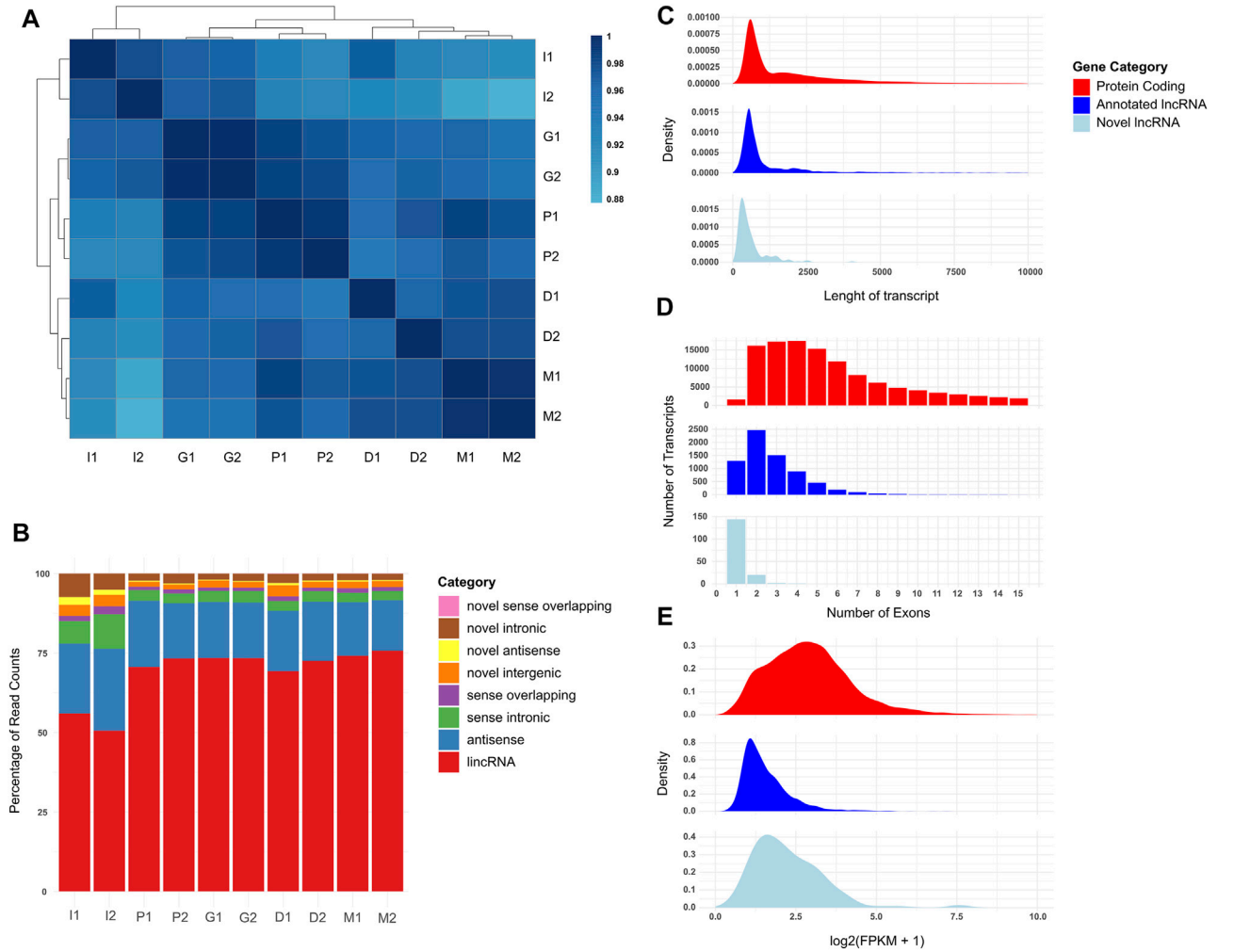

FIGURE 2 | Comparison of the coding and non-coding transcriptome profiles of the cells during the neural differentiation process. I1 and I2 are iPSC samples, P1 and P2 are cells during neural induction, G1 and G2 are neural progenitor cells, D1 and D2 are cells undergoing neural differentiation, and M1 and M2 are mature neural cells. All transcriptome profiles are filtered for genes which display an FPKM value of at least 1.0 in both replicates of at least one biological condition. **(A)** Pearson correlation heatmap of samples clustered using hierarchical clustering. Cell colors indicate Pearson correlation values of the samples indicated in the row and column. Darker cells indicate higher correlation. **(B)** Stacked bar graph of long noncoding transcriptome profiles of the samples divided by percentage of RNA-seq counts sequenced per type. **(C)** Violin plot of protein coding and lncRNA expression values of the samples. **(D)** Density graph of genes by transcript length per gene type. **(E)** Histogram of genes by exon count in canonical transcript per gene type.

datasets aligned to the same transcripts. To do so, we utilized two studies on iPSC-derived neuron transcriptomes that used a comparable sequencing depth and sequencing platforms, as well as ribosomal RNA depleted sequencing libraries (Burke et al., 2020; Solomon et al., 2021). We found that out of the 354 identified lncRNA candidates, 350 showed expression in at least one sample of the independent datasets, and 296 showed expression in one sample in both datasets (**Supplementary Files S2, S3**). When filtered for consistent expression across biological conditions, 89 of the lncRNAs showed an expression of 1 FPKM or higher in over 50% of the samples from a single biological condition in one dataset (**Supplementary Figure S3**), and 88 of them showed consistent expression in both datasets (**Supplementary Figure S4**). Our investigations into the independent datasets revealed that the majority of the novel lncRNA candidates were transcribed in other biological samples, and a number of the lncRNAs showed consistent transcription in similar biological processes across datasets.

2.3 Differential Expression of Protein Coding and lncRNA Genes Across Differentiation Steps

Afterwards, we used differential expression analysis to understand which genes in the transcriptome showed significant changes between stages of differentiation. Our findings revealed that the iPSC samples show the highest amount of differentially expressed genes (DEG), coding and

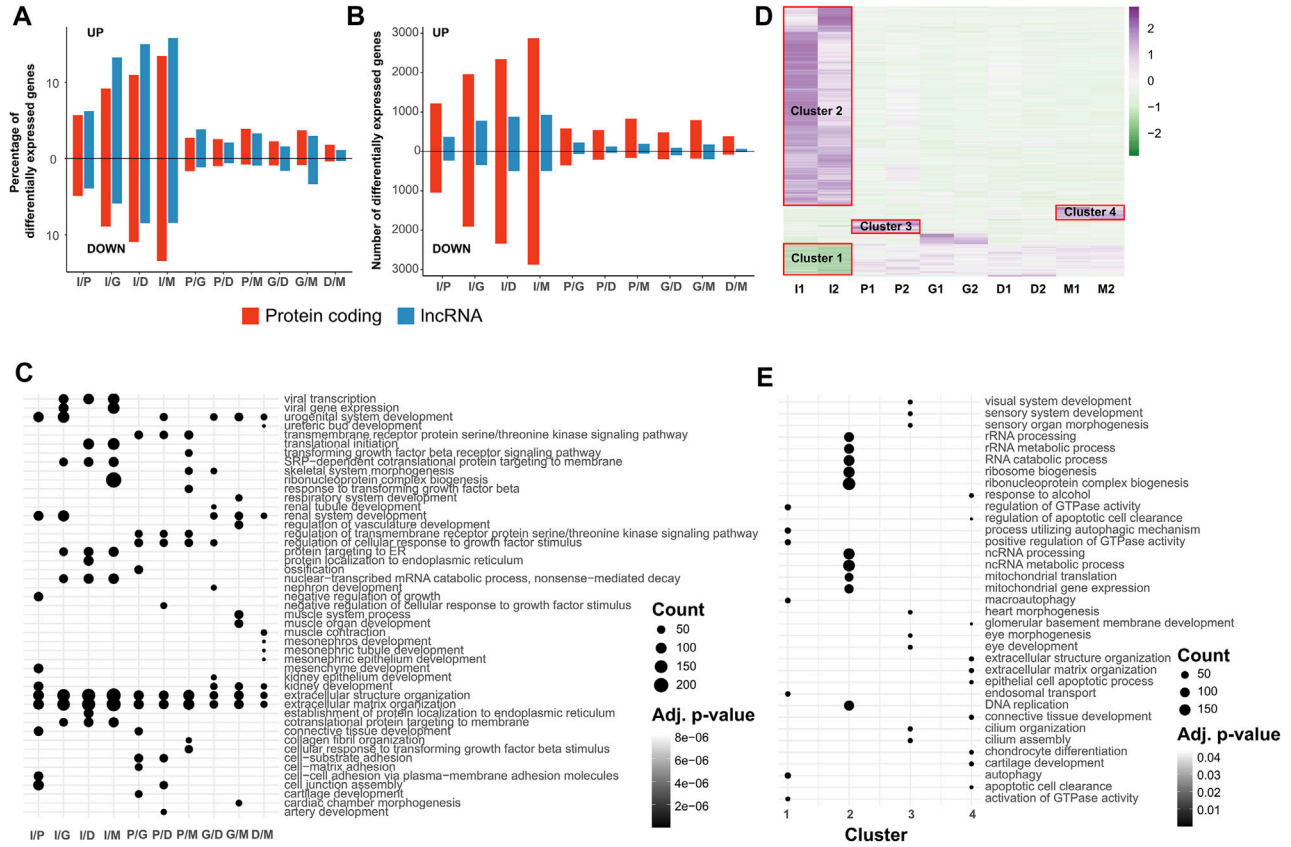

FIGURE 3 | Genes showing differential or stage-specific expression during neural differentiation from iPSC cells are implicated in biological processes. All transcriptome profiles are filtered for genes which display an FPKM value of at least 1.0 in both replicates of at least one biological condition. Differentially expressed genes are defined as those with an FDR of ≤0.05 and an absolute log2 fold change value of ≥0.6. **(A,B)** Percentage **(A)** and absolute count **(B)** of genes displaying differential expression between pairs of neural differentiation stages, divided into protein coding and lncRNA. Percentage values are calculated using the size of the transcript category as the denominator. **(C)** Dot plot of GO terms enriched in differentially expressed gene sets. Top 10 sets are selected in order of Benjamini-Hochberg adjusted *p*-values in each condition pair. Size of the dots indicate the number of differentially expressed genes associated with the GO term. **(D)** Scaled heatmap of the expression values of transcripts showing stage-specific expression according to the ROKU tissue specificity index. **(E)** Dot plot of GO terms enriched in stage specific gene sets. Top 10 sets are selected in order of Benjamini-Hochberg adjusted *p*-values in each condition pair. Size of the dots indicate the number of differentially expressed genes associated with the GO term.

non-coding, both in percentage of gene category (**Figure 3A**) and number of genes (**Figure 3B**), and also throughout the differentiation late stages having a lower amount of DEGs between each other. Between stages, the highest percentage of DEGs is found in the iPSC/induction contrast, and the lowest percentage is in the differentiation/maturation contrast, indicating that the majority of the cell fate determination and differentiation happens in the induction and progenitor expansion stages. A full list of the DEGs in each contrast is available in **Supplementary File S4**.

We also examined the DEGs in each contrast to identify which biological processes they are most strongly associated with. Using GO enrichment, we identified the top 10 biological processes for each contrast (**Figure 3C**). As expected, the most significant terms are found between iPSCs and the other stages, with key terms including those associated with stemness, development and differentiation. Contrasts between later stages show lower significance, as a result of the lower number of DEGs found between the stages. Synapse formation mediating cell-cell communication between neurons is a complex process that is regulated by wide variety of molecules and transmembrane proteins (Südhof, 2018). It is worth noting that the existence of GO terms regarding synapse assembly and axon development between iPSCs and other conditions indicates the proper induction of iPSCs toward neurons. Among the DEGs associated with these GO terms, the semaphorin genes (SEMAs), which constitute a large family of secreted ligands and transmembrane proteins (Kumanogoh and Kikutani, 2004), show changes in expression in induction and subsequent differentiation stages. For instance, SEMA3B, which plays a role in axonal guidance and positioning of the brain anterior commissure, SEMA6B, which acts as a receptor in post-crossing commissural axon guidance (Moreno-Flores et al., 2003; Julien et al., 2005), SEMA3A, which has a role in dendritogenesis (Molofsky et al., 2014), and SEMA5A, which has a bifunctional role in axon development (Kantor et al., 2004),

are upregulated during differentiation. In contrast, SEMA4D (Yamaguchi et al., 2012), which acts as an inhibitor of neuronal differentiation by promoting apoptosis, is downregulated in late stages of differentiation. Bone morphogenetic proteins (BMPs) form a large family of molecules and belong to the transforming growth factor-β (TGF-β) superfamily, which have critical roles in embryogenesis, neural induction, specification, and nervous system development (Mehler et al., 1997; Bond et al., 2012; Hegarty et al., 2013). Our results are consistent with previous studies, showing BMP signaling pathway ligands and receptors are crucial for neurogenesis. However, high levels of BMP7 and BMP4 expression were detected in the early induction stage, and increased levels of expression for several members of the BMP signaling pathway, such as BMP4, BMP6, and BMP1, were also observed across the late stages of differentiation, especially in the maturation stages. In addition, our results show the existence of BMP signaling pathway related genes among the most significant GO terms, such as connective tissue development, axon development, epithelial tube morphogenesis, and transmembrane receptor protein serine/threonine kinase signaling pathway, highlighting the importance of this signaling pathway in neuronal development. In early development stages expression of BMP4 is inhibited with Noggin to allow the neural induction (Thomsen, 1997). Reverse correlation between Noggin and BMP4 expressions in our results suggests that the gradual expression of BMP4 might play a vital role in iPSC-derived neuron differentiation. Of note, crosstalk between BMP and other signaling pathways, such as Wnt, SHH, and MAPK signaling pathway, in conjunction with extracellular matrix organization may govern the determination of cell decision (Compagnucci et al., 2014). The full list of enriched GO terms for each contrast is available in **Supplementary File S5**.

2.4 Identification and Annotation of Stage Specific Protein Coding and lncRNA Genes

As a follow-up to the identification of DEGs between stages, we identified genes with stage specific upregulation or downregulation during the entire process. As the DEG identification method only uses pairwise contrasts, and driver factors of cell fate specification and differentiation are transiently expressed or repressed (Semrau et al., 2017), it is vital to determine such stage specific expression patterns to act as markers of individual differentiation stages. To do so, we used the ROKU algorithm, a tissue specificity index used to determine which genes show increased or decreased expression in a stage specific manner across multiple samples, as described in the **Section 4**. After identifying protein coding genes with stage specific expression, we observed that the genes were divided into four clusters, as shown on the heatmap in **Figure 3D**. Cluster 1 comprised the genes downregulated in iPSCs compared to cells undergoing induction and differentiation, Cluster 2 comprised genes upregulated in iPSCs, while Cluster 3 and 4 were genes upregulated in the induction and maturation stages, respectively. The full list of genes in each cluster are available in **Supplementary File S6**. Similarly to **Figure 3C**, we also identified the top ten enriched GO terms for each cluster, according to adjusted *p*-value to determine the biological processes active during each stage (**Figure 3E**). Cluster 2, due to its large size, had the highest number of genes in its enriched terms, with 199 genes out of 2,314 in the "ribonucleoprotein complex biogenesis" gene set. In comparison, Cluster 1 only had a maximum of 27 out of 403 annotated genes in its top ten terms, in the "autophagy" and "process utilizing autophagic mechanism" gene sets, while cluster 3 and 4 both had 13 out of 140 and 159 annotated genes, respectively, in their top enriched GO terms (cilium assembly for cluster 3, extracellular matrix organization for cluster 4). In addition, the size of the gene set of the individual GO terms also affect the adjusted *p*-values, therefore genes with lower counts could be found to have lower adjusted *p*-values for their enrichment (**Supplementary File S7**). Based on the ROKU analysis, we found three distinct clusters consisting of iPSCs (clusters 1 and 2), progenitors (cluster 3) and mature neurons (cluster 4). In addition, stage specific functional terms were detected across the differentiation stages. The expression profile of iPSCs (clusters 1 and 2) and subsequent differentiated cells were clearly distinguishable and clustered by overall stage specific expression. In this regard, iPSC samples exhibited an expression profile typical of pluripotent stem cells, with NANOG, SALL4, and LIN28A all being upregulated, compared to other stages. Cluster 3 comprises genes which show increased expression in the early stage of differentiation (induction). The ROKU analysis for these genes shows they are primarily involved in the cilium assembly and organization process. Cilium is a unique cytoskeletal structure on the surface of most cells. It participates in signal transduction (Haycraft et al., 2005; Rohatgi et al., 2007), and plays an essential role during the early polarization of the neuroepithelium (Higginbotham et al., 2013), the expansion of the progenitor pool, formation of neural stem cells during nervous system development (Chizhikov et al., 2007; Spassky et al., 2008). Regulatory Factor X (RFX) transcription factors have been known to participate in the control of ciliogenesis by regulating many genes that play fundamental roles in cilia assembly, organization, and function (Thomas et al., 2010). Among these factors, RFX3 is a critical transcription factor in ciliogenesis and early brain development, where it indirectly regulates GLI3 and FGF8 to distribute neurons guidepost to morphogenesis (Benadiba et al., 2012). Our analyses demonstrated that high expression of RFX3 and GLI3 in progenitor cells appears to be informative of molecular cues throughout iPSC-derived neuron generation. In addition, any deficiency in the genes associated with ciliary causes several syndromes in humans. Unraveling of the gene network pattern during early brain development will provide an insight into the identification of the causes of such brain defects. Cluster 4 is obviously distinct, and includes genes with low expression in earlier stages of differentiation which increase at the maturation stage. The high expressions of LXN, C4A, and GAS6 during the maturation stage are consistent with previous studies, which showed a gradual elevation of LXN gene over the course of development (Arimatsu, 1994; Arimatsu et al., 1999; Arimatsu

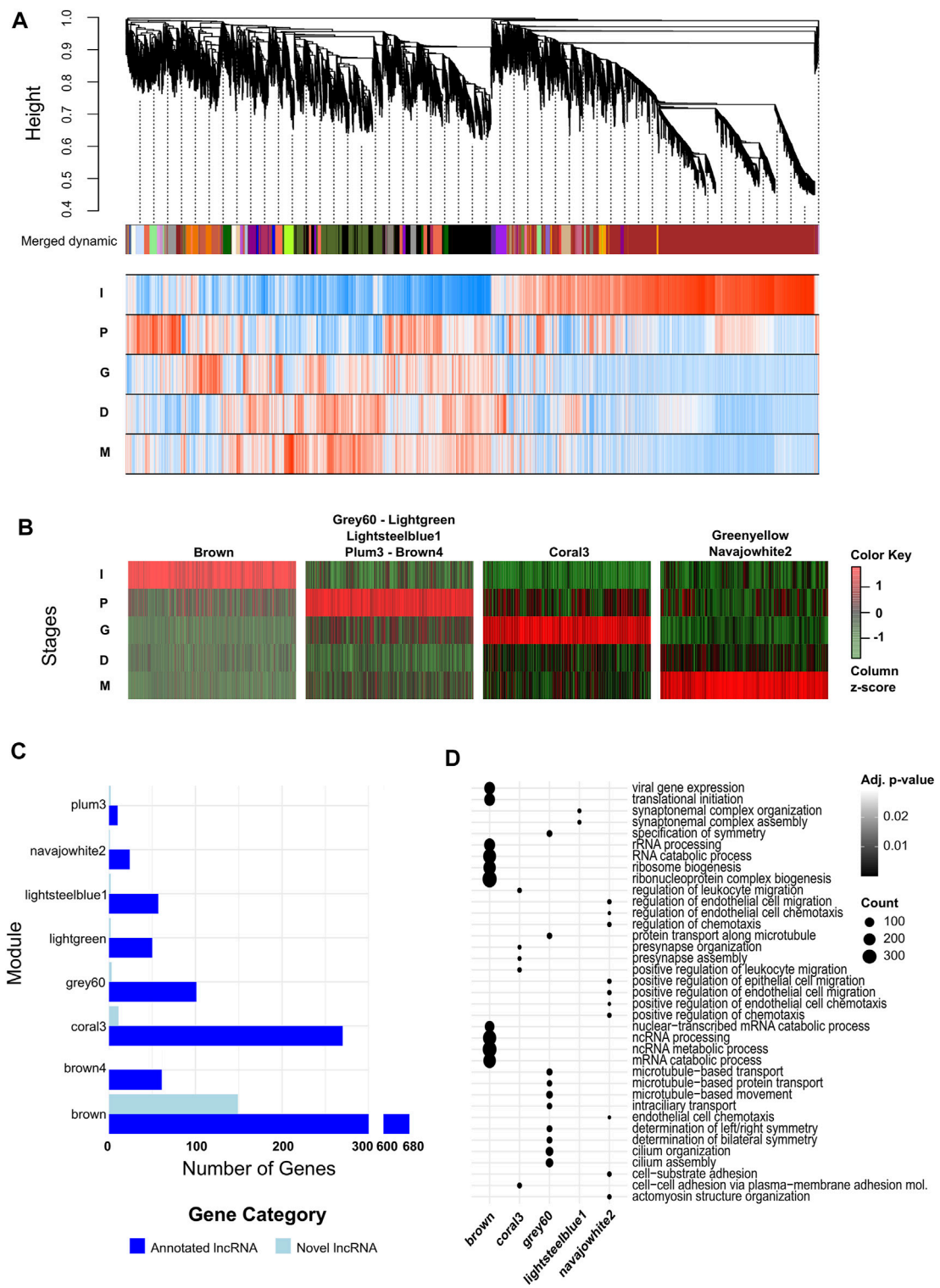

FIGURE 4 | lncRNAs expressed during different stages of neural differentiation from iPSCs are associated with protein coding genes implicated in neural development biological processes. **(A)** WGCNA dendrogram and module affiliation graph of the transcriptome and association of gene expressions with biological conditions during neural differentiation. Each branch of the dendrogram represents a single gene expressed during neural differentiation. The colored bar under the dendrogram indicate the module the gene belongs in, with each color indicating a single module. The heatmap underneath the colored bar shows stage-module correlation levels, with red cells indicating positive correlation, blue cells indicating negative correlation, and darker colors indicating stronger correlation levels. **(B)** Scaled heatmaps of genes in modules showing strong association with single maturation stage (Pearson correlation coefficient ≥0.7). **(C)** Bar graphs indicating lncRNA module membership in clusters of interest categorized into annotated and novel lncRNAs. **(D)** Dot plot of GO terms enriched in gene clusters of interest. Up to 10 sets are selected in order of Benjamini-Hochberg adjusted *p*-values in each module. Size of the dots indicate the number of genes in the module associated with the GO term.

et al., 2009). In addition, the GAS6 gene promotes the survival of neurons, and its expression starts in later embryonic stages, remaining elevated in adults (Prieto et al., 1999). The ROKU analysis for these genes revealed that iPSC-derived neuron differentiation has the potential to mimic the *in vivo* developmental process.

2.5 Co-Expression Network Analysis and Functional Annotation of Differentiation Stage Associated lncRNAs

After the identification of both individual DEGs and stage specific protein coding genes, and the annotations of those sets, we further wanted to identify the genes working in tandem during each stage, in order to annotate the potential functions of novel or otherwise poorly annotated lncRNAs. Using WGCNA, we generated a dendrogram and gene/trait association heatmap to identify co-expressed gene modules (**Figure 4A**, **Supplementary File S8**). Once the modules were identified, we further used a module/trait association matrix, using differentiation stages as traits, to identify which modules were expressed with strong correlation with individual stages. We observed nine modules strongly correlated with a stage ($r > 0.7$ and p-value < 0.05). Five of the modules (lightgreen, brown4, grey60, lightsteelblue1, plum3) were associated with the induction stage (P). Two modules (greenyellow, navajowhite2) were associated with the maturation stage (M), while the iPSC (I) and progenitor (G) stages both had a single module associated with them (brown and coral3, respectively). No modules were strongly correlated with the differentiation stage (D) (**Supplementary Figure S5**). We also plotted the expressions of the genes found in these modules across the maturation process (**Figure 4B**). The genes in each module showed a significant increase in expression in the stage the module is associated with, indicating an accurate module—stage correlation analysis. We then observed the lncRNA membership of each of the seven modules, divided into annotated and novel lncRNAs, to identify how likely each module is to predict the behavior of lncRNAs (**Figure 4C**). Modules brown and coral3 had a high number of lncRNAs compared to the remaining modules, as well as a higher number of novel lncRNAs in particular. In particular, the module brown4 had no novel lncRNAs. We futher performed GO enrichment analysis on the modules to identify the main biological processes the modules are involved in (**Figure 4D**, **Supplementary File S9**). Five of the modules were enriched for at least one biological process. The brown module, associated with the iPSC stage, was enriched for terms associated with noncoding RNA regulation, ribosome formation, and gene transcription and translation. The modules grey60 and lightsteelblue1, associated with induction (P), were enriched for terms associated with synapse formation and cytoskeletal regulation, and the module coral3, associated with progenitor cells (G), was enriched for terms involved in cell-to-cell adhesion and presynapse assembly. Finally, the module navajowhite2, associated with the maturation stage (M), was enriched for terms associated with cellular migration and chemotaxis.

Following the analysis of modules, we performed hub gene identification in our modules. Hub genes in networks have high connectivity to the other genes in the network, and are likely to be critical actors in the activity of those networks (van Dam et al., 2018). As a result, identifying any lncRNAs as hub genes during the iPSC-derived neuron differentiation process would allow us to narrow down our list of targets for future research. Out of 16,699 genes found in the identified modules, 5,163 were considered to be hub genes (kME > 0.90, **Supplementary File S10**). 89 of the hub genes were novel lncRNAs, and 707 were annotated lncRNAs. Furthermore, 55 of the novel lncRNA hub genes, and 257 of the annotated lncRNA hub genes were found in modules showing strong correlation with individual maturation stages.

Recent studies have reported that lncRNAs are involved in the regulation of cellular processes in mammalian development and disease. Nevertheless, many lncRNAs have unknown biological functions. Our WGCNA and GO analyses predict possible roles for lncRNAs in a wide range of biological processes, such as ncRNA processing, establishment of protein localization to organelles, sensory perception of bitter taste, cilium assembly, microtubule-based movement, neural tube development, and axonogenesis. Additionally, Our findings show that the AC006062.1 and AC025280.3 lncRNAs were upregulated in the differentiation and maturation stages. The high expression of these lncRNAs was accompanied with the upregulation of coding genes such as C4A and C4B in the maturation stage, both of which are hub genes (kME > 0.94) in a module associated with maturation. Furthermore, the alteration of LINC00261, C4A, and C4B following valproic acid treatment in motor neurons (Yoshida et al., 2015), as well as the clustering of AC025280.2 with neuron-related genes (Tenjin et al., 2020), suggest the possible role of these genes in neuron development. Additionally, while the expression and function of GAS6-DT in neurons and the brain remain unclear, there is evidence that GAS6-DT is involved in the upregulation of GAS6 gene expression in melanomas (Wen et al., 2019). GAS6 is also a protein-coding hub gene associated with maturation (kME > 0.90), with roles in the central nervous system (CNS) (Goudarzi et al., 2016), and highlighting a similar crosstalk between GAS6 and GAS6-DT can influence neuron development and maturation in the context of iPSC-derived neuron generation. Our results also revealed the upregulation of MIRLET7BHG (kME > 0.92) in the maturation stage of differentiation. This data is in agreement with previous reports that demonstrated the expression of MIRLET7BHG in various tissues, including the brain (Sauvageau et al., 2013). In addition, LINC00842 and LINC00857 are lncRNAs with unknown function in the brain, but LINC00842 downregulation was detected in the lung adenocarcinoma sample, compared to healthy tissue (Ding et al., 2018). In addition, LINC00857 is one of the lncRNAs dysregulated in lung cancer (Wang et al., 2016; Wang et al., 2020), and it regulates biological processes such as tumor growth, proliferation, motility, and the invasion capacity of lung cancer, in addition to acting as an oncogene in liver (Xia et al.,

2018), bladder (Dudek et al., 2018), gastric (Pang et al., 2018), and esophageal cancer models (Su et al., 2019). In our analyses, the upregulation of LINC00842 and LINC00857 throughout differentiation suggests that LINC00857 might have roles in the biological response of cells during the maturation of neuron cells. In addition to the novel lncRNA candidates in our results, we found upregulation of expression of lncRNAs known to be active during neurogenesis identified as hub genes in our study, such as MALAT1 (kME > 0.96) (Bernard et al., 2010; Lipovich et al., 2012) and TUNA (kME > 0.90) (Lin et al., 2014). Thus, these results might be used to understand the cell compositions and differentiation stages of iPSC-derived neuronal cultures and discovery of novel markers throughout brain development.

3 CONCLUSION

Understanding the transcriptome is a critical step in study of the differentiation process in multicellular organisms, as changes in the transcriptome are what allows the large variety of cells required for the formation of a multicellular organism to arise from undifferentiated cells with a shared genome. While the protein coding transcripts play an important role in cell differentiation and fate determination, our understanding of the non-coding transcriptome and its role in these processes is as of yet incomplete. As a large percentage of the genomes of higher order eukaryotes is made up of non-coding genes with functional roles in chromatin organization and the regulation of gene expression, an in-depth analysis of the full transcriptome, as opposed to the coding transcriptome, is crucial in studying processes such as the formation of neurons from undifferentiated stem cells. Such in-depth analysis of the transcriptome has been made available in the last two decades by advent of high-throughput sequencing technologies, such as RNA-seq, as well as the computational tools used in processing the sequencing data.

Here, we presented a detailed analysis of the transcriptome during multiple stages of iPSC-derived neuronal differentiation. We included comparisons and contrasts between the stages, and identify biological processes enriched during specific stages. We provided an overview of the most significant terms and co-regulated gene modules, as well as a comparison of our findings to previously established literature on cell differentiation and proliferation. In order to provide a valuable resource for future research on neural development and neuron differentiation, we further included in-depth lists of differentially expressed or stage-specific genes, and co-expressed gene modules, as well as enriched GO terms in each of these categories. Crucially, we have also provided detailed data regarding the expressions of lncRNAs during iPSC-derived neuronal differentiation, and potential differentiation-affiliated biological processes they are implicated in. The comprehensive map of the coding and non-coding transcriptome during neuronal differentiation is of great importance to future research in both developmental biology and neuroscience.

4 MATERIALS AND METHODS

4.1 iPSC Expansion

Two healthy human iPSC lines from two independent donors, represented as iPSC line WT1 (home-made) and WT2 (Cat No. #ASE-9202, Applied StemCell Inc.) were cultured and maintained as previously described (Akbari et al., 2019b). iPSCs expanded on hESC-qualified Matrigel matrix basement membrane (cat no: #354277, Corning) with mTeSR1 medium (cat no: #SC-05850, Stem Cell Technologies). Cells were passaged once a week with a 1:6 ratio, and culture medium was changed every other day following sub-culturing.

4.2 Neural Induction and Expansion of Neural Progenitor Cells

We used STEMdiff™ Neural System to generate iPSC-derived neuron cells. Production procedure comprise mainly the induction/generation, expansion, differentiation and maturation steps. In all steps of differentiation, cells were cultured in a monolayer culture system, and we did not isolate or enrich cells according to their surface markers while sub-culturing the cells. iPSCs were harvested from the mTeSR1 culture, and plated on matrigel coated plates at 200,000 cells/cm^2 in neural induction medium (NIM) supplemented with SMADi (cat no: # 08581, Stem Cell Technologies) and 10 μM Y-27632 (cat no: # 72302, Stem Cell Technologies) for 9 days. Afterwards, the generated NPCs in NIM were sub-cultured for two additional passages before starting differentiation as recommended in the manufacturer's protocol. To this end, the NPCs were detached with Accutase (cat no: # 07922, Stem CellTechnologies), seeded at 1.25×10^5 cells/cm^2 on Matrigel coated plates, and expanded in neural progenitor medium (NPM) (cat no: # 05833, Stem Cell Technologies) for the next 20 days. The samples were collected on day 0 (pluripotent), days 9–12 (induced progenitor), and days 25–28 (expanded progenitor).

4.3 Neural Differentiation and Maturation

To generate mature neuron cells, two more passages were performed during days 25–28 (first day of differentiation) and days 32–35 (first day of maturation), respectively. Neural differentiation medium (NDM) (cat no: # 08500, Stem Cell Technologies) was used to generate neuronal precursors from iPSC-derived NPCs. On days 25–28, NPCs were placed at a density of 4×10^4 cells/cm^2 on matrigel coated plates. After overnight incubation at 37°C and 5% CO_2 in the incubator, the culture medium was fully refreshed with NDM, and the process continued for 7 days. On days 32–35, generated neural precursor cells were passaged and plated at a density of 4×10^4 cells/cm^2 on matrigel coated cell culture plates. The culture medium was switched to neural maturation medium (NMM) (cat no: # 08510, Stem Cell Technologies) on the following day, and the cells were incubated at 37°C and 5% CO_2 in the incubator for

at least 1 week. The samples were collected on days 32–35 (differentiation) and days 45–47 (maturation).

4.4 Immunofluorescence Staining

Characterization of the generated cells during differentiation stages was performed using immunofluorescence staining, as previously described (Akbari et al., 2019b; Karagonlar et al., 2020). Briefly, the cells were fixed in %4 paraformaldehyde (PFA; cat no: # 158127, Merck) for 20 min at room temperature, washed three times with 1× PBS, then permeabilized using 0.5% TritonX (cat no: #28313, Thermo Fisher Scientific). After 2 hours, blocking staining was carried out using the following primary antibodies: OCT3/4 (cat no: # 75463S, Cell signaling), PAX6 (cat no: # 60433S, Cell signaling), NESTIN (cat no: # 33475S, Cell signaling), GFAP (cat no: # 12389T, Cell signaling) and β-TUBIII (cat no: # 4466S, Cell signaling). Slides were visualized using a confocal microscope (cat no: # LSM880, Zeiss).

4.5 RNA Extraction and Sequencing

Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA concentration was measured *via* NanoDrop (Thermo Fisher Scientific), and the quality was assessed using Agilent Bioanalyzer. RNA sequencing was performed at EMBL GeneCore. Briefly, the samples were prepared using NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) and the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® to generate strand-specific RNA libraries. We started with 250 ng of total RNA as input, adaptor dilution was 1:5, and we used nine cycles for the PCR enrichment of adaptor ligated DNA. Then 5-plex pools were prepared equimolarly and sequenced in a NextSeq 500 system with 40 pair-end read model. The samples were sequenced to an average depth of 100 million reads per sample.

4.6 Gene Expression Measurement

Paired-end RNA-seq reads were aligned against the GENCODE GRCh38 human genome assembly (Release 34, obtained from https://www.gencodegenes.org/) using HISAT2 (version 2.1.0) (Kim et al., 2015) with the parameters "-p 36 --dta -× -1 -2 -S". The resulting SAM alignment files were converted to BAM binary files and sorted and indexed using SAMtools utilities (version 1.9) (Li et al., 10002009). The alignment files were used to calculate the total expression levels of gene transcripts (including all known isoforms) using the featureCounts function of the R package Rsubread (version 2.4.0) (Liao et al., 2019) with the following parameters: "files = {infile.bam}, annot.ext = "{infile.gtf}", isGTFAnnotationFile = T, GTF.featureType = "exon", GTF.attrType = "gene_id", useMetaFeatures = T, countMultiMappingReads = T, isPairedEnd = T, nthreads = numParallelJobs." Identified transcripts were annotated using the GENCODE GRCh38 human transcriptome annotations (Release 34). Following expression quantification, we removed transcripts that did not have an expression of ≥1 FPKM (fragments per kilobase of transcript per million reads) in both replicates of at least one biological condition to improve detection sensitivity of differentially expressed genes.

4.7 Identification of Novel lncRNA Candidates

A number of filters were applied to transcripts that were not annotated by our reference transcriptome assembly in order to identify high confidence novel lncRNA candidates. Transcripts coded "u," "x," "o," and "i" by StringTie were selected as the initial candidate pool, which signify transcripts aligned to intergenic regions, to the antisense strand of known genes, to the sense strand of known genes with partial exonic overlap, and to the intronic regions of known genes, respectively. We further selected only transcripts longer than 200 nucleotides for the subsequent analyses. The remaining transcripts were analyzed to identify those with high coding potential and an ORF coding for longer than 100 aminoacids with TransDecoder (https://github.com/TransDecoder/TransDecoder) the "TransDecoder.LongOrfs -t" command, which were removed from the analysis. The remaining transcripts were aligned against the SwissProt manually curated protein sequence database (version 2017_08) to identify any protein domain homology, as is found in pseudogenes, using a local installment of blastx, with an E-value cutoff of 0.01 (blastx -evalue 0.01). The remaining transcripts were aligned against the Rfam database of RNA families (version v12.1) (Nawrocki et al., 2015) with the Infernal cmscan program, with an E-value cutoff of 0.01 (–E 0.01), in order to identify any housekeeping RNAs. Finally, transcripts with a human-specific coding probability of 0.8 or higher, as identified by CPAT (version 1.2.4) (Wang et al., 2013) were considered to be protein coding and removed from the list of novel lncRNA candidates (Hudson et al., 2019).

Independent iPSC-derived neuronal differentiation datasets were used to inspect the expressions of identified lncRNAs. A total of 52 raw RNA-sequencing libraries were downloaded from the Sequence Read Archive (SRA) database (Leinonen et al., 2011), pertaining to previous studies of iPSC-derived neurons (SRA Accession #s: SRP238174, SRP266877). The libraries were downloaded in FASTQ format using the fastq-dump utility of the SRA Toolkit (v.2.9.0), with the following parameters: "*--gzip--skip-technical--readids--dumpbase--clip--split-3.*" The libraries were then processed and quantified using the same methods as described above. Genes were considered consistently expressed in a dataset if it had an expression of ≥1 FPKM of more than 50% of the samples in at least one biological condition in the dataset.

4.8 Differential Expression Analysis

The differential expression statuses of protein-coding and lncRNA transcripts were analyzed using the R package edgeR (version 3.32.0) (Robinson et al., 2010). All condition pairs were examined to identify transcripts that are upregulated in one condition compared to the second, for a total of 10 combinations. Count data of the transcripts were normalized using the trimmed mean of M-values method (TMM), which were then fitted to a generalized linear model (GLM). Afterwards, the estimateDisp and glmFit functions were used to calculate the contrast statistics for the condition pairs. Genes were considered differentially expressed between two conditions if they had an adjusted *p*-value (FDR) of 0.05 or lower, and an absolute log2 (fold change) value of 0.6 or higher.

4.9 Identification of Stage Specific Expression Patterns

To identify which protein coding genes had stage-specific expression patterns, whether upregulation or downregulation, and which stages of the differentiation process they were specific to, we used the ROKU function of the R package TCC (version 1.24.0) (Sun et al., 2013). ROKU is an algorithm that analyzes the expression levels of a gene across multiple samples, whether a time-course series or discrete biological conditions, and identifies whether any of the values are outliers (Kadota et al., 2006). It then marks them with a 1 if it is upregulated in a sample compared to the other samples, and −1 if it is downregulated. Samples with nonspecific expression patterns are marked 0. Genes with consistently low expression (<1 FPKM in at least one replicate of all stages) were also marked 0 to avoid noise.

4.10 Weighted Gene Co-Expression Network (WGCNA) Analysis

To examine the potential coregulatory relationships between genes expressed during the differentiation stages, we used the R package WGCNA (version 1.69) (Langfelder and Horvath, 2008) to perform weighted gene coexpression network analysis using the FPKM expression values of transcripts and created a coexpression network. We set a soft thresholding power of 18 for the correlation network formed prior to the coexpression analysis, as recommended by WGCNA for our experimental design. The Dynamic Tree Cut algorithm of WGCNA was then applied to a hierarchical clustering of the genes using the average linkage method to identify clusters of co-expressed genes, or gene modules. A minimum module size of 30 genes was set to avoid excessive noise in module determination. The correlation of each module eigengene with the differentiation stage is calculated and significant modules associated with the stages were determined ($r > 0.7$ and p-value < 0.05). The hub genes in each module were calculated using intramodular connectivity scores (kME). Genes with a kME of 0.90 or higher were considered to be hub genes.

4.11 Statistical Analysis and Graphical Representation

We used the R statistical computation environment (version 3.6.0) for all analysis and visualization purposes. Functions of the stats package or the base R installation were used for most statistical analysis, either directly or by other packages, including hclust for Euclidean hierarchical clustering of genes and samples, prcomp for identification of principal components of the expression matrix, and cor.test for correlation calculation. clusterProfiler (version 3.18.0) (Yu et al., 2012) was used to identify and visualize the enrichment of GO terms in sets of genes of interest. Pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html) (version 1.0.12) was used with row-wise scaling of data to visualize the Z-score values of genes during differentiation. Native WGCNA functions were used to visualize the coexpression network dendrogram, module colors, and relationships of such with specific biological conditions. ggplot2 (https://cran.r-project.org/web/packages/ggplot2/index.html) (version 3.3.2) was used for all other visualization.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The RNA-seq datasets are deposited into the Sequence Read Archive (SRA), with accession number SRP304457.

ETHICS STATEMENT

This study was reviewed and approved by İzmir Biomedicine and Genome Center GOEK Ethics Committee with protocol number 2021-037.

AUTHOR CONTRIBUTIONS

MK: conceptualization, funding acquisition, formal analysis, investigation, methodology. SA: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing. DE: methodology, formal analysis, investigation, software, visualization, writing—original draft, writing—review and editing. AB: methodology, data curation, formal analysis, investigation, software, visualization. KE: conceptualization, funding acquisition, methodology, project administration, resources, validation. EE: conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation. GK: conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, supervision, validation, visualization, writing—original draft, writing—review and editing.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.727747/full#supplementary-material

Supplementary Figure S1 | Bar plot displaying the percentages of read counts of expressed transcripts in stages of iPSC-derived neuronal differentiation. lncRNAs include annotated and novel intergenic lncRNAs, sense overlapping lncRNAs, intronic RNAs, and antisense RNAs. Other RNAs include any transcript that is not protein coding or in the lncRNA category, including miRNAs, piRNAs, siRNAs, snRNAs, and snoRNAs.

Supplementary Figure S2 | Violin plot displaying the distributions of log2-transformed FPKM expression values of protein coding and lncRNA genes during iPSC-derived neuronal differentiation. Horizontal lines within the violin shapes indicate the median log2-transformed FPKM value.

Supplementary Figure S3 | Scaled heatmap of the log2(FPKM + 1) expression values of novel lncRNAs identified in iPSC-derived neuronal differentiation process consistently expressed in the samples from the SRP238174 dataset (>1 FPKM in at least 50% of one timepoint). Purple cells indicate increased expression compared to the mean, while green cells indicate decreased expression. Dataset originally described by Burke et al. (2020).

Supplementary Figure S4 | Scaled heatmap of the log2(FPKM + 1) expression values of novel lncRNAs identified in iPSC-derived neuronal differentiation process consistently expressed in the samples from the SRP266877 dataset (>1 FPKM in at least 50% of one timepoint). Purple cells indicate increased expression compared to the mean, while green cells indicate decreased expression. Dataset originally described by Solomon et al. (2021).

Supplementary Figure S5 | Module – trait relationship heatmap of coexpressed gene modules and iPSC-derived neuronal differentiation stages. Top number in each cell indicates the Pearson correlation between the module expression and the differentiation stage. Bottom number (in parentheses) indicates the *p*-value of the correlation. Red cells indicate a positive correlation, while green cells indicate a negative correlation.

Supplementary File S1 | GTF file of transcripts identified as high-confidence lncRNAs.

Supplementary File S2 | Expression matrix of novel lncRNA candidates in SRP238174.

Supplementary File S3 | Expression matrix of novel lncRNA candidates in SRP266877.

Supplementary File S4 | List of differentially expressed genes in each contrast between conditions.

Supplementary File S5 | List of biological process GO terms enriched in the DEGs of each contrast.

Supplementary File S6 | List of genes that are stage-specifically expressed during differentiation.

Supplementary File S7 | List of biological process GO terms enriched in stage-specifically expressed gene clusters.

Supplementary File S8 | List of gene memberships of co-expressed gene modules.

Supplementary File S9 | List of biological process GO terms enriched in co-expressed gene modules.

Supplementary File S10 | List of identified hub genes and their module memberships.

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Hyperoxygenation During Mid-Neurogenesis Accelerates Cortical Development in the Fetal Mouse Brain

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Oxygen tension is well-known to affect cortical development. Fetal brain hyperoxygenation during mid-neurogenesis in mice (embryonic stage E14.5. to E16.5) increases brain size evoked through an increase of neuroprecursor cells. Nevertheless, it is unknown whether these effects can lead to persistent morphological changes within the highly orchestrated brain development. To shed light on this, we used our model of controlled fetal brain hyperoxygenation in time-pregnant C57BL/6J mice housed in a chamber with 75% atmospheric oxygen from E14.5 to E16.5 and analyzed the brains from E14.5, E16.5, P0.5, and P3.5 mouse embryos and pups *via* immunofluorescence staining. Mid-neurogenesis hyperoxygenation led to an acceleration of cortical development by temporal expansion of the cortical plate with increased $NeuN^+$ neuron counts in hyperoxic brains only until birth. More specifically, the number of $Ctip2^+$ cortical layer 5 (L5) neurons was increased at E16.5 and at birth in hyperoxic brains but normalized in the early postnatal stage (P3.5). The absence of cleaved caspase 3 within the extended $Ctip2^+$ L5 cell population largely excluded apoptosis as a major compensatory mechanism. Timed BrdU/EdU analyses likewise rule out a feedback mechanism. The normalization was, on the contrary, accompanied by an increase of active microglia within L5 targeting $Ctip2^+$ neurons without any signs of apoptosis. Together, hyperoxygenation during mid-neurogenesis phase of fetal brain development provoked a specific transient overshoot of cortical L5 neurons leading to an accelerated cortical development without detectable persistent changes. These observations provide insight into cortical and L5 brain development.

Keywords: oxygen, hyperoxia, corticogenesis, neural stem cells, apoptosis, brain development, microglia, cortical layers

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Abbreviations: CC3, cleaved caspase 3; CP, cortical plate; Ctip2, chicken ovalbumin upstream promotor transcription factor-interacting protein 2; E, embryonic day; L, layer; NeuN, neuronal nuclear protein; RBFOX3, RNA binding protein fox-1 homolog 3; OSVZ, outer subventricular zone; P, postnatal stage; Pax6, paired box 6; PBS, phosphate buffered saline; Satb2, special AT-rich sequence-binding protein 2; SVZ, subventricular zone; Tbr1, T-box brain protein 1; Tbr2, T-box brain protein 2; TBST, Tris buffered saline/Tween 20; vGluT2, vesicular glutamate transporter 2; VZ, ventricular zone.

INTRODUCTION

Oxygen tension during development is known to critically affect brain development (Fagel et al., 2006; Schneider et al., 2012; Porzionato et al., 2015; Wagenfuhr et al., 2015; Lange et al., 2016; Wagenfuhr et al., 2016). Thereby, the effects depend on timing and intensity of oxygen application: while short-term hyperoxygenation is able to enhance neurogenesis and brain size, chronic hyperoxygenation can lead to adverse effects (Wagenfuhr et al., 2015; Wagenfuhr et al., 2016; Markert et al., 2020). Indeed, short-term hyperoxygenation during mid-neurogenesis of fetal mouse brain development (embryonic stages E14.5 to E16.5) leads to an immediate expansion of a distinct proliferative cell population basal of the subventricular zone (SVZ) constituting a new neurogenic cell layer similar to the outer SVZ (OSVZ), which contributes to corticogenesis by heading for deeper cortical layers as a part of the cortical plate (CP) (Wagenfuhr et al., 2015). Finally, the number of Ctip2$^+$ neurons in the deeper layer 5 (L5) of the CP projecting into various brain regions is markedly increased (Hattox and Nelson 2007; Oswald et al., 2013; Wagenfuhr et al., 2015). This phenomenon is of high interest, since alterations within the cortical L5 cell population are directly linked to diseases such as schizophrenia (Kolomeets and Uranova 2019; Mi et al., 2019), which is also linked with oxidative stress and various other factors during development (Chew et al., 2013; Górny et al., 2020). Despite these known effects of maternal hyperoxygenation with subsequent changes of the oxygen tension of brain tissue *in utero*, a pilot study applying maternal oxygenation in humans, although in a more chronic treatment scheme, shows initial morphological changes of the head, but no differences in neurodevelopmental testing of the children (Edwards et al., 2018). These results raise not only concerns about the safety of maternal hyperoxygenation therapy (Rudolph 2020) but also the questions whether and how the brain is able to better compensate for changes of neuronal plasticity during development to normalize the cortical structure.

The process of embryonic/fetal brain development is highly orchestrated through main events like proliferation, differentiation, and migration of neuronal stem cells (Noctor et al., 2001; Talamillo et al., 2003; Haubensak et al., 2004; Noctor et al., 2004) and morphological and functional shaping of cortical cell populations (White and Barone 2001; Blanquie et al., 2017). Thereby, radial glia cells located at the ventricular surface develop into cortical neurons through the Pax6, Tbr2, and Tbr1 axis where the resulting cells migrate through the cortex and form the cortical inside-out layering (Englund et al., 2005; Agirman et al., 2017). The resulting number of neurons seems to be prenatally regulated through invading microglia capable of phagocytizing and controlling the number Pax6$^+$ or Tbr2$^+$ cells (Cunningham et al., 2013). Other suggested mechanisms include a feedback signal from cortical deep layer cells to the radial glia affecting the generation of upper layer cells as well as already occurring apoptosis of neuroprecursor cells (Blaschke et al., 1996; Toma et al., 2014). After birth, apoptosis of postmitotic neurons particularly becomes prominent in the cortex where around 50% of all neurons die (Dekkers et al., 2013; Wong and Marín 2019). This mechanism likely regulates the number of cortical neurons in an area-dependent manner through their electrical activity and indicates a specific postnatal connectivity control (Blanquie et al., 2017).

Although the short-term effects of hyperoxygenation during mid-neurogenesis of fetal mouse brain development with immediately enhanced neurogenesis particularly within cortical L5 are reported, the subsequent consequences of these phenomena during later cortical development remain enigmatic. We therefore used our established model of maternal hyperoxygenation to investigate the effects of increased oxygen tension during mid-neurogenesis (E14.5–E16.5) on later cortical development until the early postnatal state (Wagenfuhr et al., 2015). Moreover, the model allows the investigation of potential mechanisms mediating the reshape of the cortical structure during late fetal and early postnatal cortical development.

MATERIALS AND METHODS

Animals and Oxygen Treatment

C57BL/6J timed-pregnant mice were housed in their home cages within a preconditioned oxygen chamber (InerTec, Grenchen, Switzerland) at 75% oxygen or room air (21% oxygen; control condition). During the whole treatment protocol, all animals were handled by the same investigator. Fetuses of both groups showed an ordinary morphology. Pregnant mice for analysis of postnatal day 0.5 (P0.5) fetuses intraperitoneally received BrdU (50 mg/kg body weight) at E14.5, the start of hyperoxia treatment, and EdU (25 mg/kg body weight) at E17.5, 1 day after the end of the oxygen treatment. All data were gathered from randomly chosen embryos or pups from at least three independent litters per group. All animals were maintained and treated with permission of the local Department of Animal Welfare (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern) (reference number 7221.3-1-043/16) and comply with the Tierschutzgesetz and Verordnung zur Umsetzung der Richtlinie 2010/63/EU from Germany. Of note, our study was initially designed including a hypoxia group (10% oxygen), but it was not possible to gather data for postnatal maternal hypoxia group as the mice tend to infanticide. To secure animal welfare and to be in line with German law, we had to cancel these experiments.

The brains of embryos and pups were dissected prior and immediately after oxygen treatment [E14.5 (for estimating the cortical volume) and E16.5] and postnatally at P0.5 and P3.5, fixed for 24 h in 4% paraformaldehyde (Merck, Darmstadt, Germany), and kept in 30% sucrose (Carl Roth, Karlsruhe, Germany) in DPBS (Thermo Fisher Scientific, Waltham, United States). Then brains were snap-frozen, sectioned coronal at 20-µm thickness using a cryomicrotome (Leica Biosystems, Nussloch, Germany), and mounted on Superfrost Plus slides (Thermo Fisher Scientific). The slides were stored at 4°C until staining.

Immunofluorescence

Slides were washed with wash buffer (Agilent, Santa Clara, United States), and heat-induced antigen retrieval was performed using 10 mM sodium citrate (Carl Roth) with 0.05% Tween 20 (SERVA, Heidelberg, Germany) for 30 min at 95°C or 2 N HCl for 30 min at 37°C for BrdU staining. After 20 min at room temperature, slides were washed with Tris buffered saline/Tween 20 (TBST), treated with TBST containing 0.2% Triton X-100 (Carl Roth) and 10% donkey serum (Merck) for 30 min, and were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit anti-NeuN (Merck, ABN78, RRID: AB_10807945), chicken anti-NeuN (Merck, ABN91, RRID: AB_11205760), rabbit anti-cleaved-caspase-3 (CC3; Cell Signaling, 9661S, RRID: AB_2341188), mouse anti-BrdU (Thermo Fisher Scientific, B35128, RRID: AB_2536432), rabbit anti-Tbr1 (Abcam, ab31940, RRID: AB_2200219), mouse anti-Satb2 (Abcam, ab51502, RRID: AB_882455), rat anti-Ctip2 (Abcam, ab18465, RRID: AB_2064130), rabbit anti-Ctip2 (Abcam, ab28448, AB_1140055), rabbit anti-Iba1 (Wako, 019-19741, RRID: AB_839504), goat anti-Iba1 (Abcam, ab5076, RRID: AB_2224402), rabbit anti-CD68 (Abcam, ab125212,RRID: AB_10975465), rat anti-CD68 (BioRad, MCA1957GA, AB_324217), or guinea pig anti-vGluT2 (Merck, AB2251-I, RRID: AB_1587626). Subsequently, slides were incubated with corresponding secondary antibodies (Thermo Fisher Scientific), and nuclei were stained with Hoechst 33258 (Merck). For EdU analysis, slides were stained using Click-iT staining kit (Thermo Fisher Scientific) as described by the manufacturer. Finally, slides were mounted with Fluoromount-G (Biozol, Eching, Germany).

Imaging and Measurements

Most images were taken with AxioObserver Z1 with Apotome using ZEN blue 2.3 software with Tiles and Position module (all from Carl Zeiss, Oberkochen, Germany). Z-stack images of microglia-targeting Ctip2$^+$ cells were taken with LSM900 with Airyscan (Carl Zeiss). Hoechst images of every sixth section with a thickness of 20 µm were taken with ×2.5 objective and subsequently used for determining the volume of the whole brain and the CP corresponding to the mouse brain atlas (Allen-Institute, 2008). Thereby, we used the corpus callosum and the lateral ventricles for orientation dorsal/between the hemispheres and for lateral the piriform region and the endopiriform nucleus. For caudal sections, we used the thinner subiculum layer in the extension of hippocampal C1 layer (excluded) for dorsal orientation (**Supplementary Figure S1**). The volume was calculated by adding up the data of each slice (midpoint type) and then multiplying by 120 (every sixth slice of 20-µm thickness).

For analysis of apoptosis, fluorescence images of the whole hemispheres from the developing parietal cortex areas were taken. CC3$^+$ cells were counted with ZEN blue 2.3 in the cortex from the dorsal to ventral site corresponding to the area described above. For double staining, CC3$^+$ were searched as described above and imaged as Z-stack with Apotome mode. For specific marker analysis, at least four images of the cortex were taken as Z-stack with 1-µm steps. The corresponding cells or VGlut2$^+$ synapses were counted in the middle focal plane using either ZEN analysis software (Tbr1, Ctip2, and Satb2) or ImageJ (BrdU, EdU, and NeuN). L5 neurons at postnatal stages were defined by high Ctip2 expression levels as described by McKenna et al. (2011). Iba1$^+$ cells and double/triple-stained cells were manually counted through the Z-stacks within the middle cortical sections in the rostro-caudal axis by the same investigator who was blinded for the treatment groups.

Statistics

All statistical analyses were performed with RGUI 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria) or SPSS version 25.0 (SPSS Inc., Chicago, IL). If not otherwise stated, statistical significance was evaluated by unpaired two-sided *t*-test or two-way ANCOVA followed by pairwise *t*-test including Bonferroni correction. Cortical volume was analyzed independent of the developmental state due to its well-known physiological large volume expansion after birth. The numbers of analyzed embryos and pups gathered from at least three independent litters are indicated by "*n*." All data are displayed as means ± s.e.m. with the numbers of analyzed embryos and pups indicated for each experiment. The significance level was set to $p < 0.05$ (two-tailed test).

RESULTS

Oxygen-Induced Cortical Expansion During Fetal Brain Development is Equalized at Early Postnatal Stage

To assess the time course and putative long-term persistency of the effects of oxygen tension on the rapidly changing and highly regulated fetal cortical development, we applied the already introduced mouse model of maternal hyperoxygenation known to reliably control tissue oxygen tension and subsequently neurogenesis within the developing fetal mouse brain (Wagenfuhr et al., 2015; Wagenfuhr et al., 2016). We thus applied maternal hyperoxygenation to time-pregnant mice at mid-neurogenesis from embryonic stage E14.5 to E16.5 and investigated cortical morphology during embryonic development from E14.5 to early postnatal stage at P3.5 (**Figure 1A**). The hyperoxia and control groups showed no abnormalities in their spontaneous or litter care behavior. All embryos and pups displayed normal morphology at all developmental stages examined, but the brains of the hyperoxia group appeared visually and quantitatively increased in their size at E16.5, but not on other developmental stages (**Figures 1B,C**). Indeed, mouse embryos of the hyperoxia group showed a 1.2-fold increase in the volume of the CP as compared with normoxic controls at E16.5 ($p = 0.002$), which persisted until birth (P0.5; $p = 0.015$), but not until P3.5 ($p = 0.653$; unpaired two-sided *t*-test; $n = 4–9$; **Figure 1D**).

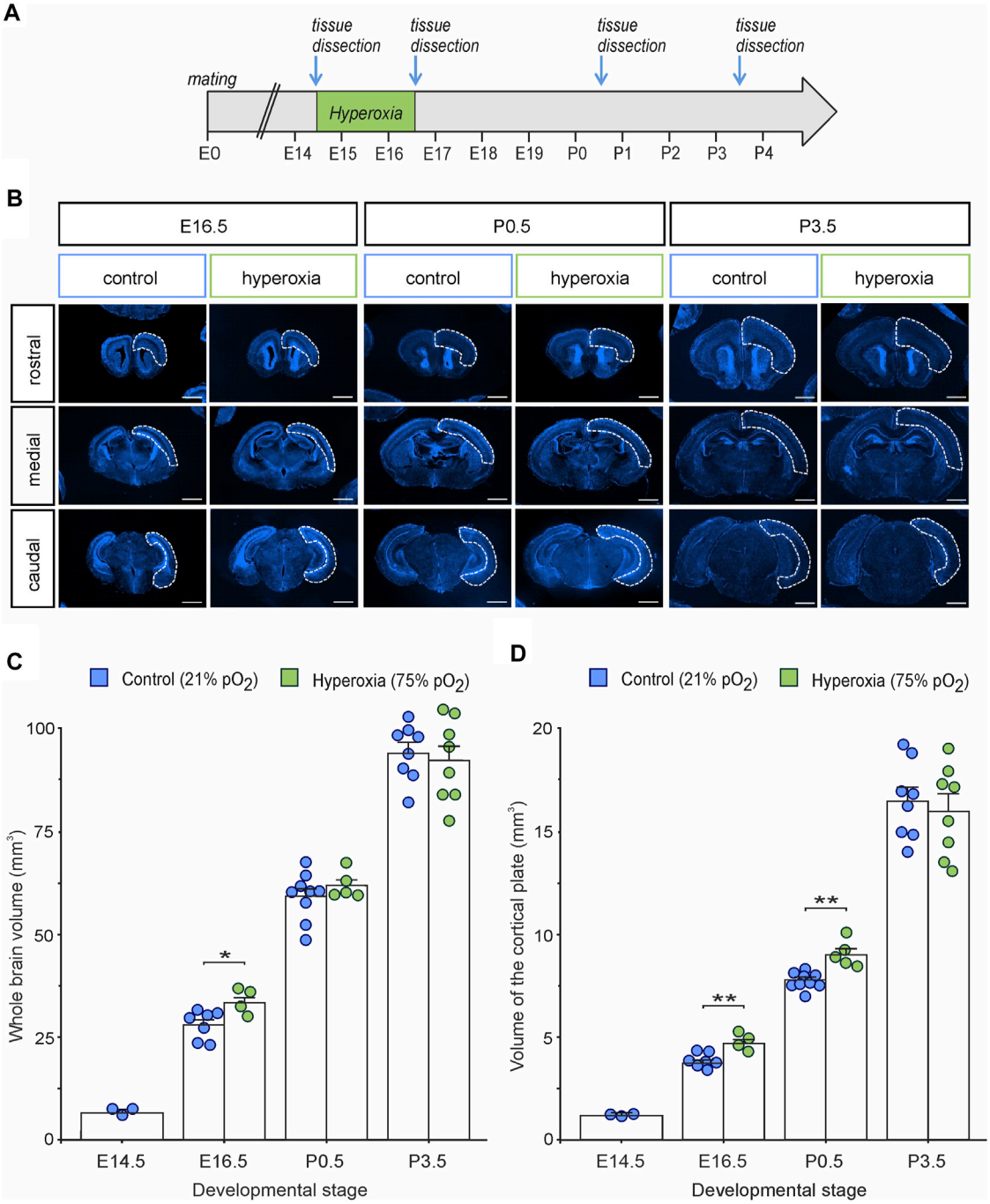

FIGURE 1 | The effects of fetal brain hyperoxia during mid-neurogenesis (E14.5 to E16.5) on volume of the cortical plate (CP) during later brain development in mice. **(A)** Experimental treatment scheme for fetal brain hyperoxygenation (by maternal application of 75% O_2) during mid-neurogenesis (E14.5 to E16.5). **(B)** Representative fluorescence images from rostral, middle, and caudal slices of E16.5, P0.5, and P3.5 brains. Slices were stained with Hoechst (blue). Dashed lines outline the cortical area used to estimate cortical volume. Scale bars, 1,000 µm. **(C,D)** Quantification of whole-brain volume **(C)** and the volume of the CP **(D)** showed increased cortical volume in hyperoxic brains at E16.5 and P0.5 (only CP) but not at P3.5 as compared to normoxic controls. Note that the value at E14.5 serves as starting point of volume just before the hyperoxic treatment, and thus, no hyperoxic condition was tested. Data are means ± s.e.m. [E14: $n = 3$; E16.5: $n = 7$ (control), $n = 4$ (hyperoxia); P0.5: $n = 9$ (control), $n = 5$ (hyperoxia); P3.5: $n = 8$ (control and hyperoxia)]. $^*p < 0.05$ and $^{**}p < 0.01$ from unpaired two-sided t-tests (non-significant comparisons are not marked for clarity).

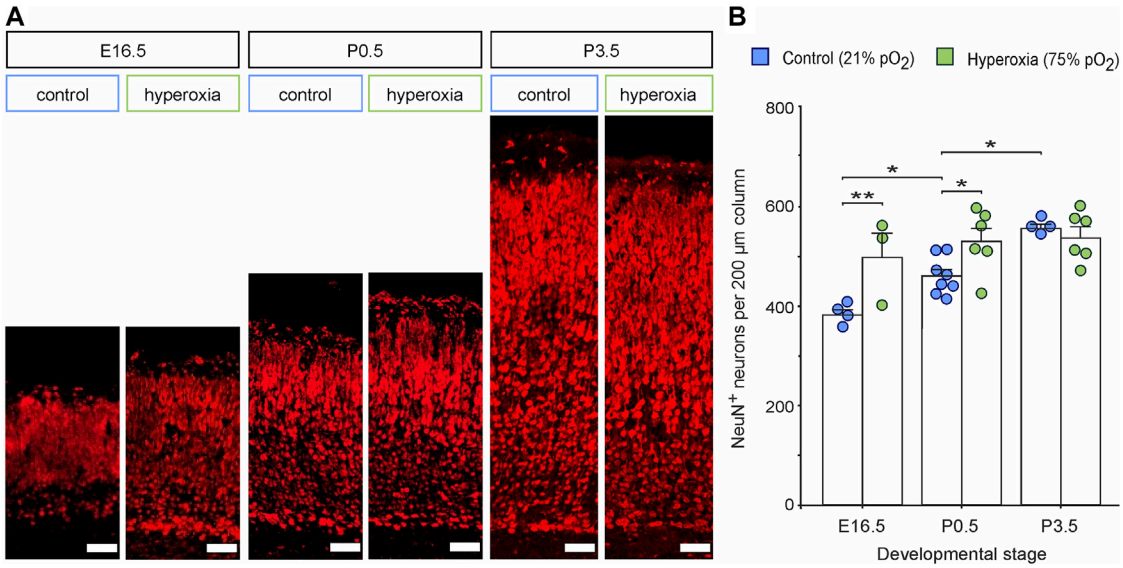

FIGURE 2 | The effects of fetal brain hyperoxia during mid-neurogenesis (E14.5 to E16.5) on the numbers of cortical neurons during later brain development in mice. **(A)** Representative fluorescence images from the CP in the middle sections along the rostro-caudal axis stained with NeuN (red). Scale bars, 50 µm. **(B)** Quantification of NeuN$^+$ cortical neurons in the brain slices. Data are means ± s.e.m. [E16.5: $n = 4$ (control), $n = 3$ (hyperoxia); P0.5: $n = 8$ (control), $n = 6$ (hyperoxia); P3.5: $n = 4$ (control), $n = 6$ (hyperoxia)]. *$p < 0.05$ and **$p < 0.01$ from two-way ANOVA with *post-hoc* two-sided *t*-test with Bonferroni correction (non-significant comparisons are not marked for clarity). For full statistics for **(B)**, see **Supplementary Table S1**.

Hyperoxygenation During Mid-Neurogenesis Accelerates But Does Not Increase Cortical Neurogenesis

To further evaluate cortical development, we used NeuN staining and analyzed the number of neurons within the middle cortical sections along the rostro-caudal axis (**Figure 2**). The hyperoxic embryos showed an increase of NeuN$^+$ neurons per volume at E16.5 with a 1.3-fold higher neuronal density as compared with normoxic control animals ($p = 0.005$), which persisted until birth (P0.5) with a still 1.2-fold higher neuronal density ($p = 0.013$), but not until P3.5 ($p = 0.581$, all from *post hoc* two-sided *t*-test with Bonferroni adjustment; **Figure 2B**). The neuronal density in the control group rose continuously between E16.5 and P3.5, while in the hyperoxia group, the maximum neuronal density seems to be already reached at earlier developmental stages at E16.5 (**Figure 2B**). Of note, neuronal density in the hyperoxia group never exceeded that in the control group, indicating an accelerated but not increased cortical neurogenesis (**Figure 2B**).

Postnatal Cortical Normalization Occurs in a Layer-Specific Manner

Whether the equalization of cortical volume and neuronal density at birth after fetal hyperoxygenation during the mid-neurogenesis phase is capable of functioning as a control mechanism for regulating neuronal layer specificity and neural circuits or whether it originates from increased raw cell numbers competitive to each other remains elusive. To shed light on this aspect, we performed a layer-specific analysis using a panel of markers Tbr1, Ctip2, and Satb2 of hyperoxic mouse embryos and pups through the developmental stages E16.5, P0.5, and P3.5 and performed a quantitative analysis for Tbr1$^+$ cells as characteristic for neurons of the SP and L6 (Hevner et al., 2001), Ctip2$^+$/Tbr1$^-$ cells representing L5 neurons (Arlotta et al., 2005), and Satb2$^+$ cells as neurons of the upper cortical layers (**Figure 3**) (Britanova et al., 2008). The number of Tbr1$^+$ cells in the CP decreased continuously from E16.5 to P3.5 (**Figure 3B**). Of note, the percentage of Tbr1$^+$ cells in the CP was reduced at E16.5, although the total number of Tbr1$^+$ cells was not affected (**Figure 3B**; **Supplementary Figure S2**).

Quantification of the percentage of L5-specific neurons (Ctip2$^+$/Tbr1$^-$ cells; (Hevner et al., 2001; Arlotta et al., 2005) showed a persistent increase within the hyperoxic group at E16.5 (1.5-fold) and P0.5 (1.6-fold; **Figure 3C**), which further supports our previous data and even further demonstrates that fetal brain hyperoxia evoked persistent effects on L5 (Wagenfuhr et al., 2015). The same layer marker panel revealed no differences in the number of Ctip2$^+$ cells at P3.5. Comparing the time course of Ctip2$^+$ cell numbers during cortical development, there was a slow drop of the percentage of Ctip2$^+$ cells within the CP between E16.5 and P3.5 in normoxic mice, but a later drop just after birth in hyperoxic mice, suggesting that the specific rearrangement of cortical L5 is postponed by hyperoxia (**Figure 3C**). We also found an increase in the absolute numbers of L5 neurons between E16.5 and P0.5. Since no more L5 neurons are generated at this time, we assume that this represents a change in the expression of Ctip2 rather than ongoing neurogenesis (McKenna et al., 2011; Toma et al., 2014).

The number of upper-layer Satb2$^+$ neurons was not affected by hyperoxia through all developmental stages (**Figure 3D**; **Supplementary Figure S2**).

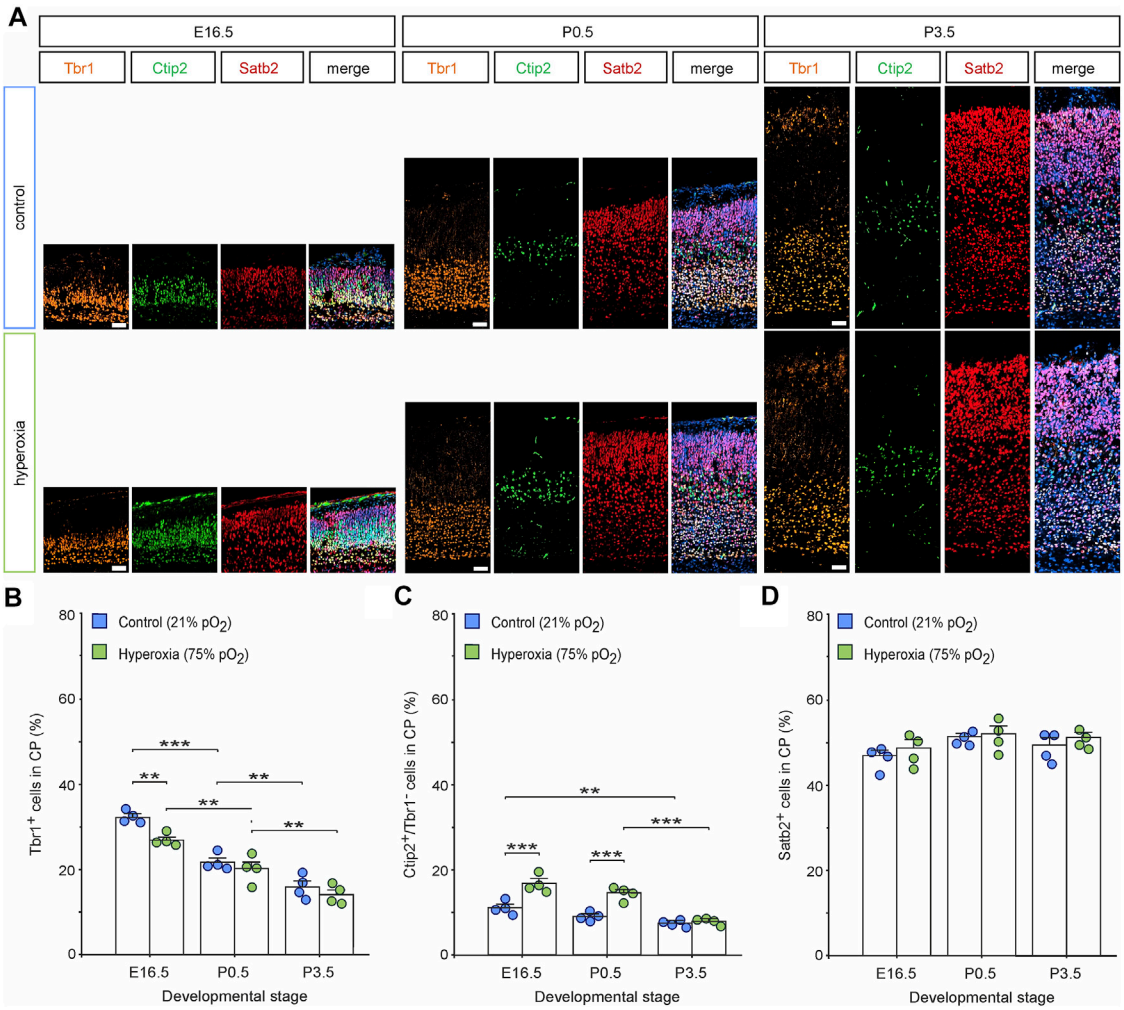

FIGURE 3 | The effects of fetal brain hyperoxygenation on layer-specific distribution of neurons. **(A)** Representative fluorescent images of Tbr1$^+$ (orange), Ctip2$^+$ cells (green), and Satb2$^+$ cells (red) from E16.5, P0.5, and P3.5 in the middle cortical sections along the rostro-caudal axis of hyperoxic and normoxic control mice. Hoechst (blue) was used to stain cell nuclei shown in the merged image. Scale bars, 50 µm. **(B–D)** Quantification of the distribution of Tbr1$^+$ cortical subplate/layer 6 neurons **(B)**, Ctip2$^+$/Tbr1$^-$ layer 5 neurons **(C)**, and Satb2$^+$ upper layer-specific neurons **(D)** showed a specific increase of layer 5 neurons at E16.5 and P0.5 but not at P3.5. Data are means ± s.e.m. (n = 4 for all animal groups). $^{**}p < 0.01$ and $^{***}p < 0.001$ from two-way ANOVA with *post-hoc* two-sided *t*-test with Bonferroni correction (non-significant comparisons are not marked for clarity). For full statistics, see **Supplementary Tables S2–S4**.

Microglia are Involved in Postnatal Cortical Normalization After Fetal Hyperoxygenation

The postnatal regulation of the CP after fetal hyperoxygenation prompted us to evaluate the possible underlying mechanisms. There are already known mechanisms capable of controlling the number of brain cells including neurons during development (Cunningham et al., 2013; Toma et al., 2014; Blanquie et al., 2017): apoptosis known to occur during early postnatal cortical development; feedback mechanisms where the increased number of neurons signals a feedback to neuronal stem cells or microglia able to phagocytose brain cells. We consequently analyzed Iba1 staining for the number of microglia, CC3 staining as an established marker for apoptosis, and time-delayed BrdU/EdU labelling for estimating the birthdate of the resulting neurons during brain development.

Immunohistochemical staining of Iba1$^+$ cells labelling resting and activated microglia (Imai et al., 1996; Morgan et al., 2010) revealed an already visually detectable increase in microglia residing in L5 of hyperoxic P0.5 mouse pups (**Figure 4A**), which was not present in E16.5 or P3.5 brains (**Supplementary Figure S3**). Quantification of these Iba1$^+$ cells revealed a specific 2.7-fold increase of microglial cells in L5 of hyperoxic mice ($p < 0.001$, *post hoc* two-sided *t*-test with Bonferroni adjustment), while the total number of Iba1$^+$ cells, other layers, and developmental stages was not affected by oxygen (**Figures 4B–E**; **Supplementary Figure S4**). Microglia within the CP were predominantly found at and after P0.5, but the invasion specifically of L5 is accelerated, although not exceeding the

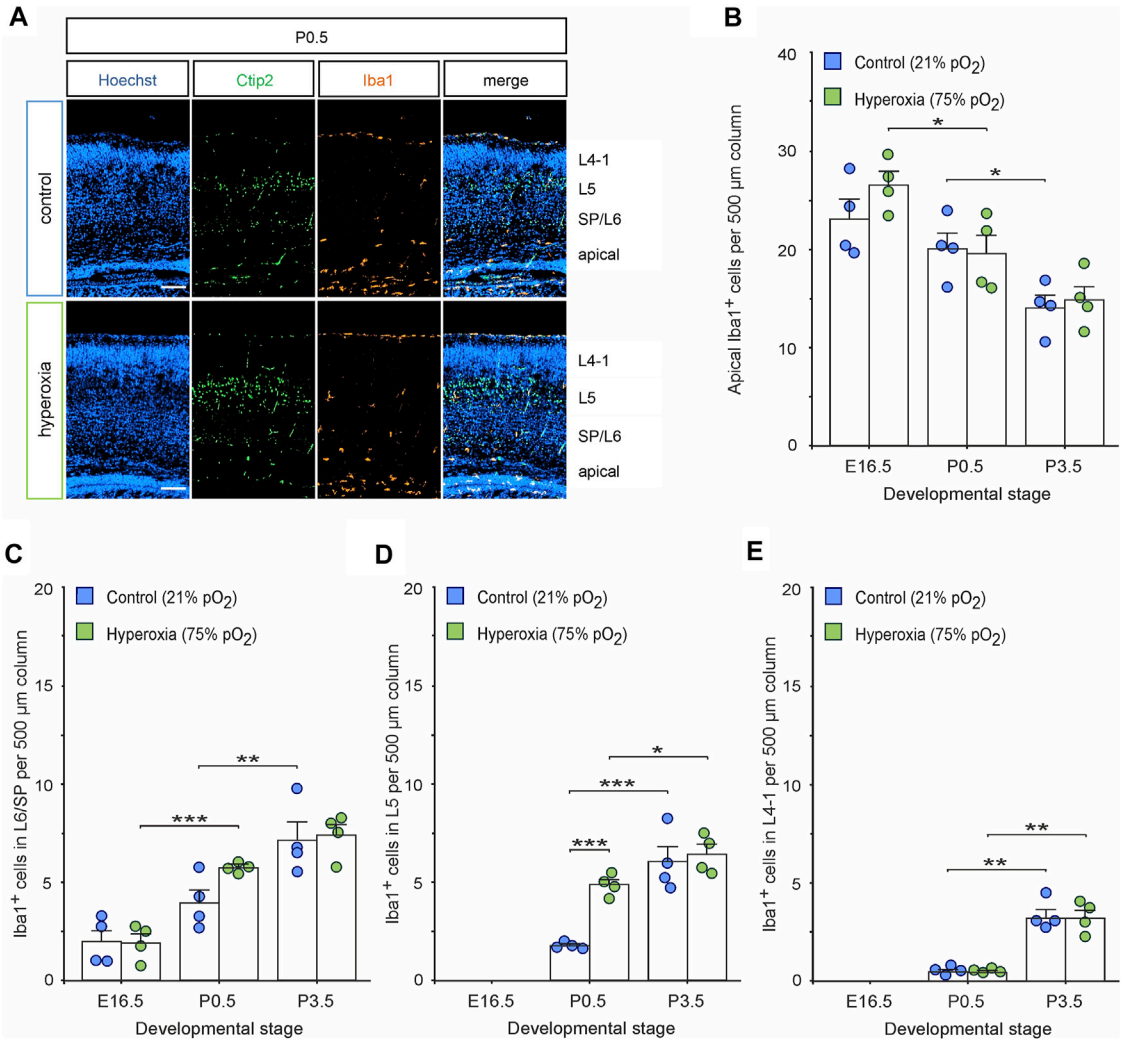

FIGURE 4 | The effects of hyperoxygenation on microglial distribution within the developing cortex. **(A)** Representative fluorescent images of Iba1$^+$ cells (orange) from P0.5 in the middle cortical sections along the rostro-caudal axis of hyperoxia-treated and control mice. Ctip2$^+$ (green) was used for layer determination, and Hoechst (blue) was used to stain cell nuclei. Scale bars represent 100 µm. **(B–E)** Quantification of Iba1$^+$ apical microglia **(B)**, within subplate/layer 6 (SP/L6) **(C)**, layer 5 (L5) **(D)**, and upper layers 4-1 (L4-1) **(E)** showed an increase of microglia within L5 at P0.5. Data are means ± s.e.m. (n = 4 for all animal groups). *p < 0.05, **p < 0.01, and ***p < 0.001 from two-way ANOVA with *post-hoc* two-sided t-test with Bonferroni correction (non-significant comparisons are not marked for clarity). For full statistics, see **Supplementary Tables S5–S8**.

number of microglia at P3.5. This is further supported by analysis of the apical and SP/L6 microglia, where the decrease of apical microglia and the successive increase of SP/L6 occurred between P0.5 and P3.5 in normoxic control mice, but already occurred between E16.5 and P0.5 in hyperoxic mice (the increase in Iba1$^+$ microglia in SP/L6 at P0.5 represents only a non-significant trend). Intriguingly, we could show that microglia in L5-targeted Ctip2$^+$ cells without morphological signs of apoptosis (**Figure 5A**). An analysis of Ctip2$^+$ and Satb2$^+$ cells targeted by microglia at time point P0.5 showed that Ctip2$^+$ cells were targeted by microglia significantly more often in the hyperoxia group than in the control group (**Figure 5B**). At the same time, there was no difference in the number of Satb2$^+$ cells targeted by microglia (**Figure 5C**; **Supplementary Figure S5**). We consequently analyzed the number of active microglia by using the combination of Iba1 and the microglia activation marker CD68 (Rabinowitz and Gordon 1991; Imai et al., 1996; Morgan et al., 2010; Jurga et al., 2020). Quantification revealed that there are indeed more active microglia in L5 and also apical, but not between these in L6/SP (**Figures 5D–H**). Further analyses revealed Ctip2$^+$ and Satb2$^+$ particles in these active microglia in L5, but—interestingly—only the number of active microglia containing Ctip2$^+$ particles was increased in hyperoxic as compared to control brains (**Figure 5I,J**).

To further evaluate whether adaptive mechanisms contribute to normalization of the number of neurons cortex after fetal hyperoxygenation, we performed a birth-dating analysis using BrdU application immediately before and EdU application 1 day

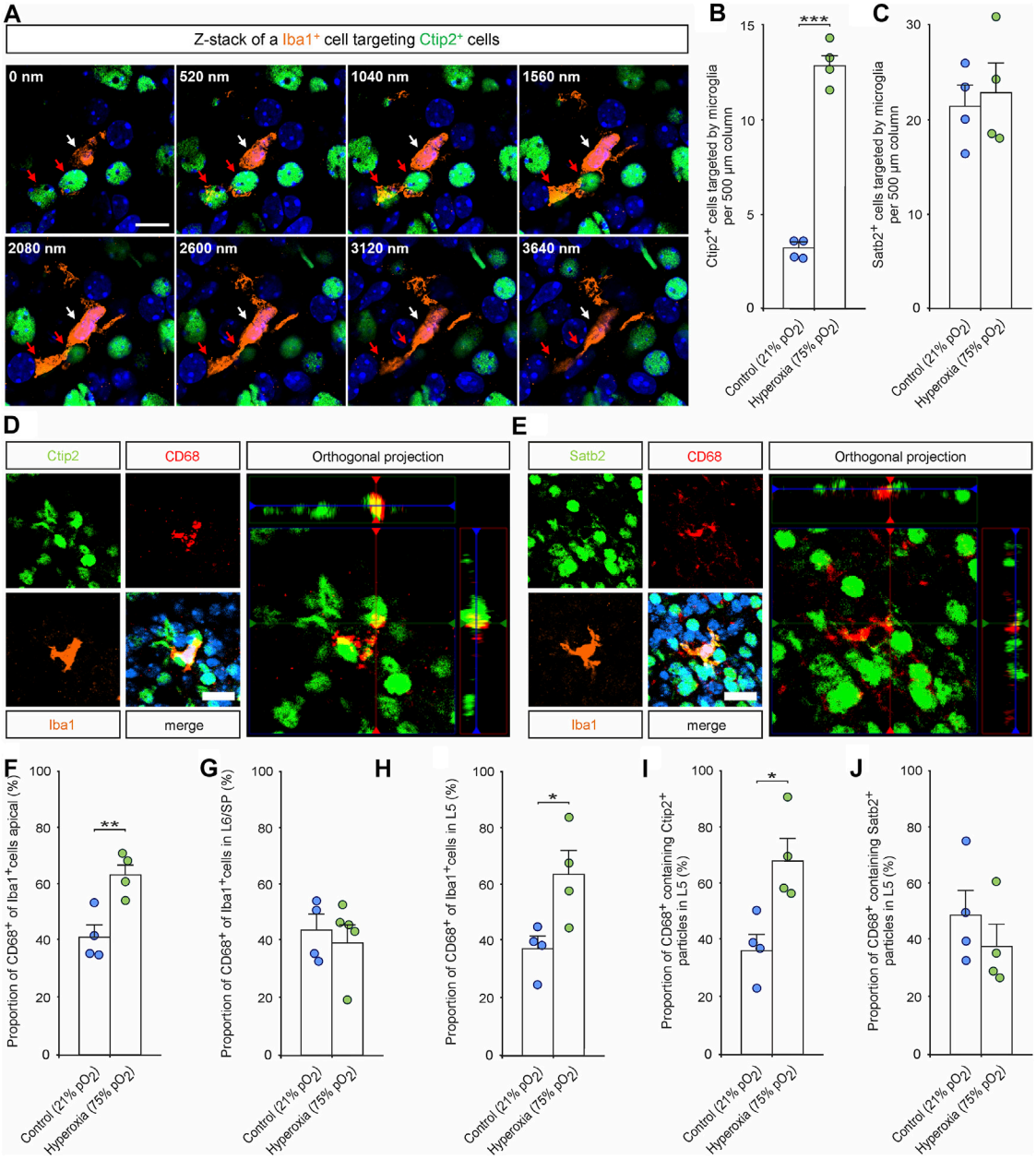

FIGURE 5 | The effects of hyperoxygenation on the layer-specific activation of microglia and their targeting and phagocytosis of neurons within the developing cortex. **(A)** Representative Z-stack images of an Iba1$^+$ microglia cell (white arrow) targeting Ctip2$^+$ cells (red arrows) in layer 5 of a P0.5 mouse cortex. Scale bars represent 10 µm. **(B,C)** Quantification of Ctip2$^+$ cells targeted by Iba1$^+$ microglia **(B)** and Satb2$^+$ cells targeted by Iba1$^+$ microglia **(C)** at P0.5. Data are means ± s.e.m. (n = 4). ***p < 0.001 from unpaired two-sided t-test (non-significant comparison is not marked for clarity). **(D,E)** Representative fluorescent images of triple staining with Iba1, CD68, and Ctip2 **(D)** or Satb2 **(E)** in the middle cortical sections along the rostro-caudal axis of hyperoxia-treated and control mice at P0.5. Orthogonal projections of CD68 and Ctip2/Satb2 show co-localization of the markers. Hoechst (blue) was used to stain cell nuclei. Scale bars represent 20 µm. **(F–H)** Quantification of CD68$^+$ microglia revealed an increase of active apical microglia **(F)** and L5 **(G)**, but not in L6/SP **(H)**. *p < 0.05, **p < 0.01, and ***p < 0.001 from unpaired two-sided t-test (non-significant comparisons are not marked for clarity; n = 4). **(I,J)** Quantification of CD68$^+$ microglia containing neuronal markers revealed an increase of microglia containing Ctip2 particles in hyperoxia-treated mice **(I)** while there is no difference regarding contained Satb2 particles **(J)**. *p < 0.05, **p < 0.01 from unpaired two-sided t-test (non-significant comparisons are not marked for clarity; n = 4).

after oxygen treatment and subsequent histological analyses of BrdU/EdU incorporation by cortical cells at P0.5 (see **Figure 6A** for experimental paradigm). Immunostaining of both markers showed that a large proportion of CP neurons were generated at E14.5 (BrdU$^+$), while cells generated at E17.5 (EdU$^+$) represent a much smaller part of the developing cortex (**Figures 6B,C**). Quantification of BrdU$^+$/EdU$^+$ cells in the CP revealed a significantly 1.4-fold increase in the number of BrdU$^+$ cells

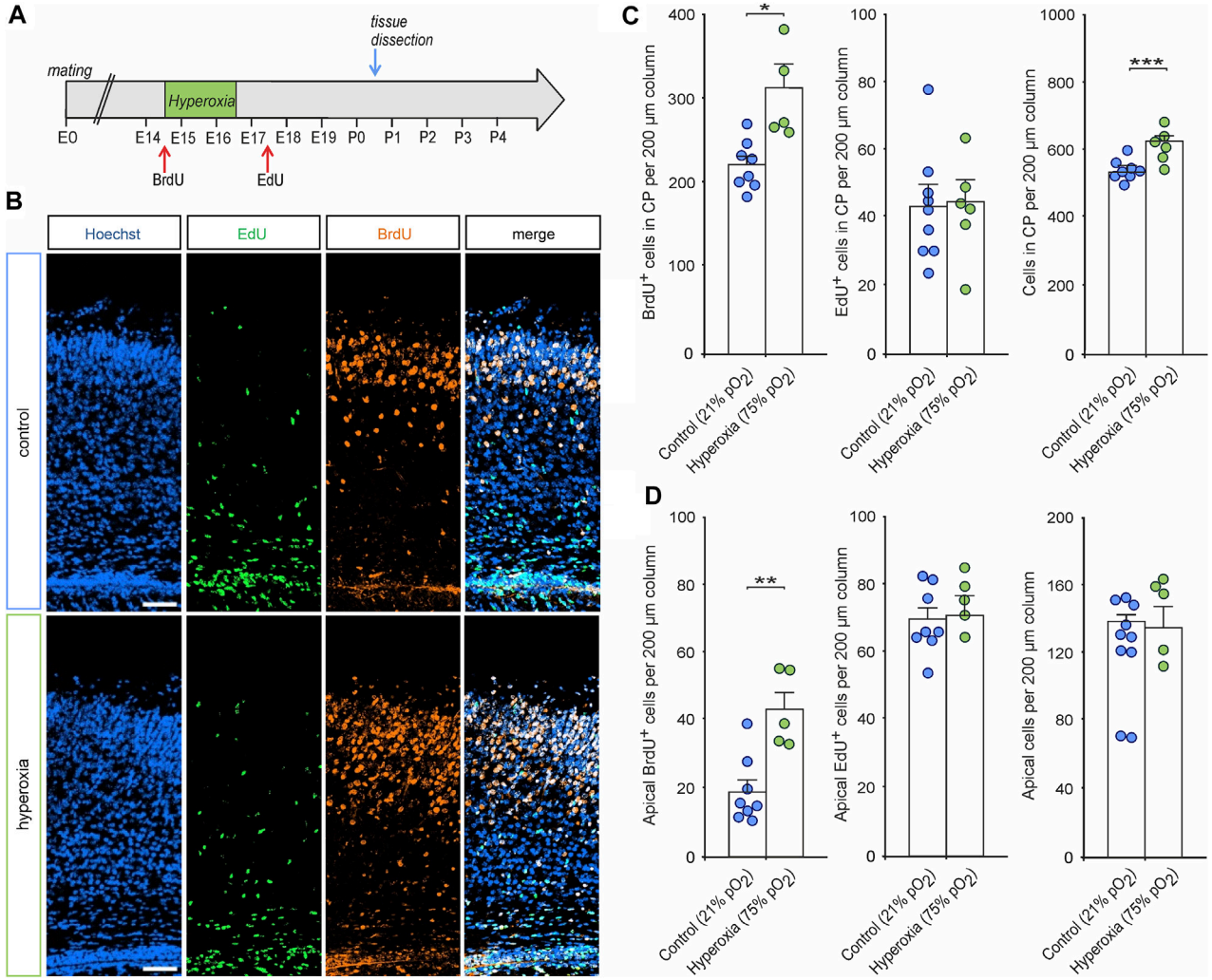

FIGURE 6 | The effects of hyperoxia on cell proliferation during and after oxygen treatment. **(A)** Experimental treatment scheme for BrdU/EdU application in fetal brain hyperoxygenation during mid-neurogenesis (E14.5 to E16.5). Mice received an intraperitoneal injection with BrdU immediately before oxygen treatment and with EdU 1 day after treatment (E17.5) and subsequently analyzed at P0.5. **(B)** Representative fluorescent images of EdU^+ (green) and $BrdU^+$ cells (orange) from P0.5 in the middle cortical sections along the rostro-caudal axis of hyperoxia-treated and control mice. Hoechst (blue) was used to stain cell nuclei. Scale bars represent 50 µm. **(C,D)** Quantification of $BrdU^+$, EdU^+, and overall cells showed an increased number of CP neurons born during hyperoxia ($BrdU^+$ cells) but not after hyperoxia treatment (EdU^+ cells) within the CP **(C)** and the apical cortex **(D)** of P0.5 mouse pups. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ from unpaired two-sided *t*-test [non-significant comparisons are not marked for clarity; $n = 8$ (control) and $n = 5$ (hyperoxia)].

($p = 0.021$) in response to hyperoxia, but not of EdU^+ cells ($p = 0.886$, both from unpaired two-sided *t*-test), as a putative later feedback reaction. Thereby, the increased rate of $BrdU^+$ cells led to an increase of the overall cells in the CP. Notably, there were more $BrdU^+$ cells left at the apical side of the cortex ($p = 0.002$), but again, no change in EdU^+ cells ($p = 0.791$, both from unpaired two-sided *t*-test; **Figure 6D**).

We further analyzed $CC3^+$ apoptotic cells within the middle cortical sections along the rostro-caudal axis showing increased apoptosis in hyperoxic P0.5 mouse pups. Immunostaining with quantification showed that the overall number of $CC3^+$ cells in the hyperoxic group is increased by 2.6-fold with apoptosis predominantly occurring in the apical regions (**Figures 7A,B**). We then evaluated whether layer 5 cells of the cortex were apoptotic through double labelling of $CC3^+$ and $Ctip2^+$, but there was almost no cell possessing both markers (<0.1% of $Ctip2^+$ cells). Since apoptosis occurred mainly apical where increased proliferation could be detected in the hyperoxia group at E14.5, we analyzed the number of $BrdU^+/CC3^+$ and $EdU^+/CC3^+$ cells (**Figures 7C–E**). Quantification revealed that indeed apical $BrdU^+$ cells were more often apoptotic while EdU^+ cells were not. Since apical active microglia were detected more often in the hyperoxia group, we finally investigated whether there are changes in elimination of apoptotic cells by $Iba1^+/CD68^+$ active microglia. However, we have neither found any increase in the number of active microglia-targeting $CC3^+$ cells nor in the number of apoptotic cells that were engulfed by microglia in hyperoxic brain when compared to controls (**Figures 7F–H**).

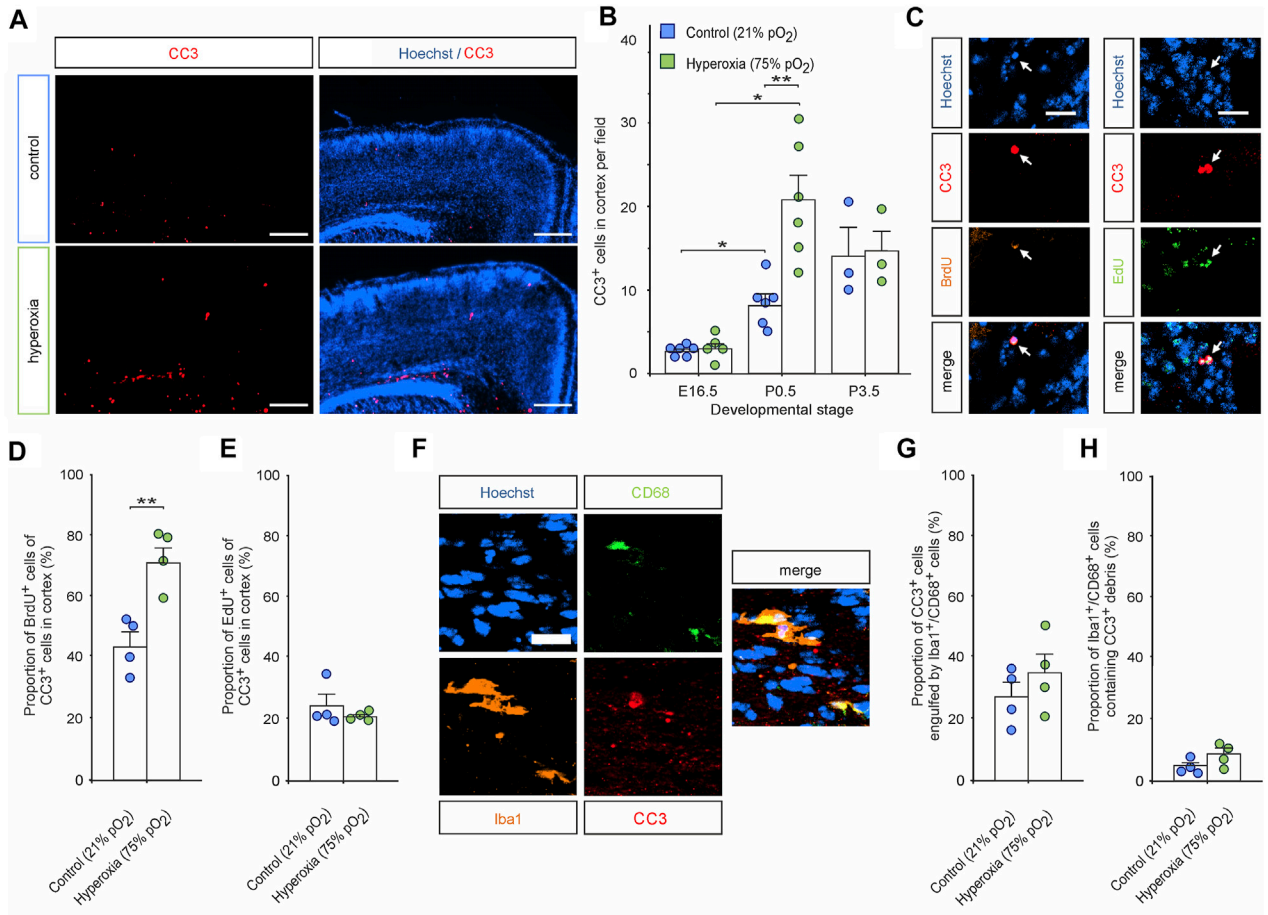

FIGURE 7 | The effects of hyperoxia on apoptosis in P0.5 mice cortex after mid-neurogenesis hyperoxygenation. **(A)** Representative fluorescent images of $CC3^+$ apoptotic cells (red) from P0.5 in the middle sections along the rostro-caudal axis of hyperoxia-treated and control mice. Hoechst (blue) was used to stain cell nuclei. Scale bars represent 200 µm. **(B)** Quantification of cortical $CC3^+$ cells shows increased apoptosis in brain slices of hyperoxia-treated mouse embryos at P0.5 but not at E16.5 or P3.5. Data are means ± s.e.m. [E16.5: $n = 6$ (control), $n = 5$ (hyperoxia); P0.5: $n = 6$; P3.5: $n = 3$]. $^*p < 0.05$ and $^{**}p < 0.01$ from robust ANOVA with *post-hoc* two-sided unpaired Wilcoxon test with Bonferroni correction. For full statistics, see **Supplementary Table S9**. **(C)** Representative fluorescent images of $BrdU^+$ (orange) and EdU^+ (green) cells double stained with CC3 in the middle cortical sections along the rostro-caudal axis of hyperoxia-treated and control mice at P0.5. Hoechst (blue) was used to stain cell nuclei. Scale bars represent 20 µm. **(D,E)** Quantification revealed that most of the apoptotic cells are $BrdU^+$ in the hyperoxia group, but not in controls **(D)**, and no differences were observed regarding apoptotic EdU^+ cells **(E)**. $^{**}p < 0.01$ from unpaired two-sided *t*-test (non-significant comparisons are not marked for clarity; $n = 4$). **(F)** Representative fluorescent image of $Iba1^+/CD68^+$ active microglia (orange/green) containing apoptotic cell debris ($CC3^+$, red) in the middle sections along the rostro-caudal axis of hyperoxia-treated mice at P0.5. Hoechst (blue) was used to stain cell nuclei. Scale bars represent 20 µm. **(G,H)** Quantification of neither the percentage of apoptotic cells engulfed by microglia **(G)** nor the number microglia engulfing apoptotic cells **(H)** revealed any differences between hyperoxia-treated vs control mice ($n = 4$).

Number of Excitatory Synapses Follows Normalization in L5

To provide first data on the effects on mid-neurogenesis hyperoxygenation on synaptic development, we analyzed the expression of the vesicular glutamate transporter 2 (vGluT2) as a common target for microglia pruning in later stages and a marker for the predominant form of excitatory synapses during early brain development (Nakamura et al., 2007; Schafer et al., 2012) (**Figure 8**). Double staining of vGluT2 and Ctip2 showed increased synaptic input into L5 in the hyperoxia animal group at P0.5: quantification in L5 showed that there were 1.7-fold more $vGluT2^+$ puncta in the hyperoxia group as compared with normoxic controls at P0.5 ($p = 0.006$), which did not persist until P3.5 (**Figure 8C**). Additionally, the number of $vGluT2^+$ puncta in L5 in the hyperoxia group at P0.5 temporary overshots that of controls at P0.5 and P3.5 ($p = 0.033$).

DISCUSSION

We present here that the short-term effects of hyperoxygenation during mid-neurogenesis of fetal mouse brain development (E14.5 to E16.5) with increased neuroprecursor cell proliferation within the SVZ/OSVZ (Wagenfuhr et al., 2015) translate into an accelerated cortical development but without

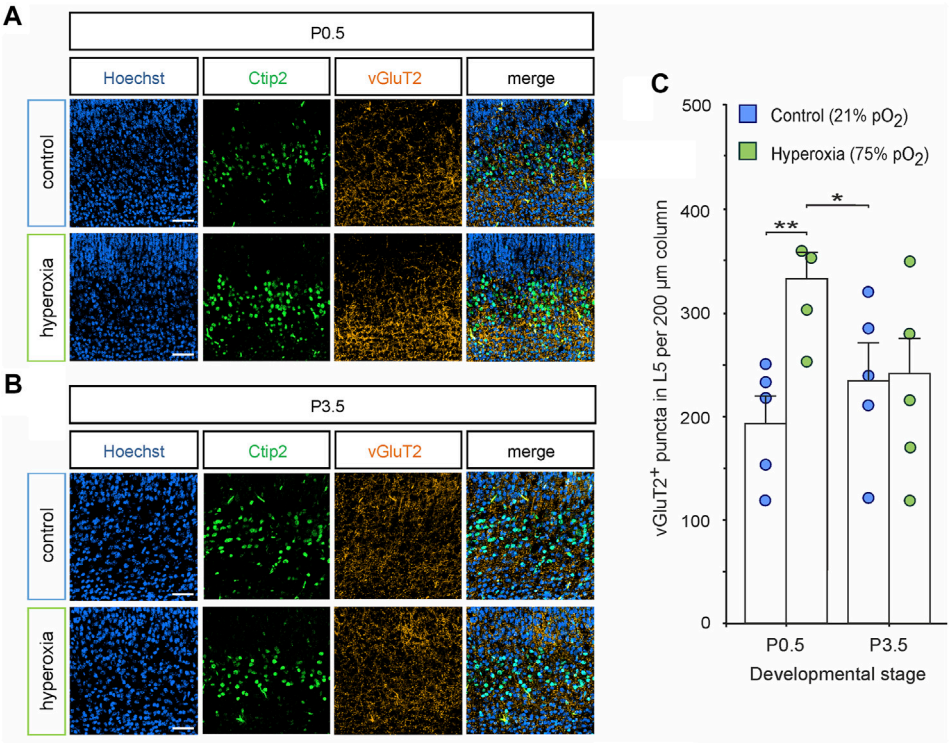

FIGURE 8 | Synaptic excitatory input into L5 is normalized together with L5 neurons. **(A,B)** Representative fluorescent images of vGluT2$^+$ synapses (orange) in L5 (Ctip2$^+$ cells, green) in the middle cortical sections along the rostro-caudal axis of hyperoxia-treated and control mice at P0.5 **(A)** and P3.5 **(B)**. Hoechst (blue) was used to stain cell nuclei. Scale bars represent 50 µm. **(C)** Quantification of vGluT2$^+$ puncta showed an increased number of puncta at P0.5, but not at P3.5. Data are means ± s.e.m. (n = 5 for all animal groups). *p < 0.05 and **p < 0.01 from two-way ANOVA with *post-hoc* two-sided t-test (non-significant comparisons are not marked for clarity). For full statistics, see **Supplementary Table S10**.

increase in cortical neurogenesis and cortical volume at early postnatal stage. Indeed, the CP is expanded through a specific overshoot amount of Ctip2$^+$/Tbr1$^-$ L5 neurons in later fetal development until birth in hyperoxic mouse cortex, which is normalized at early postnatal stage. This normalization is accompanied by an increase of microglial cells within L5 capable of targeting the respective neurons but no signs of L5 neuronal apoptosis.

We used our established model of maternal hyperoxygenation to investigate the effects of increased fetal brain oxygen tension during mid-neurogenesis (E14.5–E16.5) on later cortical development until the early postnatal state (Wagenfuhr et al., 2015; Wagenfuhr et al., 2016). Early chronic hyperoxygenation in this model causes severe reduction of neuroprecursor cell proliferation and the apical neuroprecursor cell pool (Markert et al., 2020). Contributing to this, Lange et al. (2016) reported that early hyperoxygenation from E10.5 to E13.5 is able to alter neuroprogenitor cell fate leading to a decrease of expanding neuroprogenitors. However, late or postnatal hyperoxygenation is known to cause brain damage accompanied by excessive loss of neurons (Gerstner et al., 2008; Yis et al., 2008; Tuzun et al., 2012), while short-term hyperoxygenation during mid-neurogenesis of fetal mouse brain development (E14.5 to E16.5) leads to an immediate expansion of a distinct proliferative cell population basal of the SVZ, which subsequently contributes to corticogenesis by heading for deeper cortical layers (Wagenfuhr et al., 2015). Finally, the amount of Ctip2$^+$ neurons in L5 projecting into various brain regions is markedly overshot after short-term maternal hyperoxygenation and at birth with a normalization until early postnatal stage (Hattox and Nelson 2007; Oswald et al., 2013; Wagenfuhr et al., 2015). Oxygen levels are known to directly regulate neuroprecursor cell maintenance, proliferation, and differentiation *in vitro* through the activation of several oxygen-sensitive signaling pathways (Chen et al., 2007; Pistollato et al., 2007; Giese et al., 2010; Mazumdar et al., 2010; Braunschweig et al., 2015; Mennen et al., 2020). Although the *in vitro* cell models are not directly comparable to our *in vivo* system, analyses of the fetal brain oxygen tension revealed that maternal hyperoxygenation of 75% leads to an increase of oxygen tension in the neurogenic niche of the VZ/SVZ from below 1.1% in maternal normoxic condition to oxygen levels above this threshold in hyperoxic animals, which is also supported by other colleagues (Wagenfuhr et al., 2015; Lange et al., 2016). Of note, there are no *in vivo* oxygen markers available for small laboratory animals to detect changes in oxygen levels in the range of 5%–20% to further define the tissue oxygen tension in the hyperoxic condition. However, maternal hyperoxia is unlikely to cause oxygen levels towards 20%, which is the commonly used oxygen tension in cell culture

experiments. Thus, the higher oxygen tension in hyperoxic brain tissue likely represents the *in vitro* condition of mild hyperoxia and provides a stimulating environment for maintenance and proliferation of neuroprecursor cells as demonstrated in cell culture (Chen et al., 2007; Chen et al., 2010; Santilli et al., 2010; Braunschweig et al., 2015; Qi et al., 2017) when compared to very low oxygen conditions.

The observed normalization of the brain morphology in the early postnatal stage indicates that the brain is able to compensate prenatally evoked morphological changes at least for an excess of cell population. Within the period of synaptogenesis during the first 30 postnatal days, neuronal programmed cell death or apoptosis is known to play a major role in shaping the neocortex with a peak around P5 in rodents in most studies (Southwell et al., 2012; Ahern et al., 2013; Blanquie et al., 2017). In the six-layered isocortex, the loss of neuronal density displays a layer-specific pattern and manifested itself mostly in L2–L4, whereas L1, L5, and L6 show fewer changes. We thus studied whether this physiological process is also mediating the normalization neuronal cell counts in L5 of hyperoxic mouse cortex in earlier postnatal stages by using CC3 staining (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Wong et al., 2018). Surprisingly, almost none of L5 Ctip2$^+$ neurons showed CC3 marker expression, indicating that apoptosis is not involved in controlling the number of L5 neurons after mid-neurogenesis hyperoxygenation. The rare occurrence of apoptosis in L5 in the first days after birth is however in line with previous systematic studies of layer-specific postnatal apoptosis (Verney et al., 2000; Denaxa et al., 2018). However, we found an increase in the number of CC3$^+$ apoptotic cells in hyperoxic as compared to normoxic brain at P0.5, but their location was vastly limited to the apical proliferative zone outside the CP, and they were eliminated by active microglia. This phenomenon might be interpreted as an adaptive response of the physiological apical apoptosis at birth to the hyperoxia-induced increased proliferation of precursor cells at E14.5 and at the end of mid-neurogenesis phase at E16.5 (White and Barone 2001; Wagenfuhr et al., 2015). Putative feedback mechanisms regulating neuroprecursor cell proliferation such as activity-dependent negative feedback from developing neurons (Toma et al., 2014) might be involved in cortical re-shaping after mid-neurogenesis hyperoxygenation. We thus applied the thymidine analogue EdU at a time point when it is known that the brain enlarges (E17.5) and 1 day after the oxygen treatment and analyzed EdU uptake at birth (P0.5). Although hyperoxygenation provokes strong immediate effects on the number of CP neurons born at E14.5 as described earlier (Wagenfuhr et al., 2015), cells generated after the hyperoxygenation phase at E17.5 predominantly reside on the apical side of the developing cortex and do not yet contribute to CP morphology at birth. Moreover, there is no indication for an adaption regarding the number of neurons in the CP during the post-hyperoxygenation phase.

Microglia already physiologically colonize the developing brain at E10.5 where they regulate the number of precursor cells through phagocytosis (Cunningham et al., 2013; Arnò et al., 2014; Tronnes et al., 2016). Until E16.5, microglia exclusively reside within the proliferating zones while they start to invade into the CP as late as during the early postnatal stage (Squarzoni et al., 2014). The majority of microglia in the developing cerebral cortex have an activated morphology and express markers associated with activation, and functional studies revealed that microglia regulate neuroprecursor cell number in the developing cortex by phagocytosis (Cunningham et al., 2013). Interestingly, most precursor cells targeted by Iba1$^+$ microglia in the cortical proliferative zones did not show signs of cell death or apoptosis (Fricker et al., 2012; Cunningham et al., 2013). Our data suggest a very similar mechanism during the re-shaping of the cortical layers after mid-neurogenesis hyperoxygenation: increased numbers of Iba1$^+$ microglia, which also express CD68 as a marker for active microglia (Rabinowitz and Gordon 1991; Jurga et al., 2020), target and incorporate Ctip2$^+$ L5 neurons with no apoptotic signs at the critical stage P0.5. However, the number of targeted Satb2$^+$ cells or incorporated Satb2$^+$ particles at the same time remains unaffected, suggesting a specific effect on Ctip2$^+$ L5 neurons. Nevertheless, our study is limited to immunohistochemical staining and cannot exclude other effects such as a critical change in microglial support for surviving of L5 cells (Ueno et al., 2013). In addition, the study is limited to a rather small sample size. Non-significant trends like the increased number of microglia in SP/L6 at E16.5 could potentially be relevant. Since there are no changes in microglial activity or absolute L6-specific Tbr1$^+$ neurons, this may reflect an accelerated invasion of the cortical plate where microglia migrate from apical through SP and L6 to L5 (Swinnen et al., 2013). Consequently, future functional studies with activation and depletion of microglia during late prenatal and early postnatal cortical development are warranted to investigate the exact microglia–L5 neuron interactions in cortical re-shaping after critical insults during mid-neurogenesis such as hyperoxygenation. These data in conjunction with no indications for changes in cell migration [data on cortical layering herein and Wagenfuhr et al., (2015)] or compensatory reduction or shift of precursor proliferation strongly suggest different mechanisms to normalize the overshoot amount of neuroprecursor cells depending on the brain region with CC3-mediated apoptosis as one major mechanism within the apical proliferative zone (VZ/SVZ) and microglia playing a key role in cortical L5.

To first shed light on alterations of early synaptic connectivity within the developing cortex by mid-neurogenesis hyperoxygenation, we further analyzed the expression of vGluT2 as a common marker for the predominant form of excitatory synapses during early brain development (Nakamura et al., 2007). We detected a temporary overshoot of glutamatergic synaptic input into L5 in the hyperoxia animal group at P0.5 with normalization until P3.5 in fairly accurate parallelism to the changes in cortical L5 neurogenesis. Although the underlying mechanisms of this normalization need to be determined by functional studies as outlined above, it might also be mediated through activated microglia, because microglia

have been reported to have a pivotal role in remodeling of developing synapses in the early postnatal brain (Schafer et al., 2012). To determine whether the morphological changes in response to hyperoxygenation during mid-neurogenesis translate into behavioral disruption, early postnatal behavioral testing of the pups using righting reflex test, gait analysis, and negative geotaxis test (Lubics et al., 2005; Fan et al., 2008) is urgently required in future studies. Our observations might then be of interest for investigating layer 5-specific neurodevelopmental disorders (Kolomeets and Uranova 2019; Mi et al., 2019) and their potential therapeutic/prophylactic interventions.

Together, the present data demonstrate that fetal brain hyperoxygenation during mid-neurogenesis from embryonic stage E14.5 to E16.5 accelerates cortical development in the fetal mouse brain. The cortical CP is expanded through a specific overshoot amount of L5 neurons at E16.5 and at birth in hyperoxic mouse cortex, which is subsequently normalized at early postnatal stage. This normalization is accompanied by an increase of microglial cells within L5 capable of targeting and incorporating the respective neurons with no signs of L5 neuronal apoptosis. Indeed, our data strongly suggest different mechanisms to the overshoot number of neuroprogenitor cells depending on the brain region with CC3-mediated apoptosis as the mechanism within the apical proliferative zone and microglial targeting in cortical L5. However, future functional studies on microglia using ablation and/or stimulation of microglia are warranted to finally confirm that an increased microgliosis in L5 is responsible or at least contribute to postnatal adaption to prenatal hyperoxia effects on corticogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, reference number 7221.3-1-043/16, and complies with the Tierschutzgesetz and Verordnung zur Umsetzung der Richtlinie 2010/63/EU from Germany.

AUTHOR CONTRIBUTIONS

AS created, designed, and supervised the project; analyzed the data; and wrote the manuscript. FM designed the project, performed the experiments; and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.732682/full#supplementary-material

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REVIEW
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Investigation of the Mechanisms Underlying the Development and Evolution of the Cerebral Cortex Using Gyrencephalic Ferrets

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The mammalian cerebral cortex has changed significantly during evolution. As a result of the increase in the number of neurons and glial cells in the cerebral cortex, its size has markedly expanded. Moreover, folds, called gyri and sulci, appeared on its surface, and its neuronal circuits have become much more complicated. Although these changes during evolution are considered to have been crucial for the acquisition of higher brain functions, the mechanisms underlying the development and evolution of the cerebral cortex of mammals are still unclear. This is, at least partially, because it is difficult to investigate these mechanisms using mice only. Therefore, genetic manipulation techniques for the cerebral cortex of gyrencephalic carnivore ferrets were developed recently. Furthermore, gene knockout was achieved in the ferret cerebral cortex using the CRISPR/Cas9 system. These techniques enabled molecular investigations using the ferret cerebral cortex. In this review, we will summarize recent findings regarding the mechanisms underlying the development and evolution of the mammalian cerebral cortex, mainly focusing on research using ferrets.

Keywords: cerebral cortex, development, evolution, ferret, gyrification

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INTRODUCTION

The cerebral cortex has changed significantly in the long history of mammalian evolution (Rakic, 1995; Kriegstein et al., 2006; Molnár et al., 2006; Rakic, 2009; Fietz and Huttner, 2011; Lui et al., 2011; Zilles et al., 2013; Borrell and Götz, 2014; Florio and Huttner, 2014; Kawasaki, 2014; Sun and Hevner, 2014; Poluch and Juliano, 2015; Hatakeyama et al., 2017; Kawasaki, 2017; Llinares-Benadero and Borrell, 2019; Gilardi and Kalebic, 2021). The number of neurons and glial cells in the cerebral cortex has increased, and as a result, the cerebral cortex has markedly expanded. Along with its expansion, it developed a variety of brain structures including folds (i.e. gyri and sulci) on its surface, and its neuronal circuits increased in complexity. Although it has been proposed that the expansion of the cerebral cortex and these developed brain structures are the fundamental basis for the acquisition of higher brain functions during evolution, the mechanisms underlying the formation and evolution of these brain structures are still not fully understood.

One reason for this is that the mouse brain, which is widely used for genetic analyses, does not have cortical folds, making it difficult to investigate the mechanisms using mice. Therefore, it seemed that genetic analyses using a well-developed cerebral cortex that shares similar properties with the human cerebral cortex would be important. For this purpose, several laboratories including ours are

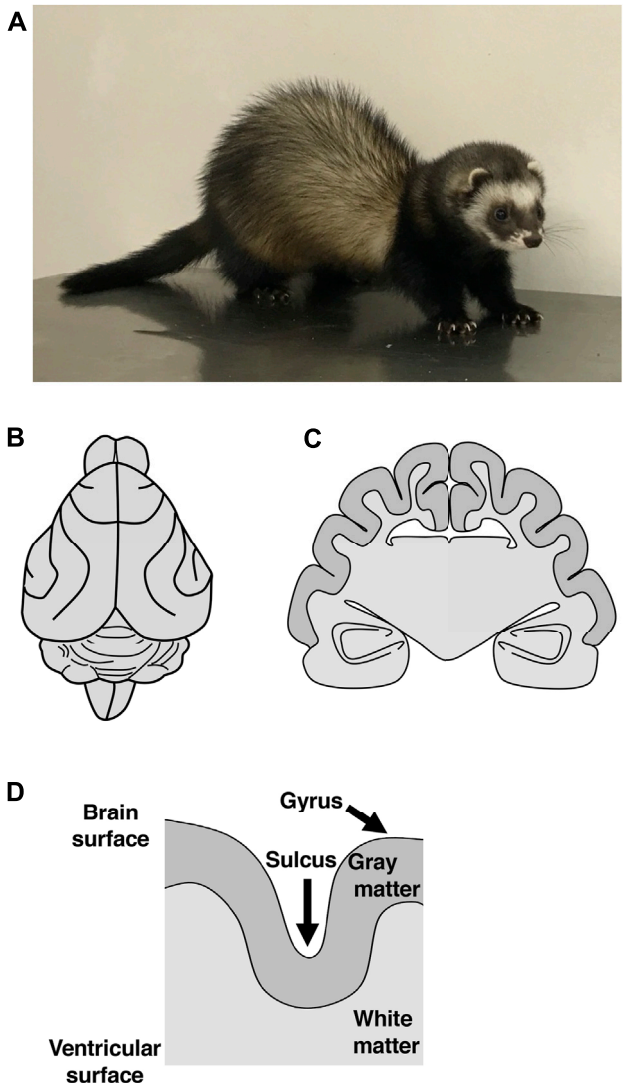

FIGURE 1 | The brain structures of the ferret. **(A)** An adult ferret. A ferret is a medium-sized carnivorous mammal with a body length of about 50 cm, a weight of 1–2 kg, and an average lifespan of 6–10 years. **(B, C)** Illustrations of a dorsal view **(B)** and of a coronal cross section **(C)** of the adult ferret brain. The ferret brain has folds on the surface of the cerebral cortex. **(D)** A schema of a cross section of the cerebral cortex in gyrencephalic animals. Both the surface of gray matter and the boundary between gray matter and white matter are curved. In contrast, the surface of white matter at the lateral ventricle does not exhibit curvature. Thus, white matter is thicker at the gyrus than at the sulcus.

using the ferret (*Mustela putorius furo*) (**Figure 1A**), a medium-sized carnivorous mammal, because it has a relatively large and developed cerebral cortex with folds (**Figures 1B,C**) (Smart and McSherry, 1986; Noctor et al., 1999; Kawasaki et al., 2004; Borrell et al., 2006; Neal et al., 2007; Fietz et al., 2010; Rowell et al., 2010). Furthermore, recent progress in genetic manipulation techniques for the ferret cerebral cortex has enabled us to investigate the molecular mechanisms underlying the development and evolution of the cerebral cortex (Borrell, 2010; Kawasaki et al., 2012; Kawasaki et al., 2013; Nonaka-Kinoshita et al., 2013; Kou et al., 2015; Tsunekawa et al., 2016; Shinmyo et al., 2017; Johnson et al., 2018; Yu et al., 2019). In this review, we first introduce recent studies that used ferrets to investigate the mechanisms underlying cortical folding, the amplification of neural progenitors and the interrelationship between them. By comparing the results of these studies with findings obtained using other animal species, we further discuss common and species-specific mechanisms of cortical folding. Our recent studies demonstrated that ferrets have developed axon fiber layers in the cerebral cortex, as is the case in monkeys and humans (Saito et al., 2019). Therefore, we also discuss the formation and evolution of neuronal circuits in the mammalian cerebral cortex.

STRUCTURAL FEATURES AND DEVELOPMENTAL PROCESSES OF CORTICAL FOLDS

One of the striking structural features of the human cerebral cortex is the large number of folds on its surface (Lui et al., 2011; Florio and Huttner, 2014; Sun and Hevner, 2014; Kawasaki, 2017; Llinares-Benadero and Borrell, 2019). Cortical folds consist of ridges called gyri and furrows called sulci (**Figure 1D**). It is believed that the acquisition of cortical folds during evolution led to the increase in the surface area of the cerebral cortex, allowing accommodation of many neurons within a limited capacity of the cranium. Cortical folds, therefore, are thought to be an important structural basis for the development of brain functions. Indeed, patients with abnormal cortical folds such as polymicrogyria and lissencephaly exhibit severe intellectual disability (Ross and Walsh, 2001; Fernández et al., 2016). It would therefore be important to elucidate the molecular mechanisms underlying the development and evolution of cortical folds and the pathogenesis of neurological diseases related to cortical folds.

The cerebral cortex is composed of six layers of gray matter, where neurons are concentrated, and white matter, which mainly consists of axons and myelin. Cortical folds are found in animal species with relatively large brains, such as humans, monkeys, cats and ferrets, whereas they tend to be absent in animal species with small brains, such as rats and mice. Gyrencephalic and lissencephalic refer to the presence and absence, respectively, of cortical folds. It would be intriguing to uncover the mechanisms that determine whether the cerebral cortex becomes gyrencephalic or lissencephalic.

Cortical folds have the following structural features (Borrell, 2018). Both the surface of gray matter and the boundary between gray and white matter are curved, and as a result, all six layers of gray matter are curved in accordance with cortical folds (**Figure 1D**). In contrast, the surface of white matter at the lateral ventricle is characterized by flatness and does not exhibit curvature (**Figure 1D**). Thus, white matter is thicker at gyri than at sulci. It would be important to examine whether these physiological features of cortical folds can be observed when performing experiments.

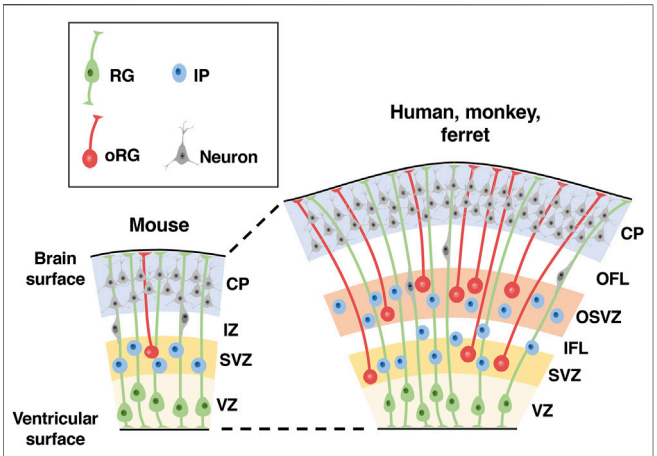

FIGURE 2 | Distribution patterns of neural progenitors in the developing cerebral cortex of lissencephalic and gyrencephalic animal species. Schemas of cross sections of the developing cerebral cortices of lissencephalic mice and gyrencephalic humans, monkeys and ferrets are shown. There are three types of neural progenitors in the developing cerebral cortex: radial glial (RG) cells, intermediate progenitor (IP) cells and outer radial glial (oRG) cells. Note that compared with the lissencephalic cortex, the gyrencephalic cortex has another region of neural progenitors, the outer subventricular zone (OSVZ), in addition to the ventricular zone (VZ) and the subventricular zone (SVZ). CP, cortical plate; IZ, intermediate zone; OFL, outer fiber layer; IFL, inner fiber layer.

Even in animals with gyrencephalic cerebral cortices, cortical folds are not present early during development, and they appear gradually as the development of the cerebral cortex progresses. Cortical folds are formed in the embryo (i.e. before birth) in cynomolgus monkeys, while cortical folding proceeds after birth in ferrets (Welker et al., 1990). Sulci formed early during development are called the primary sulci, while those formed later during development are called the secondary and higher sulci (Welker et al., 1990). In other words, when cortical folding starts during development, the cerebral cortex exhibits simple patterns of cortical folds, having only the primary sulci. As cortical folding proceeds, the secondary and higher sulci are added, making the final patterns of cortical folds. Interestingly, the positions of the primary sulci are well-conserved between genetically identical twins, but those of the secondary and higher sulci are less-conserved, suggesting that the position of the primary sulcus is determined by genetic factors, whereas other factors are involved in the formation of the secondary and higher sulci (Lohmann et al., 1999).

CORTICAL DEVELOPMENT IN GYRENCEPHALIC ANIMALS

The cerebral cortex is formed from neuroepithelial (NE) cells surrounding the lateral ventricle (Taverna et al., 2014). NE cells give rise to radial glial cells (RG cells, also known as apical progenitors, ventricular RG cells and apical RG cells), which reside in the ventricular zone (VZ) and have bipolar radial processes between the ventricle and the pial surface of the cerebral cortex (**Figure 2**) (Taverna et al., 2014). The asymmetric division of RG cells produces basal progenitors including intermediate progenitor (IP) cells and outer radial glial cells (oRG cells, also known as OSVZ RG cells, basal RG cells, intermediate RG cells and translocating RG cells), which reside in the subventricular zone (SVZ) (**Figure 2**) (Taverna et al., 2014). In addition to the VZ and the SVZ, the developing cerebral cortex of gyrencephalic animal species, namely humans, monkeys and ferrets, has another region containing abundant oRG cells, the outer SVZ (OSVZ) (**Figure 2**) (Smart et al., 2002). Only a small number of oRG cells are seen in the SVZ of mice, and it is thought that an increase in oRG cells led to the production of a large number of neurons in the cerebral cortex. Cortical neurons generated from these progenitor cells migrate to the cortical plate using the radial processes of RG cells in a birth-date-dependent inside-out manner, and newly generated neurons migrate radially past existing neurons (**Figure 2**) (Silva et al., 2019). Thus, cortical neurons in different cortical layers are generated in a temporal sequence, such that lower-layer neurons are generated before upper-layer neurons. Migrating cortical neurons extend their axonal fibers, which compose the wiring of the brain. Gyrencephalic animals have developed axon fiber layers in the cerebral cortex (Molnár and Clowry, 2012; Saito et al., 2019), as will be mentioned later.

Because the ratio of the number of upper-layer neurons to that of lower-layer neurons is much greater in humans than in rodents (DeFelipe et al., 2002), investigations of temporal plasticity in neural progenitors in the gyrencephalic cortex have been of interest. Previous studies with heterochronic transplantations showed the presence of fate-restricted progenitors in the ferret cerebral cortex (Frantz and McConnell, 1996; Desai and McConnell, 2000). When late-stage progenitors that produce upper-layer neurons were transplanted into the cerebral cortex of younger hosts, they were not competent to generate lower-layer neurons and were restricted to producing upper-layer neurons. In contrast, recent studies using the mouse cerebral cortex revealed progenitor-type-specific differences in fate plasticity (Oberst et al., 2019). RG cells can revert their temporal identity and re-enter past neurogenic states, while IP cells are committed progenitors that lack such retrograde fate plasticity. It would be important to investigate the fate plasticity of oRG cells, which predominantly produce upper-layer neurons, in the gyrencephalic cortex (Lukaszewicz et al., 2005).

HYPOTHESES ON THE MECHANISMS UNDERLYING CORTICAL FOLDING

Several hypotheses regarding the mechanisms of cortical folding have been proposed (Lui et al., 2011; Florio and Huttner, 2014; Kawasaki, 2014; Sun and Hevner, 2014; Kawasaki, 2017; Borrell, 2018; Llinares-Benadero and Borrell, 2019; Gilardi and Kalebic, 2021). One hypothesis is that cortical folding resulted from increased intracranial

pressure because cortical folds tend to be observed in animals with larger cerebral cortices but not in those with smaller cerebral cortices (Welker et al., 1990). According to this hypothesis, as the cerebral cortex expanded in the limited volume of the cranium, increased intracranial pressure caused the cortex to fold. However, because cortical folds did not disappear when intracranial pressure was reduced experimentally, this hypothesis seems unlikely. Another hypothesis is that an abundance of oRG cells is crucial for cortical folding. Indeed, the gyrencephalic cerebral cortex has many oRG cells in the OSVZ (**Figure 2**) (Smart et al., 2002). Some animal species, such as marmosets, have an OSVZ, but their cerebral cortex exhibits almost no cortical folds (Kelava et al., 2012). However, it seems plausible that oRG cells in the OSVZ are important for cortical folding because the amounts of neural progenitors in the OSVZ are positively correlated with the degree of cortical folding (Reillo et al., 2011). A third hypothesis is that the ratio of the thicknesses of the superficial and deep regions in the cerebral cortex is crucial for cortical folding (Richman et al., 1975; Kriegstein et al., 2006). If superficial regions preferentially expanded relative to deep regions, it would result in an outward convex. Consistent with this hypothesis, experiments using expandable materials successfully reproduced structures similar to cortical folds of the mammalian cerebral cortex (Tallinen et al., 2016). A fourth hypothesis is that the tension created by axons connecting neighboring cortical regions produces cortical folds (Van Essen, 1997). Based on this hypothesis, axons connecting neighboring cortical regions bind these regions to each other, and the cortex between them protrudes outward. Finally, because the morphology and gene expression patterns of neural progenitors in the SVZ and the OSVZ are diverse in animals with cortical folds, this diversity may also be related to cortical folding (Reillo and Borrell, 2012; Betizeau et al., 2013; de Juan Romero et al., 2015; Johnson et al., 2015). Cortical folding has also been associated with the frequency of neural progenitor proliferation and gene expression patterns (Reillo et al., 2011; de Juan Romero et al., 2015; Toda et al., 2016; Matsumoto et al., 2017). Although many hypotheses had been proposed, experimental investigation of these hypotheses was delayed because of the difficulty of genetic manipulation in animals with cortical folds.

INVESTIGATIONS OF CORTICAL FOLDING MECHANISMS USING MICE

Although the mouse cerebral cortex does not have cortical folds, there have been attempts to clarify the molecular mechanisms of cortical folding using mice. This is mainly because many genetic techniques, such as those used to make knockout mice or transgenic mice, are available. Early pioneering studies reported that enhancing the proliferation of neural progenitors could create a cortical fold-like curvature on the surface of the mouse cerebral cortex (Chenn and Walsh, 2002). Other studies reported that inhibition of cell death also produced curvatures of the surface of the cerebral cortex in mice (Haydar et al., 1999; Depaepe et al., 2005). Since then, introduction or knockout of various genes was reported to produce a cortical fold-like structure on the surface of the mouse cerebral cortex (Depaepe et al., 2005; Rash et al., 2013; Stahl et al., 2013; Florio et al., 2015; Ju et al., 2016; Martínez-Martínez et al., 2016; Wang et al., 2016; del Toro et al., 2017; Liu et al., 2017; Chizhikov et al., 2019; Shao et al., 2020; Han et al., 2021; Kerimoglu et al., 2021; Kyrousi et al., 2021; Shqirat et al., 2021; Wang et al., 2021). These results are intriguing because they experimentally demonstrated that curvature of the surface of the cerebral cortex can be produced by manipulating neural progenitors. It would be important to investigate the roles of these genes in cortical folding of the gyrencephalic brains, and ferrets would be an important option for these investigations.

FEATURES OF FERRETS AS AN EXPERIMENTAL MODEL ANIMAL

Ferrets are medium-sized carnivorous mammals with a body length of about 50 cm, a weight of 1–2 kg, and an average lifespan of 6–10 years (**Figure 1A**). They are thought to be a descendant of the European polecat and have been domesticated. Ferrets have the following advantages for investigating the mechanisms underlying the development and evolution of the cerebral cortex. First, by using ferrets, it is possible to analyze the mechanisms underlying the expansion and folding of the cerebral cortex and the amplification of oRG cells, as mentioned above. It would be intriguing to combine *in vivo* studies using ferrets and *in vitro* studies using human iPS/ES organoids to uncover the mechanisms that caused changes to the cerebral cortex during evolution. Second, because ferrets have been used for electrophysiological and neuroanatomical experiments, electrophysiological and anatomical information on the ferret brain is available (Cucchiaro and Guillery, 1984; Law et al., 1988; Sur et al., 1988; Hahm et al., 1991; Meister et al., 1991; Callaway and Katz, 1993; Mooney et al., 1993; Crowley and Katz, 2000; Borrell and Callaway, 2002; Huberman et al., 2003; Kawasaki et al., 2004). For example, many fundamental findings regarding neural plasticity and its critical period were discovered in studies of ocular dominance columns in the visual cortex and eye-specific segregation of retinogeniculate projections in the thalamus of the ferret visual system. This electrophysiological and anatomical information provides an important basis for interpreting the experimental results of genetic studies. Third, ferret pups are born in an immature state, making them suitable for analyzing developmental processes and molecular mechanisms. Cortical folding proceeds after birth in developing ferrets, whereas it is largely completed before birth in cynomolgus monkeys. Fourth, in addition to being used in neuroscience research, ferrets have been widely used in research in other fields, for example, in research on infectious diseases such as the influenza virus and on the mechanisms of vomiting and

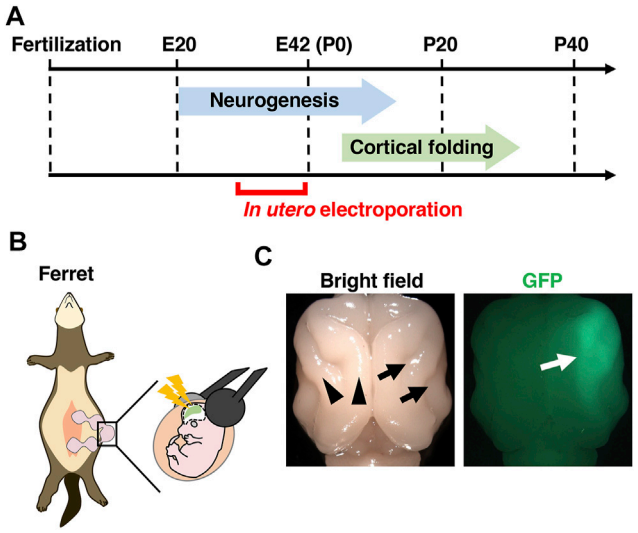

FIGURE 3 | *In vivo* genetic manipulation for the ferret cerebral cortex using *in utero* electroporation. **(A)** A schema showing the developmental timing of neurogenesis and cortical folding, and the stages when *in utero* electroporation can be performed. Neurogenesis in the ferret cerebral cortex starts around embryonic day 20 (E20) and completes around postnatal day 1 (P1) in the somatosensory cortex and P14 in the visual cortex. Cortical folding of the ferret cerebral cortex starts around P4 and completes around P30. *In utero* electroporation of the ferret cerebral cortex can be performed from around E30, when layer 5/6 neurons are generated. **(B)** An illustration of an *in utero* electroporation procedure. **(C)** Dorsal views of a ferret brain in which GFP was expressed by *in utero* electroporation. Cortical folds, i.e. gyri (black arrowheads) and sulci (black arrows), are present on the cerebral cortex. GFP signals are observed on the right side of the cerebral cortex (white arrow).

antiemetic reagents. As a result, they are easily obtained, and many researchers already have at least some familiarities with them. Finally, because knowledge regarding their breeding and mating has accumulated, ferrets are easy to raise. Despite these advantages, however, genetic techniques necessary for investigating the molecular mechanisms underlying the development and evolution of the ferret brain had been poorly available.

GENETIC MANIPULATION TECHNIQUES FOR THE FERRET BRAIN

Because of the ferret's advantages as a model animal, genetic techniques that could be applied to the ferret brain were desirable. One technique that was needed was genetic screening. Therefore, we made a custom ferret microarray for identifying genes expressed with characteristic patterns in the ferret brain (Kawasaki et al., 2004). Using this microarray, we have identified genes that are selectively expressed in magnocellular or parvocellular neurons, which are characteristically found in the well-developed visual system of higher mammals (Kawasaki et al., 2004; Iwai et al., 2013; Sato et al., 2017). Similarly, genes preferentially expressed in future gyral regions and future sulcal regions were uncovered using a ferret microarray (de Juan Romero et al., 2015). More recently, whole transcriptome RNA-seq analysis was applied to ferrets, and ferret RG cells were found to share key transcriptional features with human RG cells (Johnson et al., 2015). In addition, the ferret genome was sequenced, and annotated DNA sequence data is partially available (Peng et al., 2014). As these examples show, various genetic screening methods are now available for ferrets.

Another important technique is genetic manipulation. Previous pioneering studies reported that transgenes can be transfected into the ferret brain using postnatal electroporation and *in vivo* retroviral vector injection (Borrell, 2010; Nonaka-Kinoshita et al., 2013). Aiming to create a convenient genetic manipulation technique that could be applied to most cortical neurons in the ferret cerebral cortex, we established an *in utero* electroporation technique for the ferret cerebral cortex (**Figure 3**) (Kawasaki et al., 2012; Kawasaki et al., 2013). Using this technique, not only most cortical neurons but also neural progenitors such as RG cells, IP cells and oRG cells can be transfected. It takes only 1 hour to perform the *in utero* electroporation procedure on one pregnant ferret mother, and transfected ferret babies are born within a few weeks after the procedure, allowing transfected ferrets to be easily and rapidly obtained. Furthermore, multiple kinds of plasmids can be co-transfected by just mixing them, and various plasmids can be applied to different embryos in the same ferret mother, making it possible to conduct experiments under many different conditions simultaneously using one ferret mother. We also succeeded in gene knockout in the ferret cerebral cortex by combining *in utero* electroporation and the genome editing technology CRISPR/Cas9 (Shinmyo et al., 2017). Transgenes can also be knocked-in in ferret cortical neurons using the CRISPR/Cas9 system (Tsunekawa et al., 2016). Another approach of genetic manipulation would be to create genetically modified ferrets. Knockout ferrets have been successfully created using genome editing techniques, and they were used to uncover the roles of Aspm and Disc1 in the ferret brain (Kou et al., 2015; Johnson et al., 2018). Transgenic ferrets were also made by inserting transgenes into the ROSA26 locus using the CRISPR/Cas9 system (Yu et al., 2019). Furthermore, because ferret iPS cells have been generated (Gao et al., 2020; Yoshimatsu et al., 2021), organoid research using ferret iPS cells would be feasible. Due to these technological developments, genetic analyses of the ferret brain have become increasingly popular.

INVESTIGATION OF THE MECHANISMS UNDERLYING NEUROGENESIS AND CORTICAL FOLDING USING FERRETS

The Importance of Neural Progenitors in Cortical Folding

An increasing number of laboratories are using ferrets to analyze the molecular mechanisms of cortical folding. Pioneering studies reported the importance of neural progenitors in cortical folding. In the developing ferret cerebral cortex, pharmacological suppression of the proliferation of neural progenitors inhibited

cortical folding (Haddad et al., 1979). Conversely, stimulation of neural progenitor proliferation in the developing ferret cerebral cortex promoted the formation of cortical folds (Nonaka-Kinoshita et al., 2013; Masuda et al., 2015). Importantly, even before cortical folds are formed in the developing cerebral cortex, the amount of cell proliferation is higher in future gyral regions, whereas it is lower in future sulcal regions (Reillo et al., 2011). Consistently, progenitors are more abundant in future gyral regions than in future sulcal regions in the embryonic monkey cerebral cortex (Smart et al., 2002). These results suggest that the increase in neural progenitors in future gyral regions is involved in cortical folding. However, even if the proliferation of neural progenitors is enhanced in the developing mouse cerebral cortex, cortical folds do not necessarily form (Nonaka-Kinoshita et al., 2013). Therefore, it seems that proliferation of neural progenitors is not the only factor mediating cortical folding.

To investigate which types of neural progenitors are important for cortical folding, the distribution of oRG and IP cells in the developing ferret cerebral cortex was analyzed. oRG and IP cells were unevenly distributed, being more abundant in areas that would become gyri, but less abundant in areas that would become sulci. This result raised the possibility that oRG cells and/or IP cells are more abundantly distributed in future gyral regions, and the greater number of neurons they provide in these regions cause outward protrusions that become gyri. Recently, we found that oRG cells in the developing ferret cerebral cortex can be subdivided into two groups, HOPX-positive and HOPX-negative, according to the presence or absence of the transcription factor HOPX (Matsumoto et al., 2020). The distribution of these cells suggests that HOPX-positive oRG cells are more likely to be involved in the formation of cortical folds. It is important to note that although oRG cells are less abundant in the developing mouse cerebral cortex (Shitamukai et al., 2011), an area with relatively many oRG cells was found in the medial region of the cerebral cortex, and this area seemed similar to the developing cerebral cortex of the gyrencephalic brain (Vaid et al., 2018). Furthermore, Hopx was shown to be important for increasing the number of oRG cells in the mouse cerebral cortex (Vaid et al., 2018). Therefore, HOPX might be a key regulator for the production of oRG cells in the gyrencephalic cerebral cortex.

Mechanisms Regulating the Abundance of Neural Progenitors

A further important question is what the regulatory mechanisms upstream of neural progenitor proliferation are. To elucidate genes responsible for the proliferation of neural progenitors, we focused on human diseases which exhibit abnormal cortical folds.

Thanatophoric dysplasia is a congenital disease that shows abnormalities in bones and the brain including polymicrogyria, in which many more cortical folds are produced than normal. A pioneering study identified a mutation in the fibroblast growth factor (FGF) receptor 3 gene in polymicrogyria patients and showed that this mutation induced an activated form of the FGF receptor 3, raising the possibility that FGF signaling is involved in cortical folding and neural progenitor proliferation

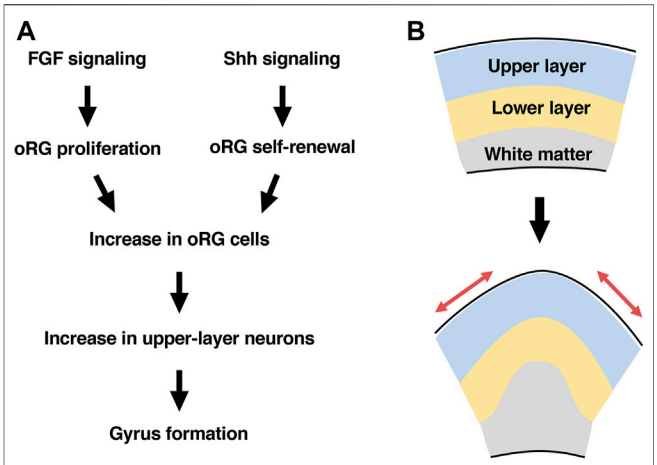

FIGURE 4 | A hypothesis about mechanisms underlying cortical folding. **(A)** FGF signaling and Shh signaling cooperate to increase oRG cells by promoting proliferation and self-renewal, respectively. The increased oRG cells preferentially produce upper-layer neurons, resulting in gyrus formation. **(B)** Gyrus formation requires the tangential expansion of the cortical surface (red arrows), which is achieved by a preferential increase in upper-layer neurons.

(Shiang et al., 1994). To directly test if FGF signaling mediates the proliferation of neural progenitors, FGF8, a ligand for the FGF receptor 3, was expressed in the developing ferret cerebral cortex using *in utero* electroporation (Masuda et al., 2015). The proliferation of oRG cells was promoted by the activation of FGF signaling, and as a result, oRG cells were increased. Consistently, when FGF signaling was inhibited by expressing a dominant-negative form of the FGF receptor 3 in the ferret cerebral cortex, the proliferation of oRG cells was reduced, and the number of oRG cells decreased (Matsumoto et al., 2017). These results indicate that FGF signaling regulates the proliferation of oRG cells.

We also investigated the roles of sonic hedgehog (Shh) signaling in the regulation of oRG cells because a previous report identified abnormality in Shh signaling in human patients with cortical fold malformations. When Shh ligand was introduced into the developing ferret cerebral cortex using *in utero* electroporation, the number of oRG cells was increased due to enhanced oRG cell self-renewal (Matsumoto et al., 2020). Consistently, Smoothened Agonist (SAG), a specific activator of Shh signaling, promoted self-renewal of oRG cells in cultured ferret brain slices (Hou et al., 2021). When Shh signaling was suppressed by introducing HhipΔC22, a dominant-negative form of the Shh signaling pathway, the number of oRG cells was decreased due to reduced oRG cell self-renewal. These findings suggest that FGF signaling and Shh signaling cooperate to increase oRG cells by promoting proliferation and self-renewal, respectively (**Figure 4A**) (Masuda et al., 2015; Matsumoto et al., 2017; Matsumoto et al., 2020).

Hippo signaling and serotonin signaling were also reported to be crucial for the proliferation and abundance of basal progenitors (Kostic et al., 2019; Xing et al., 2020). Moreover, when the human-specific gene ARHGAP11B was introduced into

the ferret cerebral cortex, it increased oRG cells (Kalebic et al., 2018). Interestingly, PALMDELPHIN was reported to increase processes of basal progenitors and stimulate their proliferation through integrin signaling (Kalebic et al., 2019). It would be important to investigate the interactions among these signaling pathways to uncover the complete picture of the mechanisms regulating the proliferation and self-renewal of oRG cells.

In addition to elucidating the mechanisms of neural progenitor proliferation and self-renewal, uncovering those of the translocation of neural progenitors is also important. In a study comparing mice and ferrets, species-specific differences in interkinesis strategies were reported (Okamoto et al., 2014). As for the molecular mechanisms, Lzts1, which is associated with microtubule components, was reported to control the delamination and generation of oRG-like cells (Kawaue et al., 2019). Because few oRG cells are observed in mice, ferrets seem useful for examining the mechanisms underlying the translocation and morphological changes of oRG cells.

Mechanisms Underlying Cortical Folding

Because both FGF signaling activation and Shh signaling activation increased oRG cells, we investigated whether FGF signaling and Shh signaling are involved in cortical folding (Masuda et al., 2015; Matsumoto et al., 2017; Matsumoto et al., 2020). When FGF signaling was activated by introducing FGF ligand into the developing ferret cerebral cortex, the number of cortical folds was increased, resulting in a polymicrogyria-like phenotype. Importantly, the increased cortical folds exhibited the critical features of physiological cortical folds. These folds contained all cortical layers (i.e. layers 1–6), and while the cortical surface exhibited additional cortical folds, the ventricular surface was smooth and without curvature. Furthermore, inhibiting FGF signaling by expressing a dominant-negative form of the FGF receptor in the developing ferret cerebral cortex attenuated cortical folding. Similarly, when Shh signaling was activated by introducing Shh ligand into the developing ferret cerebral cortex, the number of cortical folds was increased. Suppression of Shh signaling using HhipΔC22 inhibited cortical folding. These results indicate that FGF signaling and Shh signaling cooperate to induce cortical folds, presumably through regulating the number of oRG cells (**Figure 4A**) (Masuda et al., 2015; Matsumoto et al., 2017; Matsumoto et al., 2020).

Interestingly, when comparing mice and ferrets, the amount of Shh protein in the cerebral cortex was found to be higher in ferrets (Matsumoto et al., 2020). Furthermore, Gli1 expression levels, which reflect the activation of Shh signaling, were higher in the cerebral cortex of ferrets than in that of mice. These results indicate that Shh signaling is more strongly activated in the ferret cerebral cortex than in the mouse cerebral cortex and may indicate that increased Shh signaling activity during evolution led to an increase in oRG cells and the acquisition of cortical folds (Matsumoto et al., 2020).

A further important question was what mechanisms link an increase in oRG cells to the morphological changes leading to cortical folds. In detailed studies of the cerebral cortex in which FGF signaling or Shh signaling was activated to promote cortical folding, the thickness of superficial layers of the cerebral cortex was selectively increased, while deep layers were less affected (Masuda et al., 2015; Matsumoto et al., 2017; Matsumoto et al., 2020). This result is consistent with the previous hypothesis that the ratio between superficial and deep regions of the cerebral cortex is important for cortical folding (Richman et al., 1975; Kriegstein et al., 2006). In order to test this hypothesis, Cdk5 was used to selectively reduce the number of neurons in superficial layers of the cerebral cortex. Cdk5 has been reported to be responsible for human lissencephaly (Magen et al., 2015) and therefore is thought to be important for cortical folding. Consistent with the data from human lissencephaly patients, knocking out the Cdk5 gene in pyramidal neurons of the developing ferret cerebral cortex by combining *in utero* electroporation and the CRISPR/Cas9 system attenuated cortical folding, suggesting that Cdk5 in pyramidal neurons is crucial for cortical folding (Shinmyo et al., 2017). Cdk5 is required for radial migration of neurons from the ventricular surface to the brain surface, suggesting that radial migration of cortical neurons is crucial for cortical folding. Consistently, introduction of a mutated SCN3A/$Na_v1.3$ sodium channel into cortical neurons inhibited both radial migration and cortical folding (Smith et al., 2018). In order to determine to which layers of the cerebral cortex it is important for neurons to migrate, a dominant-negative form of Cdk5 was selectively introduced into either layer 2/3 neurons or layer 5–6 neurons. Interestingly, suppressing the radial migration of layer 2/3 neurons significantly inhibited cortical folding, whereas suppressing that of layer 5–6 neurons did not. This result supports the hypothesis that a preferential increase in superficial regions of the cerebral cortex relative to deep regions induces cortical folding (**Figures 4A,B**). It should be noted that cortical neurons migrate in a tangential orientation without following strict radial paths in the developing ferret cerebral cortex (Reillo et al., 2011; Gertz and Kriegstein, 2015). Therefore, it seems plausible that cortical folding requires a tangential expansion of the superficial portion of the cerebral cortex.

As mentioned above, the mechanisms of cortical folding have been intensively investigated. However, there are still many aspects that are not yet clearly understood. First, the formation of cortical folds continues even after neurons have completed their neurogenesis and radial migration during development. This suggests that mechanisms other than the proliferation of neural progenitors and radial migration of cortical neurons are also involved in cortical folding. In addition to intrinsic genetic factors, some non-genetic factors could be involved in cortical folding because patterns of cortical folds are not completely identical even between genetically identical twins (Lohmann et al., 1999). Since cortical folding proceeds after birth in ferrets, the ferret may be a useful model to study the influence of not only intrinsic genetic factors but also extrinsic environmental factors such as birth.

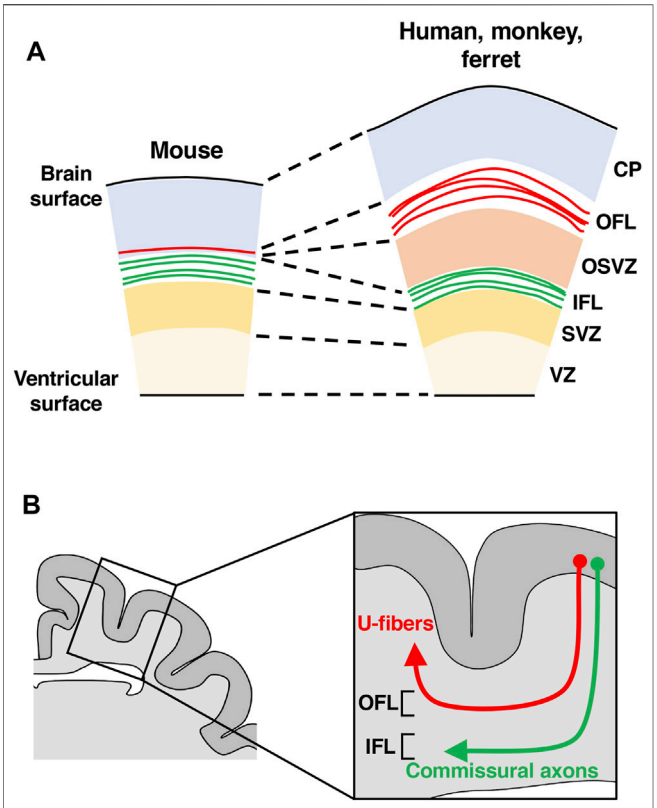

FIGURE 5 | A hypothesis about the evolution of fiber layers in the developing cerebral cortex. **(A)** Schemas of cross sections of the developing cerebral cortices of mice, ferrets, monkeys and humans. Fibers in the outer fiber layer (OFL, red) increased during evolution. **(B)** A schema of a cross section of the ferret cerebral cortex. The OFL becomes subcortical U-fibers (red), while the IFL gives rise to commissural axons (green) and subcortical projecting axons. CP, cortical plate; OSVZ, outer subventricular zone; SVZ, subventricular zone; VZ, ventricular zone.

Common and Species-specific Mechanisms Underlying Cortical Folding

An important question is whether the genetic programs controlling cortical folding found in ferrets are conserved in primates. Folds of the cerebral cortex are present in many mammalian orders, while they are absent in some species including mice. It seems likely that gyrencephalic animals such as humans and ferrets, at least in part, share common mechanisms regulating cortical folds, although ferrets are phylogenetically farther from primates than rodents. Since FGF signaling, Shh signaling and Cdk5 are involved in cortical folding in both humans and ferrets, it is plausible that the regulation of oRG cell amplification by FGF signaling and Shh signaling is conserved in both species. This idea is also supported by numerous previous studies showing that neural progenitors in gyrencephalic animals share common features that are lacking in those in mice. For example, larger amounts of neural progenitors are seen in the OSVZ of various gyrencephalic animals including humans, monkeys, ferrets and guinea pigs (Kriegstein et al., 2006; Martínez-Cerdeño et al., 2006; Hansen et al., 2010; Reillo and Borrell, 2012; Dehay et al., 2015; Hatakeyama et al., 2017). Moreover, differentially expressed genes between prospective gyri and sulci in neural progenitors of the ferret cerebral cortex exhibit a similar expression pattern to those in the developing human cerebral cortex (de Juan Romero et al., 2015). It was recently shown that microRNA miR-3607 plays a key role in the amplification of RG cells by acting as a regulator of Wnt/β-catenin signaling in ferrets and humans and that the loss of miR-3607 expression during evolution reduced the amplification of neural progenitors in the mouse cerebral cortex (Chinnappa et al., 2022). It seems possible that the common ancestor of mammals had folds of the cerebral cortex and that mice underwent a secondary loss of cortical folds (Kelava et al., 2013).

It also should be noted that although ferrets share several developed brain structures with humans, these structures in humans are often more developed than those in ferrets. Indeed, in addition to brain size, the gyrification index, which indicates the extent of cortical folds, and the thickness of superficial layers of the cerebral cortex, are larger in humans (Hutsler et al., 2005; Zilles et al., 2013). These facts suggest the emergence of primate-specific mechanisms that enhanced brain growth and cortical folding. Recent studies have identified primate-specific and human-specific genes and noncoding microRNAs that promote the amplification of neural progenitors (Arcila et al., 2014; Florio et al., 2015; Fiddes et al., 2018; Florio et al., 2018; Nowakowski et al., 2018; Suzuki et al., 2018). It was demonstrated that introduction of the human-specific gene ARHGAP11B in the ferret brain resulted in a further expansion of the cerebral cortex (Kalebic et al., 2018). Thus, the ferret is an important model organism for the investigation of common mechanisms of cortical folding among gyrencephalic animals as well as the impact of primate-specific and human-specific genomic changes on brain growth and cortical folding in the gyrencephalic cerebral cortex.

EVOLUTION AND DEVELOPMENT OF THE FIBER LAYER IN THE CEREBRAL CORTEX

In the developing cerebral cortex of humans and monkeys, in addition to the cortical plate and the germinal zones, there are two fiber layers, the inner fiber layer (IFL) and the outer fiber layer (OFL) (**Figure 5A**) (Molnár and Clowry, 2012). However, a detailed understanding of their development and evolution had remained elusive, at least partially because these two fiber layers were not recognized in the mouse cerebral cortex. Interestingly, when GFP was expressed in excitatory cortical neurons of the ferret cerebral cortex using *in utero* electroporation, GFP-positive axons were found to be accumulated in positions corresponding to where the IFL and the OFL are observed in humans and monkeys (**Figure 5A**) (Saito et al., 2019). These results suggest that ferrets, like humans and monkeys, also have the IFL and the OFL in the developing cerebral cortex and that the IFL and the OFL contain axons

derived from excitatory neurons of the cerebral cortex (Saito et al., 2019).

To investigate which neuronal circuits the OFL and the IFL become after developmental processes proceed, we expressed GFP in cortical neurons and examined the projections of GFP-positive axons corresponding to the IFL and the OFL. The IFL was found to give rise to mainly commissural and subcortical projecting axons, whereas the OFL became U-fibers (**Figure 5B**) (Yoshino et al., 2020). U-fibers are short association fibers located just below gray matter and have been found in humans and monkeys (Meynert, 1885; Nieuwenhuys et al., 1988; Schuz et al., 2002; Catani et al., 2012; Ouyang et al., 2017). U-fibers are thought to be important for functional association between neighboring cortical areas, and their abnormalities have been reported in neurodevelopmental and psychiatric disorders. MRI and histological analyses of U-fibers have been performed on human and monkey brains, but fibers corresponding to the IFL and the OFL have not been reported in mice, making an investigation of U-fibers difficult. Taken together, these results suggest that ferrets are a useful model organism for investigating U-fibers (Yoshino et al., 2020).

Because U-fibers can be visualized in ferrets by expressing GFP using *in utero* electroporation, we performed similar experiments using mice. Interestingly, a small number of GFP-positive axons that project to neighboring cortical areas were observed in mice (Saito et al., 2019; Yoshino et al., 2020). This result suggests that a small number of axon fibers corresponding to U-fibers found in humans, monkeys and ferrets also exist in mice, and these axon fibers have increased significantly during evolution, forming the OFL (**Figure 5A**, red) (Saito et al., 2019; Yoshino et al., 2020). Although these results have clarified some aspects of the development and evolution of U-fibers, many points still remain unclear. It would be important to elucidate the molecular mechanisms that regulate the formation of U-fibers and the physiological significance and pathological involvement of U-fibers. Furthermore, because U-fibers are predominantly observed in the gyrencephalic mammalian cerebral cortex, it would be intriguing to investigate the roles of U-fibers in cortical folding.

FUTURE PROSPECTS

The mammalian cerebral cortex continues to be of great interest to researchers. Our understanding of the developmental processes, functions and diseases of the cerebral cortex has been advanced mainly using mice as a model animal. Recently, the use of new animal models such as ferrets and marmosets, along with the development of various genetic techniques for them including *in utero* electroporation, genome editing and iPS/ES organoids, have expanded the scope of analyses of the cerebral cortex. It is expected that the use of new animal models will accelerate our understanding of the mechanisms underlying the development and evolution of the complex brain architecture and brain functions observed in the well-developed cerebral cortex of higher mammals, as well as those underlying the pathophysiology of diseases of the cerebral cortex. Ferrets should provide an important platform for investigating these mechanisms *in vivo*.

AUTHOR CONTRIBUTIONS

YS, THH, KS and HK wrote the manuscript.

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A Brain Region-Specific Expression Profile for Genes Within Large Introgression Deserts and Under Positive Selection in *Homo sapiens*

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Analyses of ancient DNA from extinct hominins have provided unique insights into the complex evolutionary history of *Homo sapiens*, intricately related to that of the Neanderthals and the Denisovans as revealed by several instances of admixture events. These analyses have also allowed the identification of introgression deserts: genomic regions in our species that are depleted of "archaic" haplotypes. The presence of genes like *FOXP2* in these deserts has been taken to be suggestive of brain-related functional differences between *Homo* species. Here, we seek a deeper characterization of these regions and the specific expression trajectories of genes within them, taking into account signals of positive selection in our lineage. Analyzing publicly available transcriptomic data from the human brain at different developmental stages, we found that structures outside the cerebral neocortex, in particular the cerebellum, the striatum and the mediodorsal nucleus of the thalamus show the most divergent transcriptomic profiles when considering genes within large introgression deserts and under positive selection.

Keywords: Homo sapiens, deserts of introgression, positive selection, cerebellum, striatum, thalamus, gene expression

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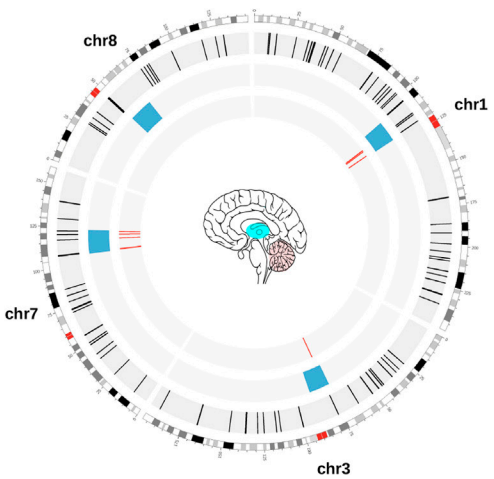
1 INTRODUCTION

The availability of high-coverage genomes from our closest extinct relatives, the Neanderthals and Denisovans, constitutes a significant advance in the range of questions one can ask about the deep history of our species (Meyer et al., 2012; Prüfer et al., 2014; Prüfer et al., 2017; Mafessoni et al., 2020). One of the main themes emerging from this progress is interbreeding. In recent years, a fairly large number of admixture events between Neanderthals, Denisovans and Sapiens populations have been postulated. A recent review (Bergström et al., 2021) considers that at least four such events are supported by strong evidence.

While it is important to ask whether our species benefited from these admixture events (so-called adaptive introgression, where alleles inherited from other hominins rose to high frequency as a result of positive selection after gene flow), it is also worth examining regions of the genomes that are depleted of alleles resulting from gene flow from other hominins (Sankararaman et al., 2016; Vernot et al., 2016; Chen et al., 2020; Skov et al., 2020; Rinker et al., 2020). Such regions are called introgression deserts (sometimes also "genomic islands of divergence/speciation" (Wang et al., 2020) and have now been identified in a range of species (Fontsere et al., 2019).

TABLE 1 | Genomic coordinates used in this study. Large deserts were retrieved from (Chen et al., 2020), and positively-selected regions from (Peyrégne et al., 2017) (see **Section 4**). The circos plot on the right shows the distribution of our regions of interest: Blue bloxes: deserts of introgression; Red lines: positively-selected regions within deserts of introgression. Colored regions within the brain represent structures that figure prominently in this study.

Set	chr	Start	End
Large deserts	1	105400000	120600000
	3	74100000	89300000
	7	106200000	123200000
	8	49400000	66500000
Positively-selected regions within deserts	1	113427676	113560554
	1	114641362	114645248
	1	119322276	119387279
	3	77027847	77034264
	7	106877730	107233808
	7	116762909	116773234
	7	120147456	120174406
	7	122320035	122406480



There are multiple reasons why genetic differences that arose after the divergence of populations may not be well tolerated (Wolf and Akey, 2018): there could be negative selection on "archaic" variants (deleterious changes on the "archaic" lineage), or positive selection on human-specific variants (adaptive changes on the human lineage), or it may be due to drift. It is reasonable to expect, and indeed has been shown, that the X chromosome constitutes such a desertic region [not only in our species (Kuhlwilm et al., 2019; Martin and Jiggins, 2017)]. This could be due to repeated selective sweeps on this chromosome: genes involved in reproduction on this chromosome might act as strong reproductive barriers between populations (Fontsere et al., 2019).

In the case of modern humans, other genomic regions are devoid of Neanderthal and Denisovan introgression, for reasons that are perhaps less obvious, and therefore worth investigating further. A recent study (Chen et al., 2020) identifies four large deserts depleted of Neanderthal introgression, partially overlapping with a previous independent study (Vernot et al., 2016). As pointed out in (Kuhlwilm, 2018; Wolf and Akey, 2018), since it is likely that there were several different pulses of gene flow between us and our closest relatives (Iasi et al., 2021), the depletion observed in these four regions must have been reinforced repeatedly, and given the size of the deserts, it is reasonable that the "archaic" haplotype was purged within a short time after the gene flow event, as predicted by mathematical modeling on whole-genome simulations (Veller et al., 2021), and as evidenced in the analysis of genome-wide data from the earliest Late Pleistocene modern humans known to have been recovered in Europe (Hajdinjak et al., 2021).

The presence of *FOXP2*, a gene known for its role in language (Lai et al., 2001; Fisher, 2019), in one of these large deserts has attracted attention (Kuhlwilm, 2018), as it raises the possibility that the incompatibility between *Homo sapiens* and other hominin in such persistent introgression deserts may point to (subtle, but real) cognitive/behavioral differences. Indeed, the presence in such deserts of not only *FOXP2* but also other genes like *ROBO1*, *ROBO2*, and *EPHA3*, all independently associated with language traits (St Pourcain et al., 2014; Wang et al., 2015; Eising et al., 2021; Mekki et al., 2022), together with an earlier observation in Vernot et al. (2016) that genes within large deserts are significantly enriched in the developing cerebral cortex and in the adult striatum, suggest a possible point of entry into some of the most distinctive aspects of the human condition (Pääbo, 2014). Such considerations, combined with independent evidence that introgressed Neanderthal alleles show significant downregulation in brain regions (McCoy et al., 2017), motivated us to focus on the brain in this study.

Specifically, we focused on the four largest genomic regions that resisted "archaic" introgression reported in (Chen et al., 2020), jointly with the most comprehensive catalog to date of signals of positive selection in our lineage (Peyrégne et al., 2017) (see **Table 1**), a combination that, to our knowledge, has not been previously studied in detail. Here, we tested if the genes that fulfill these two conditions (falling within large deserts of introgression and being under positive selection) follow particular (brain-region) expression trajectories that significantly deviate from that of other subsets of genes with evolutionary relevance or from control genomic regions. We characterized the gene expression dynamics (including genes falling within either

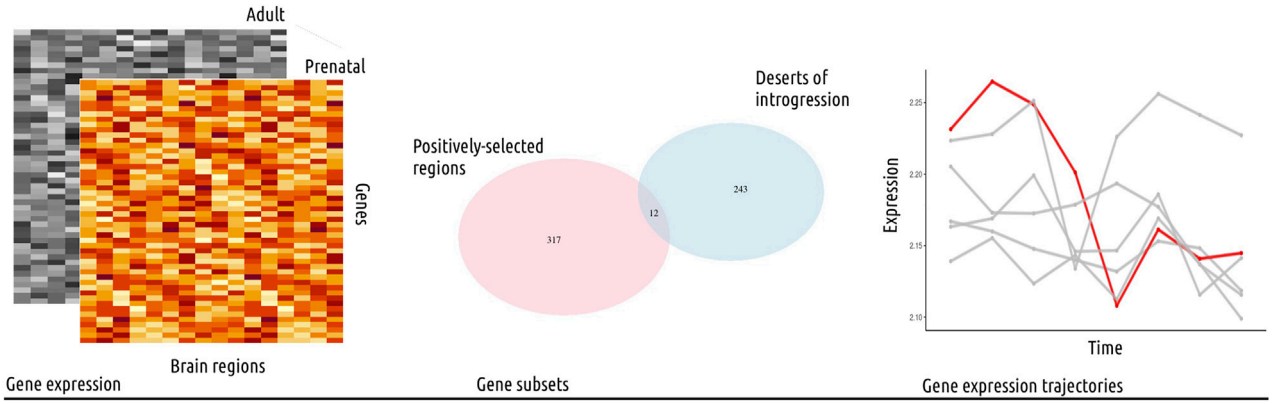


FIGURE 1 | Study outline. In this study, we used publicly available gene expression data from the psychENCODE project (Li et al., 2018), covering sixteen brain regions and eight developmental stages, from prenatal to adult (left panel). The data was analyzed following standard procedures and the normalized, log-transformed gene expression values were then used for further analysis. We focused on gene subsets from genomic regions with evolutionary-relevant properties (middle panel): 1) positively-selected regions and 2) large deserts of introgression (see also **Table 1**). We investigated the expression trajectories of genes fulfilling both of these conditions (twelve genes in total) across brain regions, and proceeded to compare their gene expression values to control genes from length and gene density-matched random regions, as well as to other subsets of genes. Specifically, we performed a Principal Component Analysis and calculated the Euclidean distance between each data point (gene expression value for a subset of genes in a particular brain region) per developmental stage to identify statistically significant differences (Wilcoxon rank sum test with Bonferroni correction) among brain regions. This analysis was complemented with a segmented regression model for a single-gene analysis of gene expression dynamics (right panel). Collectively, we found the most significant differences for the cerebellum, the striatum and the mediodorsal nucleus of the thalamus.

deserts of introgression or positively-selection regions alone) by analyzing transcriptomic data from several brain regions encompassing multiple developmental stages from prenatal to adulthood. This dataset allows for greater resolution than the Allen Brain Atlas data used in (Vernot et al., 2016), especially at early stages of development (see **Figure 1**). Three of the brain regions under study showed marked transcriptomic divergence (i.e., a statistically significant difference when compared to all other regions, based on the Principal Component Analysis-derived Euclidean distances): the cerebellum, the striatum and the thalamus. Among the genes at the intersection of regions under positive selection and large deserts of introgression, we found *CADPS2*, *ROBO2*, or *SYT6*, involved in neurotrophin release, axon guidance and neuronal proliferation, and known to be expressed in the brain regions our analysis highlights.

2 RESULTS

2.1 Genes in Large Deserts of Introgression Have Different Expression Levels Relative to the Rest of the Genome

We set out to understand whether the mean expression of genes in large deserts of introgression (Chen et al., 2020) and the positively selected regions within them (extracted from (Peyrégne et al., 2017)) is significantly different compared to the rest of the genome, using publicly available transcriptomic data from the human brain (Li et al., 2018). To this end, we selected random regions of the genome ($n = 1{,}000$), excluding the large deserts, of the same average length (i.e., 15 million base-pairs), with a possible deviation of 1 million base-pairs to account for the length variability between different deserts of introgression. To avoid genomic regions with low genetic density that might skew the results, the randomized areas were required to hold at least as many genes (265) as the desertic regions reported in (Chen et al., 2020).

The mean expression of genes lying in random regions of the genome was summarized for each brain structure (and log2-transformed). A repeated-measures two-way ANOVA shows that the mean expression of both sets of these regions is significantly different from the rest of the genome ($p < 0.01$ for both sets). A post-hoc pairwise ANOVA (with Bonferroni correction) shows the difference between a gene expression value in a brain region as derived from the control set and that obtained from the genes in our two sets of interest is significant for most structures. An outlier's Grubbs test shows that the structures with the highest and lowest mean gene expression values in large deserts of introgression and the positively-selected windows within them fall inside the expected range of variability given the data ($p > 0.01$).

2.2 The Cerebellar Cortex, the Striatum and the Thalamus Show Divergent Transcriptomic Profiles When Considering Genes Within Large Deserts of Introgression and Under Positive Selection

We then investigated the temporal progression of the expression of genes within large deserts of introgression and putative positively-selected regions analyzing RNA-seq data of different human brain regions at different developmental stages (Li et al., 2018). We found that the median expression of genes within large deserts and positively-selected regions is higher than those present in deserts alone, the former peaking at prenatal stages in neocortical areas and decreasing later on. Outside the cerebral

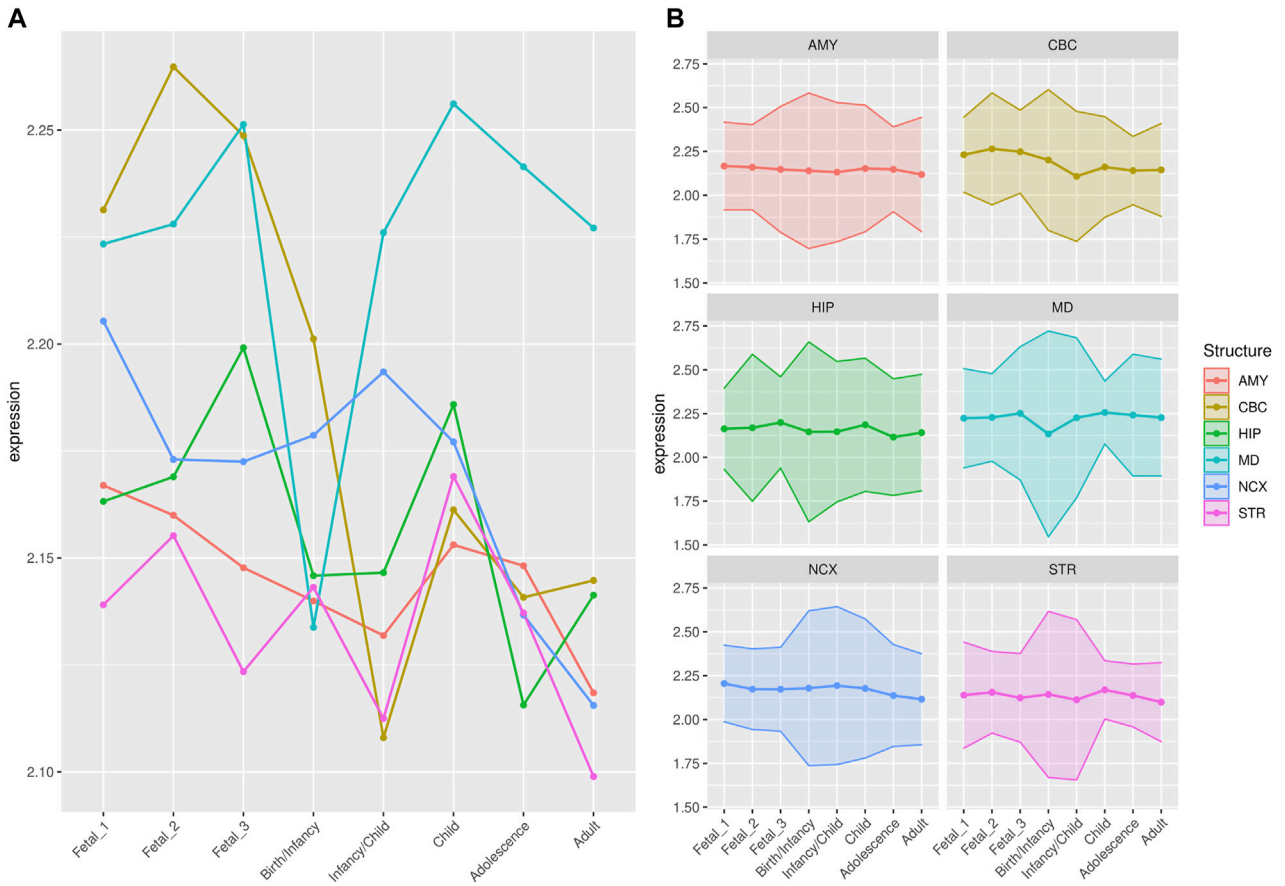


FIGURE 2 | Median expression of genes within large deserts. **(A)** Comparison of median of gene expression across developmental stages between structures and **(B)** Standard deviation per structure, for genes (n = 255) within the four large deserts of introgression. The cerebellar cortex, prenatally, and the mediodorsal nucleus of the thalamus prenatally and postnatally present the highest expression. The median expression profile of genes within deserts, per chromosome, is shown in **Supplementary Figure S12**. *Structures:* AMY, amygdala; CBC, cerebellar cortex; HIP, hippocampus; MD, mediodorsal nucleus of thalamus; NCX, neocortex; STR, striatum. *Stages:* Fetal 1: 12-13 post conception weeks (PCWs); Fetal 2: 16-18 PCW; Fetal 3: 19-22 PCW; Birth—Infancy: 35-37 PCW and 0–0.3 years; Infancy—Child: 0.5–2.5 years; Childhood: 2.8–10.7 years, Adolescence: 13–19 years; Adulthood: 21–64 years [as in (Li et al., 2018)].

neocortex, this pronounced prenatal peak is not observed and, specifically for the cerebellar cortex, the expression profile of these genes increases before birth and reaches the highest median expression from childhood to adulthood in comparison to the rest of structures (see **Supplementary Figure S1**).

In order to statistically evaluate the differences observed for each structure and developmental stage (see **Figures 2**, **3**), we performed a Principal Component Analysis and calculated the pairwise Euclidean distances between brain regions for each developmental stage using statistically significant principal components (p < 0.05) as assessed using the JackStraw analysis implemented in Seurat (Butler et al., 2018). For genes within large deserts of introgression overlapping putative positively-selected regions, we performed dimensionality reduction on the first two principal components. Due to the low number of genes at this intersection (n = 12), the second principal component did not report statistical significance. The sum of the percentage of variance explained by first and second components is around 50%. The transcriptomic profile of a brain region in a given developmental stage was considered "divergent" if the expression value of the subset of genes under consideration was significantly different (p < 0.01) in that region when compared to all other regions (performing a Wilcoxon rank sum test with Bonferroni correction).

For genes that reside in the deserts of introgression under consideration, the cerebellum stands out as the structure with the most divergent transcriptomic profile at postnatal stages, from childhood to adulthood (**Figure 4**). For genes under positive selection that are also found within introgression deserts, the cerebellum still remains as the most transcriptomically divergent structure postnatally (birth/infancy, childhood, adolescence and adulthood; see the caption of **Figure 2** for the specific time points associated to each developmental stage). Moreover, prenatally, the cerebellum again (fetal stages 1 and 2) and the mediodorsal nucleus of the thalamus (fetal stage 1; see **Supplementary Figures S2, S3**) exhibit the most significant differences in the pairwise comparisons. Previous research found that genes within large deserts are over-represented in the striatum at adolescence and

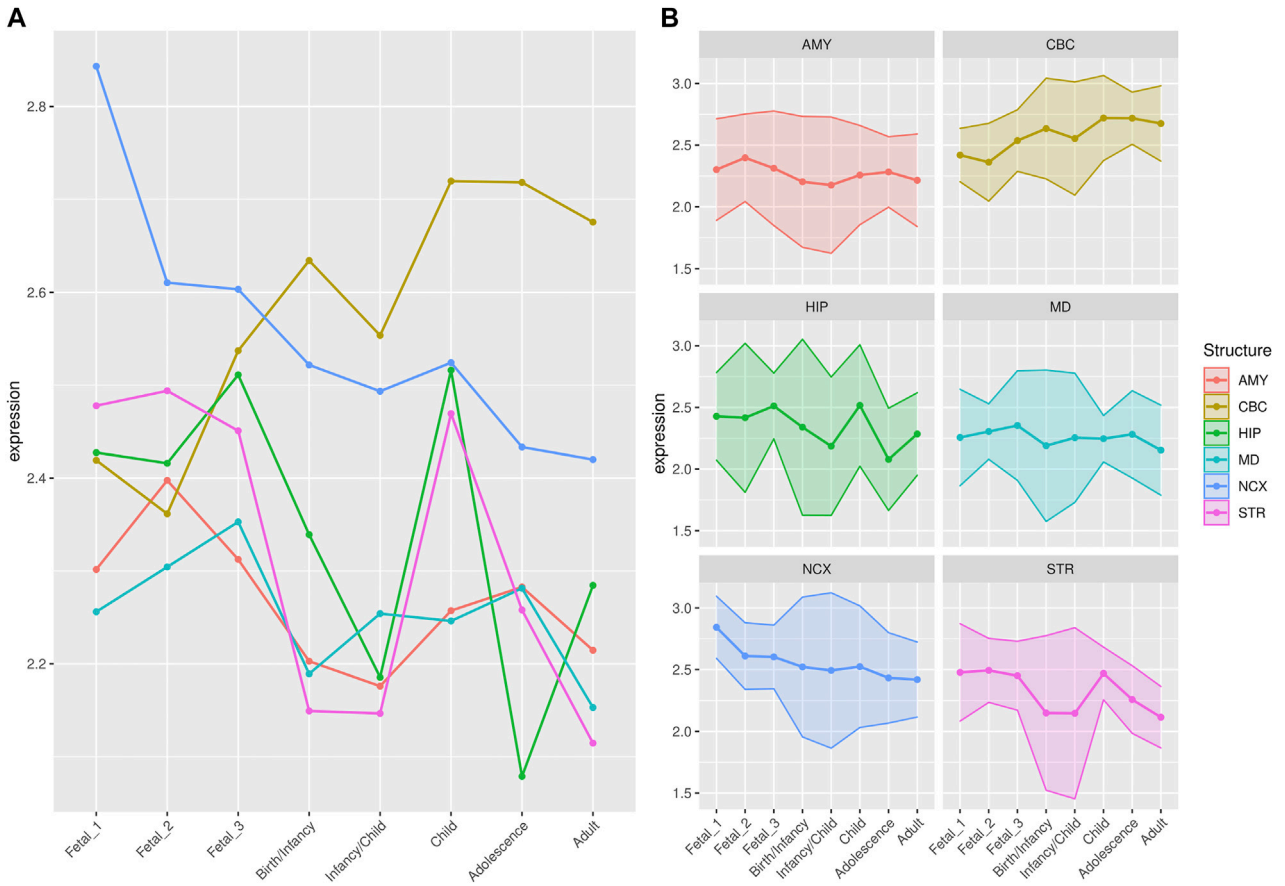


FIGURE 3 | Median expression of genes under putative positive selection within large deserts. **(A)** Comparison of median of gene expression across developmental stages between structures and **(B)** Standard deviation per structure, for genes (n = 12) within the four large deserts of introgression and under putative positive selection. Genes expressed in neocortical areas reach the highest expression at the early fetal stages, whereas the cerebellar cortex, from birth until adulthood, retains its status as the structure with the highest expression profile. The expression profile of each of the twelve genes under putative positive selection within deserts is shown in **Supplementary Figure S13**. *Structures:* AMY, amygdala; CBC, cerebellar cortex; HIP, hippocampus; MD, mediodorsal nucleus of thalamus; NCX, neocortex; STR, striatum. *Stages:* Fetal 1: 12-13 post conception weeks (PCWs); Fetal 2: 16-18 PCW; Fetal 3: 19-22 PCW; Birth—Infancy: 35-37 PCW and 0–0.3 years; Infancy—Child: 0.5–2.5 years; Childhood: 2.8–10.7 years, Adolescence: 13–19 years; Adulthood: 21–64 years [as in (Li et al., 2018)].

adult stages (Vernot et al., 2016). In agreement with this finding, we found that the transcriptomic profile of the striatum for genes within large deserts is significantly different at adolescence and adulthood but also at fetal stage 3, while for genes within deserts under putative positive selection, significant differences are found at infancy and adolescence (see **Figures 4**, **5**, and **Supplementary Figure S4**). Lastly, to disentangle the effect of set of genes within specific chromosomes, we also evaluated the expression dynamics of genes within large deserts of introgression for each of the four chromosomal regions separately (a corresponding evaluation of the twelve genes under putative postive selection within deserts is presented in the next section). Overall, and in agreement with the previous observations, the cerebellum (at perinatal and later postnatal stages for the four chromosomes) and the striatum (at adulthoood for three out of four chromosomes, and childhood for one chromosomal region) are found as the most transcriptomically divergent structures. The transcriptomic profile of the mediodorsal nucleus of the thalamus was also found to be statistically different at fetal stages for chromosome 1 and chromosome 8 (see **Supplementary Figures S5–S8**).

For the sake of comparison, we note that a similar profile postnatally was obtained for the cerebellum when subsetting for genes under positive selection not present within large introgression deserts (marked differences from childhood to adulthood; see **Supplementary Figure S9**). When evaluating the global expression profile (n = 9,358 genes), the cerebellum shows statistically significant differences also at postanatal stages (birth, infancy, childhood and adulthood) and the mediodorsal nucleus of the thalamus at fetal stage 3 and adulthood (see **Supplementary Figure S10**). All p-values can be found in the Supplementary files.

The trajectories of expression across developmental stages in genes within large deserts of introgression might be affected by positive selection. To control for this, we analyzed the contrast between a control group of genes not under positive selection but

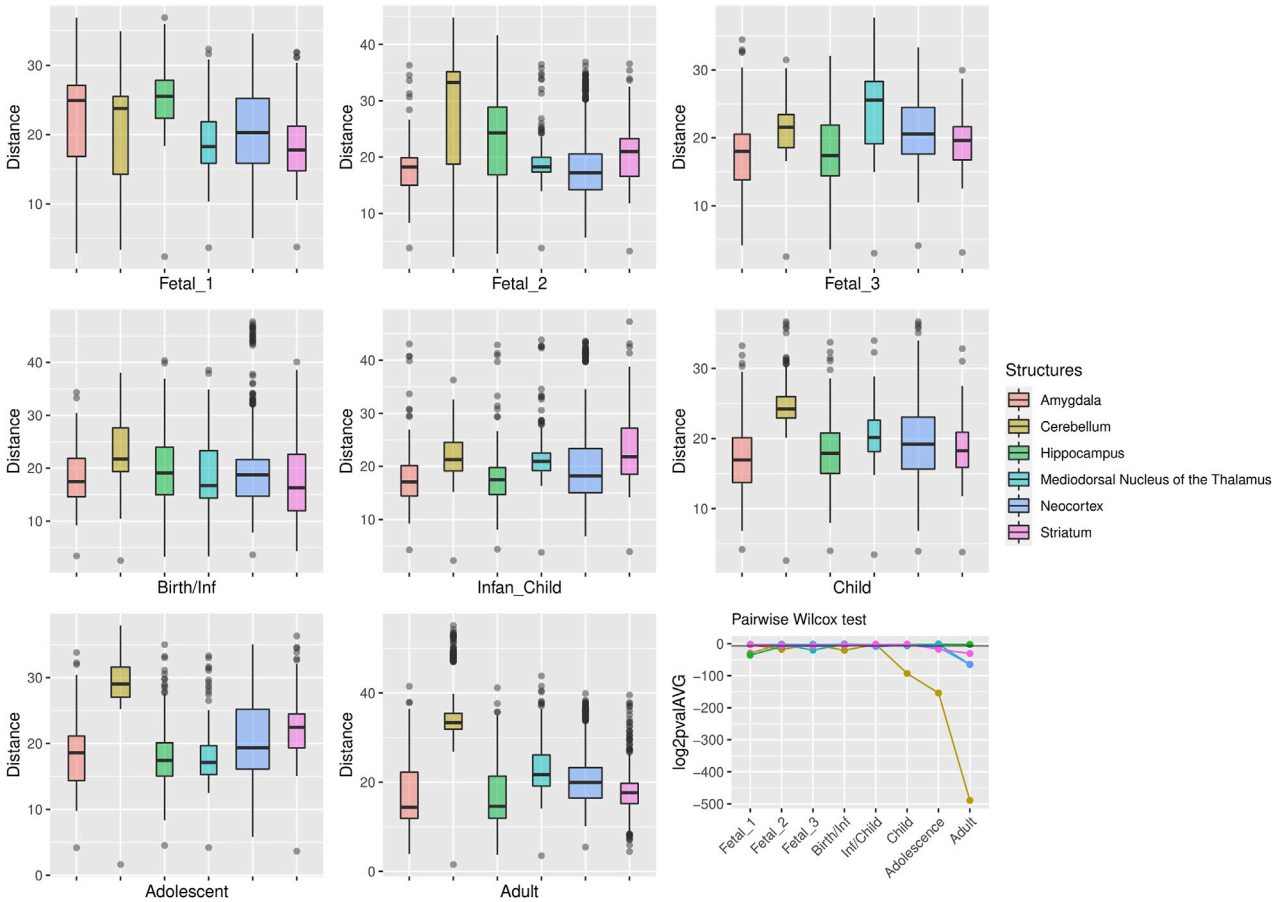


FIGURE 4 | Genes within large deserts. One structure, the cerebellum, reports the most significant differences (pairwise Wilcoxon test with Bonferroni correction) encompassing postnatal stages: childhood ($p = 1.06 \times 10^{-28}$), adolescence ($p = 4.71 \times 10^{-47}$), adulthood ($p = 5.54 \times 10^{-148}$); also at birth ($p = 5.64 \times 10^{-7}$) and fetal stage 2 ($p = 4,79 \times 10^{-6}$). Significant differences are also found for the thalamus (fetal stage 3 and adulthood), the hippocampus (fetal stages 1 and 2) or the striatum (fetal stage 3, adolescence and adulthood). The boxplots show the values of the pairwise Euclidean distances at each stage for each structure. The line graph represents the average *p*-value (log2-transformed for representational purposes) for each pairwise comparison between structures at each stage. The horizontal black line denotes $p = 0.01$.

within deserts of introgression compared to those under positive selection in these same regions. We found that, within large deserts of introgression, genes under positive selection have an overall lower expression than those in regions not under positive selection ($p = 0.0007$, Kruskal-Wallis test). A linear regression model predicts that this effect is not structure-specific ($p = 0.655$), and that overall variability in the data is not explained by between-structure differences ($p = 0.9904$, ANOVA test between fitted models that do and do not include brain regions as a variable). Expression linked to specific developmental stages diverges significantly between genes under positive selection and those that are not (0.0001, linear regression). However, a post-hoc TUKEY test (corrected for repeated measures, **Supplementary Figure S11**) reveals that this difference holds only at the fetal stages. In portions of large deserts not under selection, the fetal period of development is significantly different from most posterior stages, while in genes under selective pressures only the first fetal stage is significantly different from post-fetal stages (with a significance threshold of $p < 0.05$).

2.3 Gene-specific Expression Trajectories of Genes in the Overlapping Desertic and Positively-Selected Regions

As described in **section 4**, we included in our analyses any outlier present in the set of genes that are either within the four large deserts of introgression or under putative positive selection within large deserts, due to their potential evolutionary relevance. To evaluate in more detail the expression of specific genes, we focused on the specific trajectories of genes at the intersection of large deserts and positively-selected regions ($n = 12$ genes; **Supplementary Figure S13**), and performed a segmented regression analysis (using the Trendy package (Bacher et al., 2018)) filtering out genes with an adjusted R^2

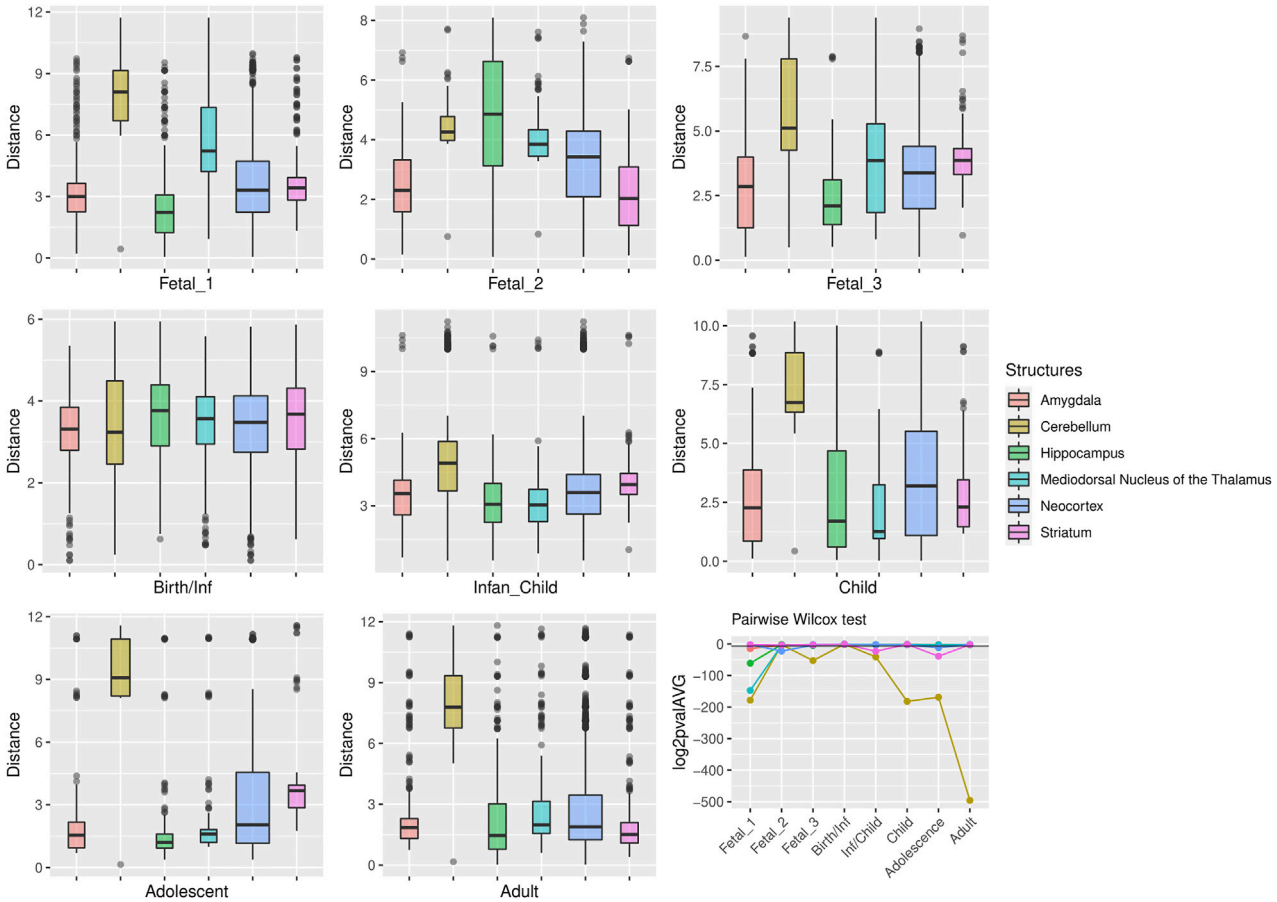


FIGURE 5 | Genes under positive selection within large deserts. The two first principal components were selected to calculate the pairwise comparisons between structures at each stage (Wilcoxon test with Bonferroni correction). Significant results were obtained for the cerebellum more prominently at childhood $p = 1.64 \times 10^{-55}$, adolescence $p = 1.45 \times 10^{-51}$, and adulthood $p = 5.83 \times 10^{-150}$. Prenatally the cerebellum and the thalamus show the most significant differences in comparison to the rest of structures, at fetal stage 1 ($p = 2.1 \times 10^{54}$ and $p = 4.47 \times 10^{-45}$, respectively). Other significant results are found at specific stages for the striatum (infancy and adolescence), neocortex (fetal stage 2), or the amygdala (fetal stages 1 and 3). The horizontal black line in the line plot denotes $p = 0.01$.

less than 0.5. As our analysis showed a marked increase of transcriptomic divergence at different developmental stages for the cerebellum, the striatum and the mediodorsal nucleus of the thalamus, we decided to focus on these structures.

For the cerebellum, *CADPS2* (chromosome 7) expression is the one that most closely mimics the observed pattern, with highest postnatal expression and a marked increased of its expression around birth and infancy (R^2 0.56; see **Supplementary Figure S13** and **Figure 6A**). This Ca^{2+}-dependent activator protein is known to regulate exocytosis in granule cells, particularly neurotrophic factors BDNF and NT-3 release, and its knockout disrupts normal cerebellar development and causes an autistic-like behavioral phenotype in mice (Sadakata et al., 2007; Sadakata et al., 2014). In addition, decreasing expression through developmental stages was also found for *SYT6* and *ROBO2* (chromosome 1 and 3 respectively; R^2 0.76 and 0.60; see **Figure 6A**). Two other genes, *KCND2* and *ST7* (both in chromosome 7), exhibited comparatively high expression postnatally, but did not pass the adjusted R^2 threshold (**Supplementary Figure S13**). Regarding the thalamus, two genes within the overlapping desertic and positively-selected regions could be fitted with an adjusted R^2 higher than 0.5: *ROBO2* and *ST7*. Both genes show higher expressions at prenatal stages, followed by a steady decline at around birth (R^2 0.65 and 0.61, respectively; see **Figure 6B**). The roles of Robo2 in the thalamus have been studied as a receptor of the Slit/Robo signaling pathway which is critically involved in axon guidance. Indeed, Robo2 is highly expressed in the dorsal thalamus and cerebral cortex in the embryonic mouse brain and, in cooperation with Robo1, is required for the proper development of cortical and thalamic axonal projections (López-Bendito et al., 2007).

Lastly, for the striatum, three genes within the overlapping desertic and positively-selected regions could be fitted with an adjusted R^2 higher than 0.5. *ST7*, *ROBO2* and *SYT6* follow a V-shape profile with higher expression at prenatal stages, a decrease around birth, and increasing levels during later postnatal stages (R^2 = 0.75, 0.57, and 0.53, respectively; see **Figure 6C**). While the role of *ST7* in neurodevelopment remains to be elucidated, Robo2 is a receptor of the Slit/Robo

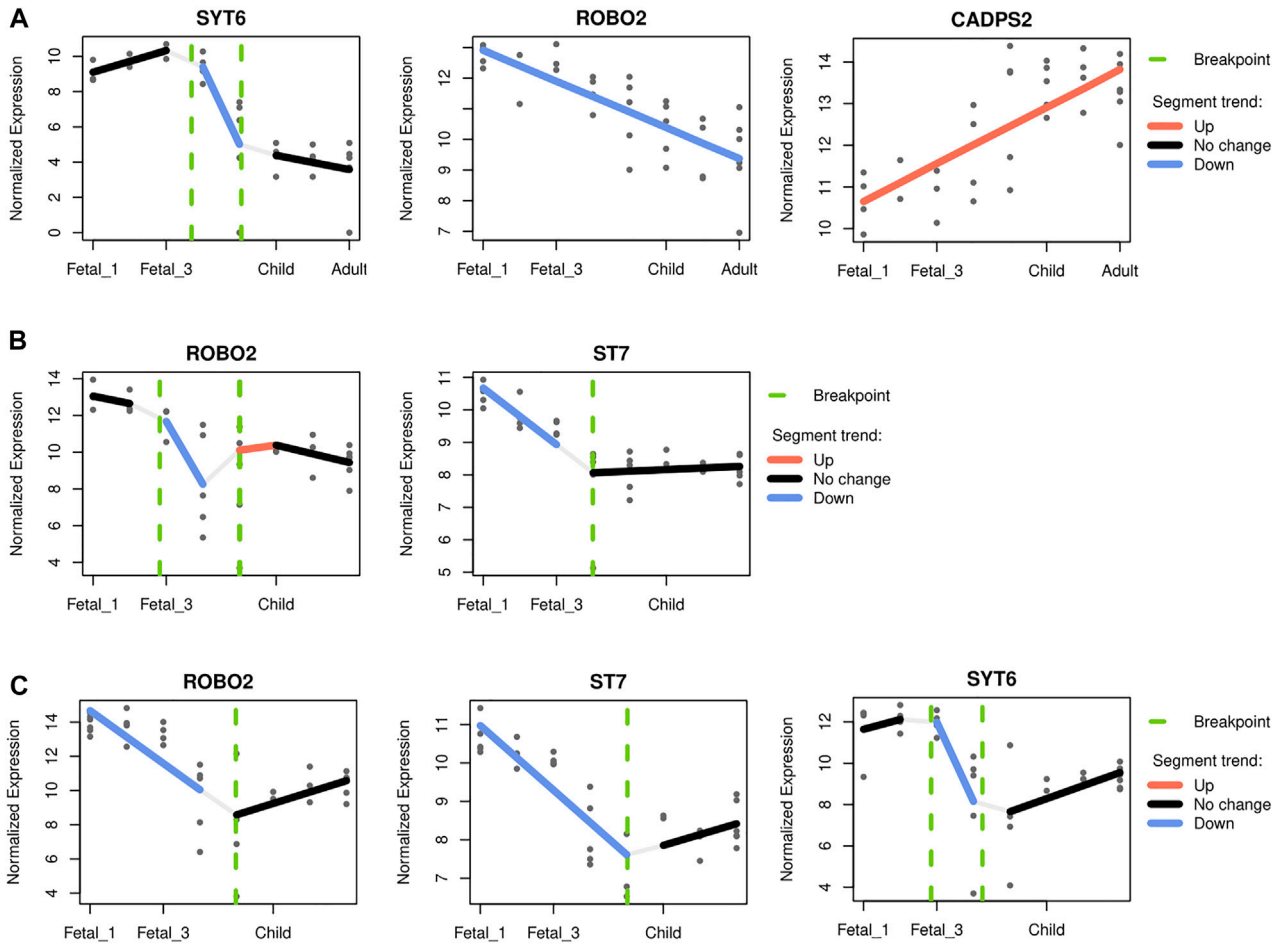


FIGURE 6 | Gene-specific trajectories. A segmented regression analysis (Bacher et al., 2018) was performed to characterize the expression dynamics of genes under putative positive selection within introgression deserts, across developmental stages. Only genes with an adjusted R^2 above 0.5 were considered. **(A)** The cerebellum and the profile of *SYT6*, *ROBO2* and *CADPS2*. The profile of *CADPS2* was found to closely recapitulate the pattern observed for the cerellebum increasing from prenatal to postnatal stages (see **Figure 3**). **(B)** The mediodorsal nucleus of thalamus, and *ROBO2* and *ST7*, both genes with higher prenatal expression declining at around birth. **(C)** The striatum and *ROBO2*, *ST7* and *SYT6*. The three genes follow a similar expression dynamics peaking at early fetal stages but declining afterwards, and increasing again postnatally.

signaling pathway which is critically involved in axon guidance (López-Bendito et al., 2007), but also in the proliferation and differentiation of neural progenitors with possible different roles in dorsal and ventral telencephalon (Andrews et al., 2008; Borrell et al., 2012). *Syt6* is another synapse-related gene expressed in the developing basal ganglia (Long et al., 2009), and in fact linked to the distinctive expression profile of this structure (Konopka et al., 2012). Additionally, *Syt6* shows a similar expression profile in the cerebellar cortex although at lower levels (see **Figure 6C**), a region where *Syt6* has been found, in mice, to be differentially expressed in a *Cadps2* knockout background (Sadakata et al., 2017).

3 DISCUSSION

There are two main findings to take away from our study: the importance of structures beyond the cerebral neocortex in the attempt to characterize some of the most derived features of our species' brain, and the fact that some of the strongest effects in these regions takes place at early stages of development. In this way our work provides complementary evidence for the perinatal globularization phase as a species-specific ontogenic innovation (Gunz et al., 2010), and also provides new evidence for the claim that brain regions outside the neocortex (cerebellum, thalamus, striatum) significantly contribute to this phenotype (Boeckx and Benítez-Burraco, 2014; McCoy et al., 2017; Neubauer et al., 2018; Gunz et al., 2019; Weiss et al., 2021).

To our knowledge this is the first study to reveal the effect of the cerebellum in the context of large introgression deserts. For the striatum, previous studies have already highlighted the relevance of this structure: genes carrying Neanderthal-derived changes and expressed in the striatum during adolescence exhibit a higher McDonald-Kreitman ratio (Mafessoni et al., 2020). In addition, using a different range of introgressed regions and gene expression data from the Allen Brain Atlas (with lower temporal resolution than the database used in this study), it had already

been noted (Vernot et al., 2016) that genes within large deserts are significantly enriched in the striatum at adolescence and adult stages, which converges with the life stages highlighted from our analysis using the most recent report of genomic regions depleted of archaic variants (Chen et al., 2020).

Naturally, the functional effects of these divergent developmental profiles for the cerebellum, the prenatal thalamus or the striatum remain to be understood, particularly in the context of the possible differences among *Homo*-species concerning regulation of the genes highlighted in this study. This is especially relevant in light of emerging evidence that selection against DNA introgression is stronger in regulatory regions (Vilgalys et al., 2021), which in addition have been found to be over-represented in putative positively-selected regions in *Homo sapiens* (Peyrégne et al., 2017; Petr et al., 2019). The fact that early developmental stages are critical holds the promise of using brain organoid technology to probe the nature of these differences, since such *in vitro* techniques best track these earliest developmental windows (Muchnik et al., 2019; Mostajo-Radji et al., 2020; Kyrousi and Cappello, 2020). Our level of analysis (mRNA-seq data, informed by paleogenetic studies) can be complemented with other *omics* data to finely resolve cell-type specificities of the genes considered here across brain areas, as with the use of single-cell RNA-seq data, or to infer gene regulatory networks (from differentially accessible and methylated regions and chromatin immunoprecipitation data) that underlie the divergent gene expression trajectories observed.

The fact that FOXP2 expression is known to be particularly high in the brain regions highlighted here (Lai et al., 2003) may help shed light on why *FOXP2* is found in one of the large introgression deserts in modern human genomes. As pointed out in (Kuhlwilm, 2018), this portion of chromosome 7 is not a desert for introgression in other great apes, nor did it act as a barrier for gene flow from Sapiens into Neanderthals. As such, it may indeed capture something genuinely specific about our species.

4 METHODS

Analyses were performed using R (R Core Team, 2019). Putative positively-selected regions were retrieved from the extended set of sweep regions in Peyrégne et al. (2017), built from two independent recombination maps using a Hidden Markov-based model applied to African and Neaderthal/Denisovan genomes. Coordinates for (large) deserts of introgression were retrieved from Chen et al. (2020), and genes within these two sets of regions were obtained using the BioMart R package version 2.42.1 (Durinck et al., 2009), using the respective genomic region coordinates as input and filtering by protein-coding genes.

mRNA-seq analysis. Publicly available transcriptomic data of the human brain at different developmental stages was retrieved from (Li et al., 2018) and analyzed using R (full code can be found at https://github.com/jjaa-mp/desertsHomo). Reads per kilo base per million mapped reads (RPKM) normalized counts were log-transformed and then subsetted to select genes either in large deserts of introgression or in both deserts and putative positively-selected regions. The complete log-transformed, RPKM normalized count matrix was subsetted to select genes with median expression value > 2, as in (Li et al., 2018), while no median filtering was employed for the subsets of genes within deserts and positively-selected regions, due to the potential relevance of the outliers in these specific regions for the purposes of our study. To assess transcriptomic variability between brain regions accounted for by genes either in large deserts or in deserts and positively-selected regions, we performed principal component analysis and calculated the pairwise Euclidean distances between brain regions for each dataset [following (Li et al., 2018)]. We then statistically evaluated such differences at each developmental stage using pairwise Wilcoxon tests with Bonferroni correction. Significant differences were considered if $p < 0.01$. Our analysis based on statistically significant principal components did not make it possible for us to use the Allen Brain Atlas data for comparisons with the psychENCODE project dataset used in this study, due to the more limited resolution, especially at prenatal stages, offered by the former.

To evaluate the expression profile of genes from our regions of interest in comparison to other regions of the human genome, we generated sets of random regions of the same length and gene density (that do not overlap with the genomic coordinates of deserts on introgression). These served as control regions for comparisons of mean expression values using two-way repeated measures ANOVA, implemented in R. ANOVA tests were performed taking mean expression values as dependent value, with structure names as subject identifiers and the different regions of interest (datasource) as between-subjects factor variable. Posthocs tests were performed similarly but with the mean expression data grouped by the datasource, obtaining an ANOVA table for each structure, with a Bonferroni correction to account for repeated measures. The stage-version of the ANOVA grouped subject identifiers by stage. Two Kruskal-Wallis tests were used, one designed to detect whether non positively-selected genes in deserts of introgression have different mean expression levels than genes that are both in deserts and in positively-selected windows; and the second to determine whether any particular brain structure has a particularly different expression mean than the rest, regardless of selection. We also used two two-level linear mixed-effects regression models, to compare non-positively selected genes and positively selected genes within introgression deserts. These models consist of repeated measures of expression on different brain structures in three different groups: control, deserts of introgression, and deserts with selection signals. The same model applies when stages are taken into account, replacing structure identifiers. Tukey's test was then used to fit the model.

Gene-specific expression trajectories. The R package Trendy version 1.8.2 (Bacher et al., 2018) was used to perform segmented regression analysis and characterize the expression trajectories of genes within both deserts of introgression and putative positively-selected regions (12 genes). The normalized RPKM values [from (Li et al., 2018)] in the form of a gene-by-time samples matrix was used to fit each gene expression trajectory to an optimal segmented regression model. Genes were considered if their

adjusted R^2 was >0.5. In addition, a maximum number of breakpoints (significant changes in gene expression trajectory) was set at 3, minimum number of samples in each segment at 2, and minimum mean expression, 2.

The permutation tests using gene expression data from (Li et al., 2018) were done using the regioneR package version 1.26.1 (Gel et al., 2016) at n = 1,000.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://github.com/jjaa-mp/desertsHomo.

AUTHOR CONTRIBUTIONS

Conceptualization: CB, AA, JM and RB; Data Curation: AA, JM and RB; Formal Analysis: AA, JM and RB; Visualization: CB, AA, JM and RB; Writing—Original Draft Preparation: CB, AA, JM, RB; Writing—Review and Editing: CB, AA, JM and RB; Supervision: CB; Funding Acquisition: CB.

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Vascular Regulation of Developmental Neurogenesis

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Evolutionary studies indicate that the nervous system evolved prior to the vascular system, but the increasing complexity of organisms prompted the vascular system to emerge in order to meet the growing demand for oxygen and nutrient supply. In recent years, it has become apparent that the symbiotic communication between the nervous and the vascular systems goes beyond the exclusive covering of the demands on nutrients and oxygen carried by blood vessels. Indeed, this active interplay between both systems is crucial during the development of the central nervous system (CNS). Several neural-derived signals that initiate and regulate the vascularization of the CNS have been described, however less is known about the vascular signals that orchestrate the development of the CNS cytoarchitecture. Here, we focus on reviewing the effects of blood vessels in the process of neurogenesis during CNS development in vertebrates. In mammals, we describe the spatiotemporal features of vascular-driven neurogenesis in two brain regions that exhibit different neurogenic complexity in their germinal zone, the hindbrain and the forebrain.

Keywords: neurogenesis, blood vessels, neurovascular communication, hindbrain, forebrain

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INTRODUCTION

In the course of phylogenic evolution, the nervous system precedes the appearance of the vascular system. The first organisms that developed a primitive nervous system were diploblasts, i.e., cnidarians (jellyfish, anemones, corals) and ctenophores (jelly comb) (Hartenstein and Stollewerk, 2015; Arendt et al., 2016). These animals exhibit a diffuse nerve net and lack a vascular system, as oxygen and nutrient demands can be met by simple diffusion (Monahan-Earley et al., 2013). Millions of years later, more complex organisms emerged, the triploblasts, which have bilateral symmetry and a tubular nervous system. With their increased complexity and body size, it became necessary to develop a circulatory system to transport fluid throughout the whole organism. This incipient circulatory system evolved into a blood vascular system.

Interestingly, the nervous system also precedes the vascular system during embryogenesis. The neural tube, the origin of the central nervous system (CNS) in bilaterian animals, is formed by neuroepithelial cells derived from the ectoderm (Hartenstein and Stollewerk, 2015) and is avascular *ab initio* (James et al., 2009). In vertebrates, neuroepithelial cells that form the neural tube initially undergo symmetric divisions in synchrony with interkinetic nuclear migration [see reviews (Miyata, 2008; Taverna and Huttner, 2010)]. This process of cell proliferation serves to amplify the pool of progenitor cells before the onset of neurogenesis (Subramanian et al., 2017). Next, from embryonic day (E) 10.5 onwards in mouse (Haubensak et al., 2004), neuroepithelial cells divide asymmetrically to generate radial glial cells (RGCs). RGCs are neuronal progenitor cells with cell-renewal and

neurogenic capacity (Malatesta et al., 2000; Noctor et al., 2001), representing the cellular source for almost all neural lineages of the CNS [see review (Gotz and Huttner, 2005)]. RGCs are morphologically similar to neuroepithelial cells, although they are more elongated [see review (Arai and Taverna, 2017)]. Both cell types exhibit apical-basal polarity and span their processes across the neural tube. The apical end-feet contact the lumen of the neural tube whereas their basal fiber anchors at the pial surface. Moreover, RGCs are not only the source of neuronal progenitors and neurons, but the basal processes of these cells are also used as scaffolds for the migrating newborn neurons (Rakic, 1971; Nadarajah and Parnavelas, 2002).

Neurovascular interactions happening before the onset of neurogenesis have not been described; however, following the closure of the neural tube, angioblasts (endothelial progenitor cells) are recruited from the pre-somitic mesoderm to surround the neural tube with a vascular mesh, termed perineural vascular plexus (PNVP) (Kurz et al., 1996; Ambler et al., 2001). These primitive vessels then sprout radially into the neural tube developing a ramified vascular network, called intraneural vascular plexus (INVP). In mouse, PNVP establishment occurs between E8.5 and E10, followed by the emergence of INVP at around E10.5 [see reviews (James and Mukouyama, 2011; Segarra et al., 2019)], therefore the onset of neurogenesis is timely harmonized with neural tube vascularization. Moreover, initial experiments in chick embryos showed a stereotypical pattern in the formation of the INVP (Feeney and Watterson, 1946), indicating that sprouting angiogenesis into the primitive neural tube is guided by neural-derived cues. This hypothesis was demonstrated later, and neural-derived vascular endothelial growth factor A (VEGF-A) was shown to be one of the major signals orchestrating neural tube vascularization (Hogan et al., 2004; James et al., 2009).

In the developing CNS, vessels establish an intimate relationship with RGCs. On one side, the vascular sprouts of the INVP align with the RGCs processes (Noctor et al., 2001; Gerhardt et al., 2004; Ma et al., 2013). On the other side, the long basal processes of the RGCs contact the pial surface irrigated by vessels of the PVNP while RGC somatas and their short apical processes lie on the ventricular side of the neural tube, where the INVP further develops and forms the periventricular plexus (PVP) [see review (Peguera et al., 2021)]. Developmental neurogenesis is a multi-step process that encompasses several waves of cell division, followed by migration and differentiation of neuroblast cells, and culminates with the integration of mature neurons into the neural circuit (Bjornsson et al., 2015). The vasculature, which intermingles and develops symbiotically with the growing CNS, may help to guide and coordinate the different stages of neurogenesis.

Communication between vessels and neural cells is bidirectional. Several studies have deciphered which neuronal cues regulate CNS vascularization [reviewed in (Paredes et al., 2018)], but less is known about the instructive role of endothelial cells in modulating neuronal processes. Emerging data reveal that the vasculature plays a functional role in CNS development, including embryonic neurogenic niches. In this review, we focus specifically on the influence of the vasculature along the neurogenic journey and its potential control of neuroblast cell division, migration and differentiation during CNS development.

LESSONS FROM *IN VITRO* SYSTEMS

Co-culture systems of neural stem cells (NSCs) and endothelial cells have helped to demonstrate the influence of the endothelium on the neurogenic niche (Shen et al., 2004; Sun et al., 2010; Vissapragada et al., 2014). Most of these studies suggest that endothelial cells induce the proliferation of NSCs to increase the undifferentiated pool of neurons. For example, Shen et al. showed that soluble factors secreted by endothelial cells promote the symmetric division of NSC, whereas NSC undergo differentiation in the absence of endothelial cells (Shen et al., 2004). Also, an enhanced proliferation of NSC was observed when neuronal progenitors were co-cultured with embryonic brain endothelial cells from PVP origin, therefore reproducing the embryonic neurogenic niche *in vitro* (Vissapragada et al., 2014). Interestingly, variations in the co-culture conditions could trigger different effects on the neuronal progenitors: whereas soluble factors led to NSCs self-renewal, direct contact with the endothelium promoted neuronal progenitor cell differentiation (Gama Sosa et al., 2007). This divergent response provides an indication of the complexity of the neurogenic dynamics *in vivo* and the diversity of signaling mechanisms that may be derived from the interplay with the endothelium.

VASCULAR-GUIDED NEUROGENESIS IN NON-MAMMALIAN VERTEBRATES

Non-mammalian models have been crucial in the study of neuronal development (Marder, 2002). In zebrafish, the avascular mutant *cloche* is a powerful model to investigate the neurovascular interactions during development. *Cloche* zebrafish have a dysfunctional heart, which impairs blood circulation, as well as lack blood cells and most of the vasculature (from 20 to 26-somite stage) (Stainier et al., 1995; Liao et al., 1997). In the hindbrain, blood vessels develop in close association with subsets of neuronal clusters in early stages (48–72 h post-fertilization, hpf), but the absence of vessels in *cloche* has no impact on local neurogenesis (Ulrich et al., 2011). However, in other brain regions at the same developmental stage, such as the cerebellum's upper rhombic lip and the optic tectum, the axonal scaffolds were reduced in *cloche*, presumably because their development requires blood flow and/or signals from the surrounding vessels (Ulrich et al., 2011). Interestingly, Taberner et al. demonstrated using *cloche* mutants that blood flow is necessary for cranial sensory neural differentiation (54–72 hpf) in the statoacoustic ganglion via activation of genes related to oxygen metabolism (Taberner et al., 2020). Besides blood flow, blood-borne signals may potentially influence the neurovascular niche during development. Indeed, in *Xenopus laevis*, neuronal progenitors that line the ventricle and extend their radial processes to establish contact with the pial surface are able to internalize circulating dextran through their end-feet (Lau et al.,

2017). However, no relationship was found between neural progenitor's end-feet-blood vessel contacts and their cell division rate.

Direct contact between endothelial cell and neural progenitor also seems to regulate neuronal development. In early cranial sensory neurogenesis of the statoacustic ganglion in zebrafish (30–36 hpf), direct interaction of endothelial cells and neuronal progenitors regulate their proliferation (Taberner et al., 2020). Thus, loss of vasculature in *cloche* correlates with a neuroblast increase in this region, indicating that endothelium-neuroblast contacts negatively regulate neurogenesis by keeping neuroblasts quiescent. Those contacts are mediated by the endothelial cell cytoneme, a thin actin-based cellular extension specialized for cell-cell communication that binds to cranial sensory neuroblasts and communicates via Dll4-Notch signaling pathway (Taberner et al., 2020).

In zebrafish retina, *cloche* mutants also lack vasculature and show prominent defects in cell proliferation, survival, organization and differentiation (30–72 hpf) (Dhakal et al., 2015). These defects in retinogenesis were independent from hypoxia, but *cloche* mutants did not allow to differentiate the role on retinal neurogenesis between endothelial cells, blood-borne factors and/or circulating blood cells. To address this, Dhakal et al. used three different mutant models characterized by: 1) absence of endothelial cells, 2) lack of blood flow and 3) no erythroid lineage cells. This strategy revealed that factors derived directly from endothelial cells are major key players in cell proliferation and differentiation in the retina; although circulating factors might also play a role in these processes (Dhakal et al., 2021). Interestingly, the ciliary marginal zone, where the retinal neurogenic niche resides, is severely affected in the absence of endothelial cells (Dhakal et al., 2021). Consistent with this, blood vessels associated with retinal stem cells in the ciliary marginal zone were shown to be required to maintain them in proliferative stages (Tang et al., 2017). In the developing rat retina, *in vitro* and *in vivo* studies also support that endothelial cells regulate the cell self-renewal of retinal progenitor cells via the epigenetic regulator *Hmga2* (Parameswaran et al., 2014).

Taken together, all data suggest that the role of the vasculature in neurogenesis is very variable depending on the region and developmental stage. Blood vessels may govern diverse mechanisms leading to different responses, from the balance between proliferation and quiescence to differentiation.

CONTRIBUTION OF THE VASCULATURE TO DEVELOPMENTAL NEUROGENESIS IN MAMMALS

Vascular regulation of developmental neurogenesis has been studied in the neurogenic niches of the hindbrain and the forebrain in the embryonic mouse (Karakatsani et al., 2019). The hindbrain gives rise to the cerebellum, pons, and medulla oblongata; whereas the forebrain differentiates into the diencephalon and the telencephalon, which generates neurons that populate the vast neocortex and the subcortical structures (such as hippocampus and basal ganglia).

The hindbrain is the most functionally and developmentally conserved region in the evolution of the vertebrate brain (Krumlauf and Wilkinson, 2021). In contrast, the evolution of the neocortex across vertebrates is variable and shows differences in tissue structures, for example number of neocortical layers (Briscoe and Ragsdale, 2019). Cortical neurogenesis is evolutionary conserved in mammals; however, the cerebral cortex is also characterized by a wide variability in volume and folding complexity across species. This could be related to a prolonged neurogenic period that correlates with the duration of gestation, exposing the developing neocortex to maternal environment for a longer period of time. This includes a whole variety of circulating factors, such as hormones, that are delivered by the blood vessels and the cerebrospinal fluid system and potentially influence neurogenesis (Montiel et al., 2013; Stepien et al., 2021).

Neurogenesis in the Developing Hindbrain

In the hindbrain, vessels from the PNVP (which later becomes the meningeal vasculature) penetrate radially into the neural tissue towards the ventricular zone, where they turn and anastomose to form the PVP at around E10 and onwards (**Figure 1A**) (Fantin et al., 2013). Subsequently, lateral sprouts emerge and anastomose to form a more complex plexus.

Interestingly, direct neurovascular contacts are described in the germinal zone of the hindbrain surrounding the ventricle. Confocal microscopy and 3D reconstructions suggested that hindbrain neural progenitor processes and end-feet directly contact PVP blood vessels (Tata et al., 2016). In support of a neurovascular communication, a spatiotemporal congruency was found between the sprouting of vessels in the PVP and the peak of neural progenitor proliferation. Moreover, endothelial deletion of Neuropilin1 (NRP1), a co-receptor of VEGF-A, resulted in premature differentiation of neural progenitor independent of VEGF signaling and hypoxia (**Figure 1B**) (Tata et al., 2016). This suggests that PVP vasculature directly regulates neurogenesis.

The hindbrain is the premise of the cerebellum, which mostly develops postnatally. In the cerebellum, glutamatergic neurons called granule cells originate in the upper rhombic lip. During embryonic development, granule cells proliferate and migrate anteriorly to cover the entire dorsal cerebellar surface, where they create a postnatal secondary neurogenic niche, the external granule layer (EGL) (Consalez, 2021). This migration process is mediated by the interaction of C-X-C motif chemokine 12 (CXCL12), expressed by the leptomeninges, and its receptor CXCR4, expressed by the migrating progenitor cells (Zhu et al., 2004; Hagihara et al., 2009). Later, CXCL12 signaling is suggested to arrest neuronal progenitors at the pial surface in the EGL (Zhu et al., 2004; Vilz et al., 2005; Consalez, 2021). At perinatal stages, the EGL actively proliferates in mice until the third postnatal week. During the first postnatal week (**Figures 1C,D**), the cerebellar cortex is poorly vascularized, resulting in low O_2 tension that increases expression of the hypoxia-inducible factor *Hif1α* (Kullmann et al., 2020). HIF1α on the one hand, negatively regulates the partitioning-defective (*Pard*) gene complex via *Zeb1*, which prevents granule cell polarization and consequent migration and, on the other hand, promotes the attachment of the proliferating granule cell progenitors

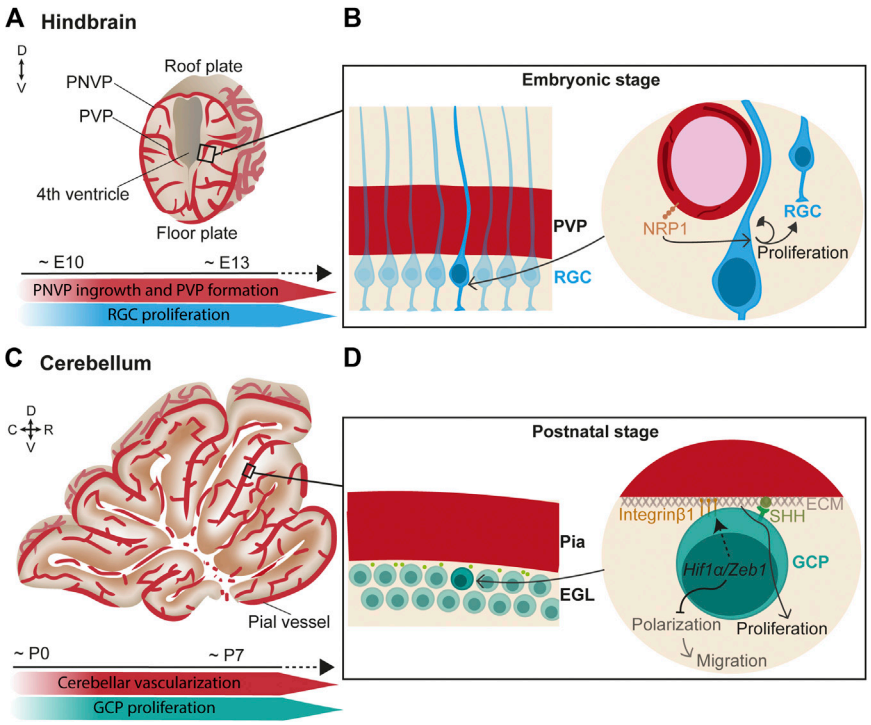

FIGURE 1 | Endothelial cell signaling regulates neurogenesis in the hindbrain/cerebellum. **(A)** (Top) Scheme of a coronal view of the hindbrain at the embryonic stage. Vessels from the perineural vascular plexus (PNVP) ingress into the neural tissue in a perpendicular manner towards the ventricle where they form the periventricular vascular plexus (PVP). Then, lateral sprouts emerge and form a more complex vascular network. (Bottom) Timeline of concurrent hindbrain embryonic vascularization and radial glia cells (RGC) proliferation. **(B)** (Left) Closer view of the ventricular zone containing a layer of radial glial cells (RGC) that extend their basal fibers towards the pial surface and the apical short processes contact the ventricle. (Right) RGC basal fibers directly contact the PVP vessels. In this scenario, endothelial NRP1 signaling maintains RGC proliferation through a mechanism independent of hypoxia and VEGF. **(C)** (Top) Scheme of a sagittal view of the cerebellum at the first postnatal week. (Bottom) Timeline of concurrent cerebellar postnatal vascularization and granule cell progenitors (GCP) proliferation. **(D)** (Left) Granule cell progenitors (GCP) reside under the pial vessels in the cerebellum and form the external granule layer (EGL). (Right) In hypoxic conditions, *Hif1α/Zeb1* favors the GCP attachment to the extracellular matrix (ECM) of the pial surface through Integrin β1 expressed at the GCP membrane. In parallel, *Hif1α/Zeb1* inhibit GCP polarization and subsequent migration. Components of the pial ECM (laminins) enhance SHH signaling which, in turn, promotes GCP proliferation.

to the pial extracellular matrix via Integrin β1, which keeps them in the germinal zone (**Figure 1D**) (Kullmann et al., 2020). Moreover, components of the extracellular matrix on the pial side of the EGL, particularly laminins, enhance the response to sonic hedgehog (SHH), the best-studied morphogen that induces granule cell proliferation (Pons et al., 2001; Consalez, 2021) (**Figure 1D**). As cerebellar vascularization progresses, *Hif1α* expression is downregulated, and granule cells can detach from the pia and prepare for migration (Kullmann et al., 2020). Granule cells extend their axons while migrating. In this process, the interaction of Discoidin domain receptor 1 (DDR1) expressed in the granule cells with collagen secreted at the pial surface is essential for their axonal formation (Bhatt et al., 2000).

All in all, although relief from hypoxia is an important factor controlling progenitor cell division, other vascular-mediated signaling pathways directly contribute to hindbrain/cerebellum neurogenesis.

Neurogenesis in the Developing Forebrain

Located on the edge of the telencephalic lateral ventricles, RGCs divide symmetrically or asymmetrically to expand the pool of progenitor cells, giving rise to either two RGCs or one RGC and one intermediate progenitor cell (IPC), respectively (see review (Taverna et al., 2014)). Progenitor cells continue to divide asymmetrically to give rise to neurons. The forebrain germinal zone is layered in two: the ventricular zone (VZ) where RGC somatas reside and the sub-ventricular zone (SVZ), above the VZ, where the newly born IPC accumulate from E12.5 (Paridaen and Huttner, 2014; Bjornsson et al., 2015). In some species (e.g., humans), the SVZ highly amplifies the pool of progenitors and is considered to be the evolutionary basis for neocortex expansion. Dorsal and ventral telencephalon give rise to excitatory and inhibitory neurons respectively, and both of these telencephalic regions exhibit a VZ and SVZ.

Simultaneously to the neurogenic process, forebrain vascularization starts ventrally and progressively extends towards the dorsal forebrain (Lange et al., 2016; Karakatsani et al., 2019; Puelles et al., 2019) (**Figure 2A**). Vessels grow from the PNVP towards the ventricle following a spatiotemporal pattern. Penetrating vessels invade the ventral forebrain already at around E10.5 whereas sprouts in the dorsal region are delayed about 1 day (Lange et al., 2016; Segarra et al., 2018).

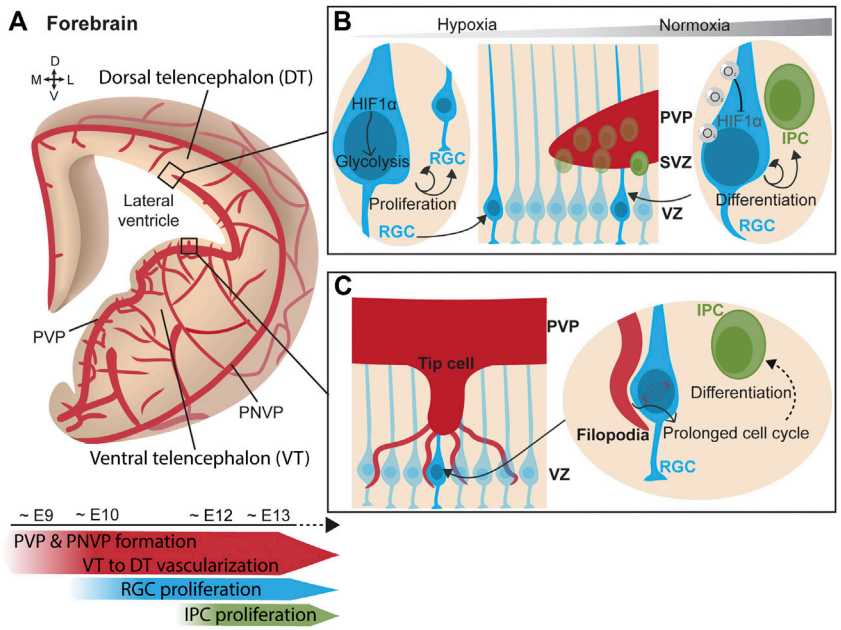

FIGURE 2 | Vasculature influences neurogenesis in the forebrain. **(A)** (Top) Scheme of a coronal view of the forebrain at the embryonic stage, showing its vascularization from the perineural vascular plexus (PNVP) and the periventricular vascular plexus (PVP) in a progressive manner from the ventral telencephalon towards the dorsal telencephalon. (Bottom) Timeline of concurrent telencephalic embryonic vascularization and neurogenesis. **(B)** (Middle) Ventricular zone (VZ) contains a layer of radial glial cells (RGC), which generate intermediate progenitor cells (IPC) that form the subventricular zone (SVZ). (Left) In a poorly-vascularized and hypoxic environment, RGCs express HIF1α which triggers glycolysis and RGC proliferation. (Right) As oxygenation progresses with increased vascularization, RGC adapt to hypoxia relief. Oxygen supply from blood vessels mediates HIF1α degradation and promotes RGC asymmetric division generating IPCs. **(C)** (Left) Tip cells from ingrowing PVP blood vessel extend filopodia that directly contact RGC somatas in the VZ. (Right) Endothelial cell filopodia adhere to RGC. This direct contact prolongs the mitotic phase of cell division in RGC and favors early neuronal differentiation.

Unlike the hindbrain, an additional angiogenic source vascularizing the PVP has been identified in the forebrain. The PVP vascularization originates from a basal vessel at the telencephalic floor that branches from the basal ganglia primordium (Vasudevan et al., 2008). This vascular plexus encompassing the ventricle begins in the ventral telencephalon around E9 and progresses ventral-to-dorsal and lateral-to-medial between E10-E11 towards the dorsal telencephalon, merging simultaneously with the penetrating sprouts from the PNVP (Vasudevan et al., 2008). Confocal microscopy and 3D reconstruction of telencephalon slices show that blood vessels are omnipresent in the telencephalic neurogenic niches and form a rich PVP. This has also been observed in humans, where the ventral telencephalon is vascularized at early mid-gestation (Di Marco et al., 2020). Altogether, these findings strongly suggested that blood vessels play a critical role in embryonic neurogenic niches.

Initially blood vessels were solely described as providing a nutrients and oxygen to tissues. Following this idea, the putative contribution of blood vessels to neurogenesis was first explored through the prism of oxygenation and circulating factors. Haigh et al. elegantly laid the groundwork by inducing telencephalic devascularization and hypoxia after deleting neuronal VEGF-A, resulting in a decreased neuronal proliferation in the VZ-SVZ (Haigh et al., 2003). Vascular-specific *Gpr124* KO mice exhibit hypoxia in the VZ region. The consequent induction of *Hif1α* in this mouse model was found to maintain the proliferative state of RGCs by triggering glycolysis and to inhibit their neuronal differentiation (Lange et al., 2016). In other words, in the early stages of development, the supply of oxygen through blood vessels is poor, which makes neurogenic niches highly hypoxic. Hypoxia maintains RGCs in a proliferative state and stimulates angiogenesis. When the tissue becomes more vascularized, hypoxia is alleviated and HIF1α signaling downregulated. This results in reduction of neural progenitor cell expansion and in their differentiation into IPCs (Bjornsson et al., 2015; Lange et al., 2016) (**Figure 2B**). This effect of hypoxia is reminiscent to the observations in the postnatal cerebellum, since in both regions the increase in vascularization regulates neuronal differentiation. While hypoxia was the first indication of a vascular contribution to embryonic neurogenesis, it became clear that the vascular system regulates neural progenitors using other strategies.

Endothelial cells are capable of secreting factors, called angiocrines, that are crucial for regulating neurogenesis. For instance, conditionally deleting *Vegf* in endothelial cells led to several developmental defects in the embryonic telencephalon (Li et al., 2013), such as: impaired angiogenesis, abnormal localization of proliferating neuronal progenitors outside of the neurogenic niche of the dorsal telencephalon, disrupted radial glia scaffold, and defective radial migration of post-mitotic pyramidal neurons. Moreover, the tangential migratory stream

of inhibitory neurons towards the dorsal telencephalon was also altered by the endothelial deletion of *Vegf*, leading to focal misplacement of neurons in the marginal zone even above the pial surface, disturbed axonal tracts, and defective cortical lamination (Li et al., 2013). Reelin is a neuronal guidance cue secreted by Cajal-Retzius cells in the marginal zone of the neocortex (Tissir and Goffinet, 2003). Deletion of the Reelin effector molecule *Dab1* in endothelial cells also resulted in several defects in forebrain cytoarchitecture, such as invasion of migrating pyramidal neurons in the marginal zone, altered positioning of neurons in the cortical layers, and disrupted adhesion of RGC processes to the pial surface (Segarra et al., 2018). However, no defects were detected in the pool of neuronal progenitors in the germinal zone, suggesting that lack of Reelin signaling in the endothelial cells preferentially impacts on neurovascular interactions at the pial surface rather than at PVP vasculature. Indeed, endothelial cells from the PVP exhibit differential gene expression compared with cells from the pial surface, suggesting that the endothelium selectively guides the tangential migration of inhibitory interneurons along the pial or the periventricular streams (Won et al., 2013). Furthermore, secretion of the neurotransmitter Gamma-Aminobutyric Acid (GABA) by endothelial cells contributes to long-distance tangential migration of inhibitory interneurons from the ventral telencephalon to their final position in the neocortex (Li et al., 2018). Deletion of endothelial GABA release not only disrupted the tangential migration of interneurons, but also increased the number of proliferating progenitor cells in the SVZ of the ventral telencephalon. In addition, RNA sequencing revealed dysregulation of crucial neurogenesis-related genes when endothelial-specific GABA secretion was deleted embryonically (Li et al., 2018).

Javaherian and Kriegstein observed that IPCs, which express the marker Tbr2, were preferentially distributed along the developing blood vessels in the SVZ. Via VEGF-overexpression after *in utero* electroporation, they induced the overgrowth of blood vessels and triggered the aberrant migration of $Tbr2^+$ cells towards the ectopic blood vessels. Moreover, mitotic progenitors were preferentially located to branch points, where tip cells are present during branching morphogenesis (Javaherian and Kriegstein, 2009). Tip cells are specialized endothelial cells that extend filopodia to sense migratory guidance cues in their environment and mediate new contacts (Gerhardt et al., 2003). These findings suggested that IPC interact with blood vessels by contacting tip cells. Ten years later, Di Marco *et al.* elegantly confirmed this hypothesis by describing direct contacts between vascular tip cell filopodia and apical neural progenitors of the lateral ganglionic eminence in both mouse and human embryos (**Figure 2C**). In the same study, and thanks to series of cell birth-dating experiments in mouse models with enriched and depleted vascular filopodia, endothelial cell filopodia were shown to extend the mitotic phase of RGCs and this triggered an earlier neural differentiation while limiting the amplification of the pool of progenitor cells (Di Marco et al., 2020). In addition, RGCs establish direct contacts with the periventricular vasculature via their apical end-feet in the ventral telencephalon. Tan et al. reported that the anchorage of RGC end-feet to periventricular blood vessels is mediated by Integrin β1 (Tan et al., 2016). Deletion of Integrin β1 specifically in RGCs halved the anchoring of the end-feet and reduced the number of mitotic RGC in the VZ of the medial ganglionic eminence. Interestingly, Integrin β1-mediated RGC anchoring was critical in defining the proportion of parvalbumin and somatostatin interneurons, the two major types of neocortical interneurons (Tan et al., 2016). Thus, the vasculature of the neurogenic niche is able to regulate the proliferation state of RGCs via direct cell-cell contacts. Moreover, Integrin β1 is also required for the attachment of basal RGC processes to the pial surface (Graus-Porta et al., 2001) by binding to laminins on the meningeal surface (Radakovits et al., 2009). While anchoring of RGC end-feet to pial vessels is not required for RGC proliferation, it is crucial for radial migration of excitatory neurons and possibly their differentiation (Haubst et al., 2006). Consistent with this, deletion of *Dab1* in endothelial cells impaired the deposition of Laminin-α4 on the vasculature, which disrupted the binding of RGC processes via Integrin β1 and, consequently, altered the proper positioning of pyramidal neurons in the neocortical layers (Segarra et al., 2018).

At late embryonic stage the VZ decreases in size while the SVZ expands, and this increase continues perinatally (Brazel et al., 2003). The SVZ located at the anterior part of the lateral ventricle gives rise to neuroblasts that migrate along the rostral migratory stream to the olfactory bulbs. Remarkably, neuroblasts generated postnatally in the SVZ prematurely leave the rostral migratory stream and migrate towards the cortex using cortical blood vessels as scaffolds. In this way, a fraction of GABAergic interneurons is added to the lower cortical layers (Le Magueresse et al., 2012). Moreover, at early postnatal stages vessels progressively align longitudinally along the developing rostral migratory stream and, interestingly, neuroblast proliferation was significantly associated with the vicinity of vessels (Nie et al., 2010).

The meninges, which are initially vascularized by the PNVP and become highly irrigated by the leptomeningeal vessels during development, support the tangential migration of the Cajal-Retzius cells via CXCL12/CXCR4 interactions during embryonic development (Borrell and Marín, 2006). In addition to providing extracellular matrix components, metabolites, and growth factors that regulate neurogenesis (Siegenthaler et al., 2009; Choe et al., 2012), meninges have been shown to harbor cells that express neural precursor markers during development, suggesting that meninges may themselves represent a neurogenic niche (Bifari et al., 2015; Nakagomi and Matsuyama, 2017). Neuronal progenitors in the meninges are generated during embryonic development. They have characteristics resembling RGCs and migrate perinatally into the brain parenchyma where they differentiate into cortical neurons (Bifari et al., 2017). These meningeal neuronal progenitors migrate from the leptomeninges through the meningeal substructures below the hippocampus towards the lateral ventricle. The meningeal-derived neuroblasts maintain a close association with the vasculature during this journey, although a direct signaling from the vasculature remains to be elucidated.

CONCLUDING REMARKS

Neurogenesis is the driving force behind CNS development. This process does not only respond to intrinsic signals from neuronal progenitors but it is also governed by the influence of the cellular milieu in the germinal zones, of which the endothelial cells are an important component. Indeed, several animal models with vascular deficits support the notion that perturbations in the vasculature have an impact on the neurogenic process. Hypoxia produced by insufficient vascularization modulates the expansion versus the differentiation of the pool of progenitors. Interestingly, vessels also exert an active role in neurogenesis, either by directly contacting neuronal progenitors or by releasing factors that modulate neurogenesis. A spatiotemporal analysis of putative molecular players in the course of neurogenesis would be relevant since unique pathways can be involved in different neurogenic niches throughout brain development. Furthermore, vascular heterogeneity could play a role in directing neurogenesis, considering that endothelial cells from PVP and PVNP express different genes (Won et al., 2013), and even transcriptional differences were found among dorsal and ventral vessels from the PVP (Vasudevan et al., 2008).

Furthermore, vessels act as conduits of blood-borne substances. These substances can reach the neurogenic niches if they are permeable to the blood-brain barrier, which is formed at embryonic stages (Daneman et al., 2010). Moreover, the choroid plexus is a vascularized structure that develops in the ventricles concomitantly to developmental neurogenesis. The choroid plexus releases molecules into the embryonic cerebrospinal fluid (CSF), which is known to contain a myriad of factors involved in neurogenesis (see review (Fame and Lehtinen, 2020)). These molecules have to cross the blood-CSF barrier to reach the ventricles. Therefore, neurogenesis can also be regulated by selective transport of molecules through the barriers within the CNS, however this field of research still remains poorly explored. In addition, it has to be considered that blood circulating maternal factors also influence the embryonic neurodevelopment in mammals.

All in all, these findings open the possibility that some neurodevelopmental defects may originate in the vascular system, either indirectly through deficits in oxygen and molecule delivery, as observed in preterm infants, or through direct endothelial-mediated signaling. In this regard, it has been observed that prematurely born rabbits exhibit an excessive pool of interneuron progenitors in the ganglionic eminence and this can be reversed by treatment with the blood-borne hormone estrogen (Tibrewal et al., 2018). Moreover, Zika virus infection in mice has been shown to cause defects in angiogenesis that are concomitant with abnormal brain development (Garcez et al., 2018). These examples suggest that the vasculature could be envisaged as a target as well as a vehicle to pharmacologically treat some neurodevelopmental disorders.

Although some advances have demonstrated that the vasculature plays a relevant role in various steps along the neurogenic process by influencing neuroblast proliferation, differentiation and migration, the molecular portfolio that orchestrates this communication between the nervous and the vascular systems remains rather elusive. Novel technologies based on omics studies as well as refined gene editing approaches will certainly contribute to unveiling these molecular players, and thus potential therapeutic targets, in the near future.

AUTHOR CONTRIBUTIONS

All authors have made a substantial, intellectual, and written contribution to the work. JV produced the figures implementing suggestions from all authors. All authors approved the manuscript for publication.

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REVIEW
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Progenitor-Based Cell Biological Aspects of Neocortex Development and Evolution

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During development, the decision of stem and progenitor cells to switch from proliferation to differentiation is of critical importance for the overall size of an organ. Too early a switch will deplete the stem/progenitor cell pool, and too late a switch will not generate the required differentiated cell types. With a focus on the developing neocortex, a six-layered structure constituting the major part of the cerebral cortex in mammals, we discuss here the cell biological features that are crucial to ensure the appropriate proliferation vs. differentiation decision in the neural progenitor cells. In the last two decades, the neural progenitor cells giving rise to the diverse types of neurons that function in the neocortex have been intensely investigated for their role in cortical expansion and gyrification. In this review, we will first describe these different progenitor types and their diversity. We will then review the various cell biological features associated with the cell fate decisions of these progenitor cells, with emphasis on the role of the radial processes emanating from these progenitor cells. We will also discuss the species-specific differences in these cell biological features that have allowed for the evolutionary expansion of the neocortex in humans. Finally, we will discuss the emerging role of cell cycle parameters in neocortical expansion.

Keywords: basal process, apical process, centrosome, primary cilia, adherens junction, spindle orientation, delamination, cell cycle

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1 INTRODUCTION

The neocortex is a six-layered neuronal structure that is part of the cerebral cortex of the brain. The neocortex is unique to mammals and is evolutionarily the newest part of the mammalian brain. Its importance lies in the facts that this part of the brain has expanded the most during human brain evolution and is associated with complex and higher order brain functions like cognitive abilities and language. Development of the neocortex is based on spatio-temporally restricted transcriptional programs that unfold in a sequential manner and are a predominant factor for the neural progenitor cell proliferation, differentiation, migration and specification of different neuronal subtypes in the neocortex (Telley et al., 2019; Vaid and Huttner, 2020; Ruan et al., 2021; Bandler et al., 2022). In addition, specific cell biological processes underlie the proper development of the mammalian neocortex and influence these transcriptional programs.

In recent years, advancements in microscopy, image analysis, molecular cell biology and other cell biological techniques have uncovered key aspects of the cell biological processes like cell polarity, mitotic spindle and cleavage plane orientation, cell cycle length, dynamics of junctional proteins, delamination etc., that occur at different developmental time points and ultimately lead to an expansion of the neocortex. Several new players and the molecular details of how their networking

regulates these processes have also been identified. In this review, we will first discuss the diversity of the stem and progenitor cells that are found in the developing neocortex across different mammalian species. We will then proceed to specifically illustrate the cell biological features that are associated with these different stem and progenitor cells, and how these features influence the proliferation, cell fate, morphology and migration of these cells (**Figure 1**).

2 NEOCORTICAL DEVELOPMENT AND PROGENITOR CELL TYPES IN DEVELOPING NEOCORTEX

With the onset of neurogenesis, the neuroepithelial cells (NECs) differentiate into a glial cell population, referred to as apical radial glial cells (aRGCs, also referred to as ventricular radial glia), which give rise to other glial and non-glial progenitor cell types that eventually generate all the neocortical projection neurons. Specifically, the various progenitor cells in the developing neocortex reside in two germinal zones—i) the ventricular zone (VZ), the primary germinal zone; and ii) the subventricular zone (SVZ), a secondary germinal zone. In species with an expanded neocortex, and especially in gyrencephalic species, the SVZ gets further subdivided into an inner SVZ (ISVZ) and an outer SVZ (OSVZ) (Smart et al., 2002), with the OSVZ becoming the most prominent proliferative zone in these species (Smart et al., 2002; Fietz et al., 2010; Hansen et al., 2010; Borrell and Reillo, 2012). An OSVZ-like zone has also been reported in the lissencephalic mouse and rat neocortex at later stages of embryonic neurogenesis (Martínez-Cerdeño, 2012; Vaid et al., 2018). Within these germinal zones, based on the location of the nucleus at mitosis, the progenitor cells can broadly be divided into two principal classes, i) apical progenitors (APs), which undergo mitosis at the ventricular surface of the VZ (**Figure 1**); and ii) basal progenitors (BPs), which undergo mitosis in the SVZ (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Fietz et al., 2010; Hansen et al., 2010; Shitamukai et al., 2011; Wang et al., 2011; Betizeau et al., 2013).

At the cell biological level, APs (i.e., NECs, aRGCs) remain integrated into the apical adherens junction (AJ) belt throughout their cell cycle, their nucleus undergoes apical-to-basal and basal-to-apical migration in concert with the cell cycle (interkinetic nuclear migration, INM), and their mitosis at the ventricular surface reflects the presence of an apical primary cilium throughout interphase. aRGCs retain their basal process at mitosis. This is similar to mouse E10.5 NECs (Kosodo et al., 2008) but in contrast to early human NECs, which have been reported to retract the basal process at mitosis (Subramanian et al., 2017). Furthermore, a subtype of APs called short neural precursors (SNPs) or apical intermediate progenitor cells (aIPCs) have been identified in developing mouse neocortex that retract their basal process at mitosis such that it remains as a small truncated process within the VZ (Gal et al., 2006). Recently, aRGCs have also been reported, during mid-neurogenesis in the developing human neocortex, to exist as a subtype with a truncated basal process; however, unlike mouse SNPs/aIPs, the basal process of human truncated aRGCs terminates in the OSVZ (Nowakowski et al., 2016). Perhaps just a coincidence, but it is interesting to note that in both mouse and human, SNPs/aIPs and aRGCs with a truncated basal process, respectively, appear around the time when about 1/3 of neurogenesis is completed (E12.5 in mouse and GW16.5 in human) (Gal et al., 2006; Nowakowski et al., 2016). Furthermore, regarding the truncated aRGCs, these progenitor cells can provide a scaffold for newborn BPs to ensure that the latter progenitor cells reach, and seed, the OSVZ. In addition to these various types of APs, the developing dorsal telencephalon of gyrencephalic species has been reported to also harbor yet another cell type called subapical progenitors, where the cells are integrated into the AJ belt and maintain a basal process that contacts the basal lamina (like conventional aRGCs), but undergo mitosis in the VZ at a position basal to the ventricular surface (Pilz et al., 2013).

BPs originate in the VZ by divisions of aRGCs. The newly generated BP retracts its apical process from the ventricular surface and AJ belt and moves to the SVZ (**Figure 1** please see *Delamination* below). BPs are further divided into two main types—basal intermediate progenitor cells (bIPCs) and basal radial glial cells (bRGCs, also referred to as outer radial glia). bIPCs are multipolar cells and are the prominent BP type in mouse (Miyata et al., 2001; Haubensak et al., 2004; Noctor et al., 2004), where they have limited proliferative capacity (see below for a definition of this term) and usually undergo only 1-2 rounds of symmetric divisions for their amplification in the SVZ before undergoing symmetric consumptive division to generate neurons (Noctor et al., 2004).

In contrast, bRGCs are the prominent BP type in species with an expanded neocortex (Lukaszewicz et al., 2005; Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Kelava et al., 2012; Betizeau et al., 2013; Lamonica et al., 2013), but are rare in lissencephalic species like mouse (Shitamukai et al., 2011; Wang et al., 2011). Interestingly, a recent study demonstrated an abundance of bRGC as high as that is found in gyrencephalic species in the developing mouse medial neocortex towards the end of neurogenesis (Vaid et al., 2018). At the cell biological level, bRGCs are characterized by radial processes. They typically extend a basal process (maintained at mitosis) that may contact the basal lamina; in addition, they may extend an apically directed process that, however, lacks contact with the ventricle (Lukaszewicz et al., 2005; Fietz et al., 2010; Hansen et al., 2010; Borrell and Reillo, 2012; Betizeau et al., 2013; Kalebic et al., 2019). bRGCs have high proliferative capacity. We define the term "proliferative capacity" as the ability of a given neural progenitor type to undergo multiple rounds of either symmetric proliferative or asymmetric self-renewing divisions, which results in an increased number of daughter cells. For example, about 40% of bRGCs in developing macaque neocortex have been shown to undergo symmetric proliferative divisions, generating up to six daughter cells per bRGC (Betizeau et al., 2013). An increase in the relative

population of bRGCs has been shown to induce/increase cortical folding (Stahl et al., 2013).

3 CELL BIOLOGICAL ASPECTS OF THE PROGENITOR CELL TYPES IN DEVELOPING NEOCORTEX

3.1 Apical Plasma Membrane and Basal Process

3.1.1 Apical Domain

The apical-most surface of the NECs and aRGCs that directly faces the ventricles constitutes the apical domain of the plasma membrane of these cells. This domain can be visualized as a cadherin–negative, prominin-1–positive segment of the plasma membrane (Kosodo et al., 2004). Despite being a small area (**Figure 1**) (corresponding to only 1–2% of the total plasma membrane), the apical plasma membrane provides crucial polarity cues that influence the cell fate of the dividing cell (Please see below the sections on *Primary cilium and centrosomes*, *Adherens junctions* and *Delamination*) and serves as a docking site for several signaling ligands through their receptors that are expressed on its surface facing the ventricular lumen (Taverna et al., 2014). Symmetric proliferative divisions of NECs prior to neurogenesis and of aRGCs during neurogenesis typically exhibit a vertical cleavage plane, which results in an equal distribution of the apical membrane to the two daughter cells.

In contrast, an oblique or even horizontal cleavage plane during neurogenesis that bypasses the AJ belt, which would result in the distribution of the apical membrane to only one of the daughter cells, predicts an asymmetric, self-renewing plus BP-genic aRGC division (Kosodo et al., 2004; Noctor et al., 2008; Kawaue et al., 2019). Fate-wise asymmetric aRGC division can also occur when the cleavage plane does not bypass, but bisects—albeit not necessarily equally—the apical domain (**Figure 1**). In such asymmetric divisions of aRGCs, the two daughter cells may inherit size-wise nearly equal portions of the apical domain, that however are unequal with regard to the fate determination of the two daughter cells . Specifically, it has been proposed that the asymmetric inheritance of a small sub-domain of the apical plasma membrane may be linked to a proliferative vs. neurogenic fate of the daughter cells. To address this issue, Shitamukai et al. (2011) visualized the inheritance of the apical domain using ZO-1-EGFP and PAR3-EGFP, both of which in epithelial cells are known to be localized also to the AJs (Itoh et al., 1993; Takekuni et al., 2003). Therefore, the readout of apical domain inheritance in the Shitamukai et al. (2011) study included a significantly larger area than just the apical plasma membrane. In contrast, Kosodo et al. (2004) used the cadherin-negative segment of the apical domain as a readout and showed that the inheritance of this very small portion of the apical domain correlated with the asymmetric divisions of aRGCs.

In extreme, rare cases, however, when the cleavage plane is parallel to the ventricular surface, the apical daughter cell inheriting the complete apical domain, and no basal domain, becomes postmitotic (Shitamukai et al., 2011). These latter results indicate that the inheritance the apical domain alone is not sufficient for the daughter cell to retain aRGC fate (please see below for the role of basal process in cell fate and proliferation capacity).

3.1.2 Basal Domain

The basolateral membrane accounts for the major fraction of the plasma membrane of NECs, aRGCs and bRGCs. On its basal-most end, a structure called the basal endfoot makes direct contact with the basal lamina in the case of NECs and canonical aRGCs, and may do so in the case of bRGCs (Haubst et al., 2006; Taverna et al., 2014). The basal lamina is a sheet of extracellular matrix (ECM) composed mainly of type IV collagen, nidogen, members of the laminin family and heparan sulphate proteoglycans, such as perlecan and agrin (Erickson and Couchman, 2000), and is enriched with a variety of growth factors (Kazanis and Ffrench-Constant, 2011; Wade et al., 2014). The basal endfoot contacting the basal lamina is a highly dynamic structure (Yokota et al., 2010) that can transduce signals from the ECM-rich basal lamina (Jeong et al., 2013; Singer et al., 2013). The basal endfoot has also been shown to spatially restrict several mRNAs and RNA binding proteins, which may be involved in transducing pro-proliferative signals (Tsunekawa et al., 2012; Pilaz et al., 2016).

3.1.3 Basal Process

Concomitant with the transition of NECs to aRGCs, the initally cuboidal NECs become more elongated and, keeping pace with the increasing cortical wall thickness, their basal-most segment, referred to as the basal process, becomes very thin and grows in length, spanning the neuronal layers to reach the basal lamina (Taverna et al., 2014). Most RGCs (both aRGCs and bRGCs) retain their basal process during mitosis (Miyata et al., 2001; Noctor et al., 2001; Fish et al., 2006; Fietz et al., 2010; Betizeau et al., 2013), and only a subset retracts it at mitosis (Gertz et al., 2014). These data suggest that from the onset of neurogenesis onwards, basal process retention through mitosis serves some important function. Originally being thought to serve primarily as a scaffold for neurons and other cells to migrate on (Rakic, 1972; Noctor et al., 2001; Noctor et al., 2004; Silva et al., 2019), the basal process has now emerged, in addition, as an active subcellular compartment involved in signaling and cell fate specification and especially as a key cell biological feature conferring high proliferative capacity to the bRGCs leading to the evolutionary expansion, and likely the gyrification, of the neocortex (Uzquiano et al., 2018; Kalebic and Huttner, 2020), discussed below in more detail).

Regarding the basal process of aRGC, live-imaging experiments in mouse have shown that the basal process is asymmetrically inherited during mitosis (Miyata et al., 2001) and that the daughter cell inheriting the basal process usually maintains an aRGC cell fate (Konno et al., 2008; Lamonica et al., 2013). In addition, for both aRGC and the bRGC divisions, the daughter cell that does not inherit the basal process can regrow it after division (Miyata et al., 2001; Betizeau et al., 2013), and active Notch signaling has been shown to induce this regrowth (Shitamukai et al., 2011). These results support the notion that the inheritance of the basal process is not necessary to remain an

aRGC or bRGC. For future research, it will be important to investigate if additional mechanisms exist that underlie the regrowth of a basal process.

3.1.4 Basal Process Branching

The basal process may show several small branches along its length (Kalebic et al., 2019). In addition to serving as a scaffold for migrating projection neurons, the long primary basal process and its branches allow the interaction with the surrounding ECM and various other cell types, e.g., with interneurons and blood vessels. This adds to the diversity of signals that the progenitor cells bearing such long basal processes can experience, and likely to their increased proliferative capacity. An inter-species comparison of BP morphology has shown that the branching index of the processes in BPs (the total number of all processes divided by the number of primary processes) increases from mouse to ferret to human (Kalebic et al., 2019). Furthermore, it was shown that the paralemmin family member PALMDELPHIN (PALMD), *via* integrin signaling, promotes the process growth of BPs, and this increase in process number and branching index is directly related to their proliferative capacity (Kalebic et al., 2019). These findings establish a strong role of increased surface area in the proliferative capacity of BPs.

Among the bRGCs, in addition to an increase in the overall branching index of the basal process, the basal process has been shown to display diversity in its morphology. Specifically, in addition to the previously described morphotypes (Betizeau et al., 2013), new morphotypes with 2 basal processes were identified specifically in gyrencephalic species (Kalebic et al., 2019). These bifurcated basal processes have been shown split either nearby the cell body or away from the cell body. These new morphotypes are particularly interesting in light of the notion that the basal process is a key feature of highly proliferative bRGCs and therefore a crucial element in cortical evolution (Smart et al., 2002; Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Betizeau et al., 2013; Lamonica et al., 2013; Kalebic et al., 2019). Kalebic et al. (2019) also showed that PALMD can increase the basal process number of bRGCs in gyrencephalic species but not in lissencephalic species. This is an interesting finding because it suggests an evolutionary difference in the basal process-generating molecular machinery between gyrencephalic and lissencephalic species. An interesting line of future research will be to compare the proliferative capacity of these different bRGC morphotypes and link it to the corresponding morphology. Along this line, bRGCs with both basal and apically directed processes have been shown to have a higher proliferative capacity than bRGCs with either an apically directed or a basal process only (Betizeau et al., 2013).

3.1.5 Basal Process Splitting

During cell division the basal process of mouse E10.5 NECs has been shown to get split before anaphase onset and to then be inherited either symmetrically or asymmetrically between the two daughter cells (Kosodo et al., 2008). As this basal process splitting during NEC division involves anillin and the cytokinesis machinery, it is unlikely to be mechanistically related to the basal process branching of bRGCs discussed above.

3.1.6 Mitotic Somal Translocation

The basal process also plays role in another cell biological event associated specifically with bRGCs—mitotic somal translocation (MST), an actin-myosin–driven fast translocation of the nucleus along the radial fiber before cytokinesis (Hansen et al., 2010; Betizeau et al., 2013; Gertz et al., 2014; Ostrem et al., 2014). MST has been proposed to play a role in the evolutionary expansion of the neocortex because the frequency of bRGCs undergoing MST and the frequency a pial-directed trajectory (which can likely expand the OSVZ) has been shown to increase from ferret to macaque to human (Betizeau et al., 2013; Gertz et al., 2014; Ostrem et al., 2014).

3.1.7 Fanning of Basal Processes

In its role as the scaffold for the migrating neurons, the basal process of the bRGCs has further gained an evolutionary importance as it has been shown that during the generation of the supragranular layers in primates, the aRGC basal process no longer contacts the pial surface (referred to as truncated aRGC). Rather the aRGC basal process instead terminates in the OSVZ [(Nowakowski et al., 2016) and the references therein], and the neurons destined for the supragranular layers therefore migrate along the bRGC basal process (Nowakowski et al., 2016). An additional evolutionary feature related to the bRGC basal processes that can directly influence the gyrification in the developing neocortex is the observation that these basal processes have been shown to fan out during development, and this fanning has been shown to be necessary to promote the tangential dispersion of the migrating neurons, which allows a significant growth in the surface area of the developing neocortex (Reillo et al., 2011; Lewitus et al., 2013).

3.2 Mitotic Spindle and Cleavage Plane Orientation

As mentioned earlier, aRGCs, like NECs, are polarized cells, and their apical-basal polarity is critical to the cell fate of their daughter cells. The cleavage plane orientation upon cell division determines how the cellular components, especially the polarity-related ones, will be distributed between the two daughter cells. The cleavage plane orientation is determined by the orientation of the mitotic spindle. It is therefore not surprising that a premature neuronal differentiation and cortical disorders such as lissencephaly or microcephaly are associated with mutations in genes that have a role in mitotic spindle orientation or mitotic spindle organization (Feng and Walsh, 2004; Fish et al., 2006; Gauthier-Fisher et al., 2009; Garcez et al., 2015).

In developing mouse neocortex, symmetric proliferative divisions of NEC have been shown to exhibit a vertical cleavage plane, that is, parallel to their apical-basal axis, distributing the cellular components equally between the two daughter cells (Kosodo et al., 2004). With the onset of cortical neurogenesis and its progression, the cleavage plane orientation

of aRGCs may be either vertical or oblique, with the frequency of oblique cleavage plane orientation increasing with the progression of neurogenesis (**Figure 1**) (Haydar et al., 2003; Konno et al., 2008; Wang et al., 2009; Asami et al., 2011; Shitamukai et al., 2011). In the developing mouse neocortex, such oblique aRGC divisions have been shown to generate BPs, both bIPs and bRGCs (Wang et al., 2009; Asami et al., 2011; Shitamukai et al., 2011; Wong et al., 2015). Interestingly, oblique or even horizontal orientations of the aRGC cleavage plane can be associated with the generation of bRGCs also in gyrencephalic species (Shitamukai et al., 2011; Lamonica et al., 2013; Pilz et al., 2013; Gertz et al., 2014). In line with the much higher proportion, among the BPs, of bRGCs in human than mouse, the frequency of such oblique and horizontal aRGC cleavage plane orientations is significantly higher in humans than in rodents (Lamonica et al., 2013; Pilz et al., 2013). Additionally, loss of function mutations causing spindle randomization have been shown to cause an increase in the generation of bRGCs in embryonic mouse neocortex (Shitamukai et al., 2011). These results raise the possibility that a downregulation of the machinery ensuring a horizontal mitotic spindle, and hence a vertical cleavage plane orientation may have contributed to neocortex expansion during evolution.

In this context, it has previously been demonstrated that an LGN-dependent decrease specifically in the astral microtubules reaching the basal or the apical region of the cell cortex (especially the basal region) triggers a change from vertical to oblique spindle orientation, leading to the shift from symmetric to asymmetric aRGC divisions in embryonic mouse neocortex (**Figure 1**) (Mora-Bermudez et al., 2014).

Another interesting feature associated with the mitotic spindle is its highly dynamic nature during metaphase. The mitotic spindle of APs has been shown to rotate, even making several turns, before it comes to rest just prior to the onset of anaphase (Adams, 1996; Haydar et al., 2003). This implies that the tethering of the astral spindle microtubules to the actin cytoskeleton at the cell cortex is not very strong during most of metaphase. One possible explanation for this spindle rotation could therefore be the active and ongoing rearrangement of the actin configuration at the cell cortex with which the astral microtubules eventually have to establish a strong contact. Another speculative explanation is that the duration of this spindle rotation provides a short plastic period to the dividing cell to allow it to sense its environment for the last time before the division and re-orient the cleavage plane appropriate for the environment at the time of cleavage.

3.3 Primary Cilium and Centrosomes

Primary cilia are non-motile cilia. They consist of a microtubule-based cytoskeletal structure surrounded by ciliary membrane, which in epithelial cells like NECs and aRGCs is an extension of the apical plasma membrane. The primary cilium of aRGCs protrudes into the lumen of the ventricle to receive, and transduce, the signals from signaling molecules, such as Wnt and Shh, that are present in the ventricular fluid (Corbit et al., 2005; Eggenschwiler and Anderson, 2007; Rohatgi and Scott, 2007; Gerdes and Katsanis, 2008; Goetz et al., 2009; Lehtinen and Walsh, 2011; Louvi and Grove, 2011; Oberst et al., 2019). In addition to serving as an antenna for such signals, the components of the primary cilium of NECs and aRGCs play essential role in various other cell biological processes like INM, mitotic spindle formation, the mode of cell division, and the stability of the apical AJ belt, which will be discussed below.

In NECs and aRGCs at interphase, the mother centriole of the centrosome (the older one of the two centrioles inherited upon the birth of the cell) constitutes the basal body of the apical primary cilium (Kumar and Reiter, 2021; Wilsch-Bräuninger and Huttner, 2021) and is therefore tethered to the apical plasma membrane (**Figure 1**). During the cell cycle of NECs and aRGCs, the apical primary cilium is not disassembled, and the mother centriole hence not detached from the apical cell cortex, until early prophase. In other words, the mother centriole remains tethered to the apical plasma membrane until mitosis onset. Moreover, the nucleus of a NEC or aRGC is located at a non-apical position within the VZ during interphase due to apical-to-basal INM. Hence, the mother centriole can only function, as part of a centrosome, as mitotic spindle pole in cell division if the nucleus migrates towards this centrosome for mitosis via basal-to-apical INM. SUN-domain and KASH-domain proteins link the microtubule appendages of the centrosome to the nucleus and transduce the contracting forces from the microtubules to the nucleus during the basal-to-apical migration of the nucleus (Zhang et al., 2009).

What about the second centrosome required to form a proper mitotic spindle? The two centrioles (one of which is the basal body of the apical primary cilium) separate and duplicate during the G1/S phase. The two new pairs of centrioles—the mother centriole with its duplicate and the daughter centriole with its duplicate—then form the two centrosomes required to build a proper mitotic spindle. During late G2/early prophase, the primary cilium gets resorbed by the cell, and the mother centriole switches its role from being the basal body to serve, along with its duplicate, as one of the mitotic spindle poles. From the resorbed components of the primary cilium, the mother centriole retains a large part of its distal and subdistal appendages (Breslow and Holland, 2019; Tischer et al., 2021) and remains associated with a remanent of the ciliary membrane; these three components—mother centriole, associated ciliary membrane remnant, and duplicated centriole—undergo endocytosis prior to this centrosome becoming a mitotic spindle pole (**Figure 1**) (Paridaen et al., 2013). Following cytokinesis, these additional components associated with the mother centriole accelerate the re-establishment of the—typically apical—primary cilium in the daughter cell inheriting the mother centriole, which allows for a faster responsiveness to stem cell fate-promoting factors in the environment, notably the ventricular fluid (Anderson and Stearns, 2009; Wang et al., 2009; Piotrowska-Nitsche and Caspary, 2012; Paridaen et al., 2013).

In the non-aRGC daughter of an asymmetric aRGC division, which typically is a BP, from the very beginning of neurogenesis, the re-establishment of the primary cilium shows a key cell biological difference when compared to the re-establishment of the apical primary cilium in the aRGC daughter. In these

newborn BPs, instead of generating an apical primary cilium, the inherited centrosome generates a basolateral primary cilium, very close (but basal to) to the apical AJ belt (**Figure 1**) (Wilsch-Bräuninger et al., 2012). This basolateral positioning of the primary cilium is the first observed cell biological indicator of BP delamination, and is likely to prevent this cilium from receiving macromolecular signals from the ventricular lumen, which do not cross the AJ belt. The genetic programs that specifically regulate the basolateral positioning of the primary cilium have not yet been elucidated and therefore remain an open field for future research.

Recent studies have shown an emerging role of centrosome-associated proteins in the delamination of BPs (see below) by regulating the interaction between the cytoskeleton and AJs, which eventually affects the stability of the AJs. For example, in BP-genic APs and newborn BPs, the AT-hook protein AKNA localizes to subdistal appendages on the mother centriole. By influencing the actin re-modeling and AJ stabilization, AKNA regulates the apical constriction and the delamination of the newborn BP (Camargo Ortega et al., 2019). Similar to AKNA, another centriolar protein, Talpid3, which localizes to the distal end of the mother centriole (Yin et al., 2009; Kobayashi et al., 2014; Wang et al., 2020), has been shown to maintain the integrity of the AJ by modulating microtubule stability (Wang et al., 2020).

3.4 Adherens Junctions

As mentioned above, aRGCs, the cells that directly or indirectly give rise to all the projection neurons of the neocortex, maintain an apicobasal polarity throughout cortical development. This apicobasal polarity of aRGCs is crucial for proper cortical development, as it has a direct influence on aRGC morphology, architecture of the ventricular surface, aRGC size, mode of aRGC division, and radial BP migration (Chenn and Walsh, 2002; Machon et al., 2003; Woodhead et al., 2006; Stocker and Chenn, 2015; Veeraval et al., 2020). The apical belt of AJs, the cadherin-based cell–cell adhesion complexes, demarcates the border between the lateral and apical plasma membrane and is a key player in maintaining the apicobasal polarity of the aRGCs. This is so because aRGCs lose functional tight junctions during neural tube closure (Aaku-Saraste et al., 1996) and therefore rely solely on the AJ belt to maintain their polarity and tissue architecture. Mutations in key junctional proteins, leading to a failure of AJ assembly, have pleotropic effects, leading to loss of aRGC polarity (Lien et al., 2006; Kadowaki et al., 2007; Kim et al., 2010; Katayama et al., 2011; Cappello et al., 2012; Yamamoto et al., 2013; Gil-Sanz et al., 2014; Taverna et al., 2014; Schmid et al., 2014; O'leary et al., 2017; Rakotomamonjy et al., 2017).

Interactions between polarity proteins and AJ components facilitates AJ assembly. Thus, Lgl1 directly binds to and promotes the internalization of N-cadherin (Jossin et al., 2017). The Par3 protein, which recruits Par6, aPKC and Cdc42 to form the Par3/Par6/aPKC/Cdc42 polarity complex is localized to the apical cell cortex (Manabe et al., 2002; Kosodo et al., 2004; Cappello et al., 2006; Costa et al., 2008). aPKC phosphorylates and deactivates Lgl1 and excludes the Lgl/Dlg/Scribble polarity complex from the apical cell cortex, and therefore this complex gets restricted to the apical-most region of the lateral membrane, promoting internalization of N-cadherin at this lateral membrane domain. aPKC-mediated phosphorylation of Lgl1 also inhibits the N-cadherin-Lgl1 interaction (Jossin et al., 2017), and therefore N-cadherin accumulation and AJ formation gets restricted to the basolateral-apical boundary.

AJs influence well-known cell fate determination signals and *vice versa*. Thus, Notch, a key stem cell determinant, associates with the cadherin complex and is localized to AJs. Conversely, AJ assembly has been shown to be required for Notch activation (Del Bene et al., 2008; Bultje et al., 2009; Ohata et al., 2011; Hatakeyama et al., 2014). Numb, a known inhibitor of Notch signaling (Frise et al., 1996; Spana and Doe, 1996; Rasin et al., 2007), also directly interacts with cadherins, is localized to cadherin-positive recycling endocytic vesicles at AJs, and is required for the maintenance of AJs (Rasin et al., 2007).

Recently, the AJ component Afadin has been shown to have a role in mitotic spindle orientation. Afadin deletion was shown to increase oblique aRGC divisions, which subsequently increased the level of BPs (Rakotomamonjy et al., 2017). Further support for Afadin's role in mitotic spindle orientation was reported in other epithelial systems (HeLa cells and human colorectal adenocarcinoma cell line Caco-2), where binding of Afadin to F-actin and LGN has been shown to promote symmetric proliferative divisions (Carminati et al., 2016).

3.5 Cell Delamination

Delamination is the process by which a cell, typically a newborn BP, loses its apical plasma membrane and its contact with the AJ belt and retracts its apical endfoot. BP delamination is therefore the first step in, and a requirement for, the migration of BPs to the SVZ. Since the generation of BPs has an immense influence on cortical expansion, BP delamination is an extremely important, and—mechanistically and temporally—tightly regulated, cell biological event in the developing neocortex.

Dynamic changes in the microtubule–actin–AJ configuration at the apical endfoot, (constriction of the AJ belt, downregulation of cadherin expression, etc.) are key events associated with delamination, which are mediated by transcriptional suppression of AJ-related components and by other posttranscriptional cascades to regulate cell adhesion and cytoskeletal architecture.

Upon asymmetric aRGC division, depending on the mitotic spindle and hence cleavage plane orientation (please see *mitotic spindle*), the daughter cell destined to delaminate, typically a newborn BP, may be born with or without inherited AJs and with or without apical domain. If a newborn, not yet delaminated BP has inherited AJs, the AJ components are actively suppressed to disassemble the AJs prior to delamination. Loss of cadherin, a crucial component of AJs, has been shown enhance cell delamination, increasing the production of both bIPs and bRGCs (Itoh et al., 2013; Martinez-Martinez et al., 2016). Moreover, the daughter cell inheriting less of the apical membrane and less of the AJ components experiences a downregulation of the Notch signaling, which leads to the stable expression of proneural genes like Ngn2 (Vaid and Huttner, 2020). Ngn2 promotes the expression of insulinoma-associated 1 (Insm1), Scratch 1 and

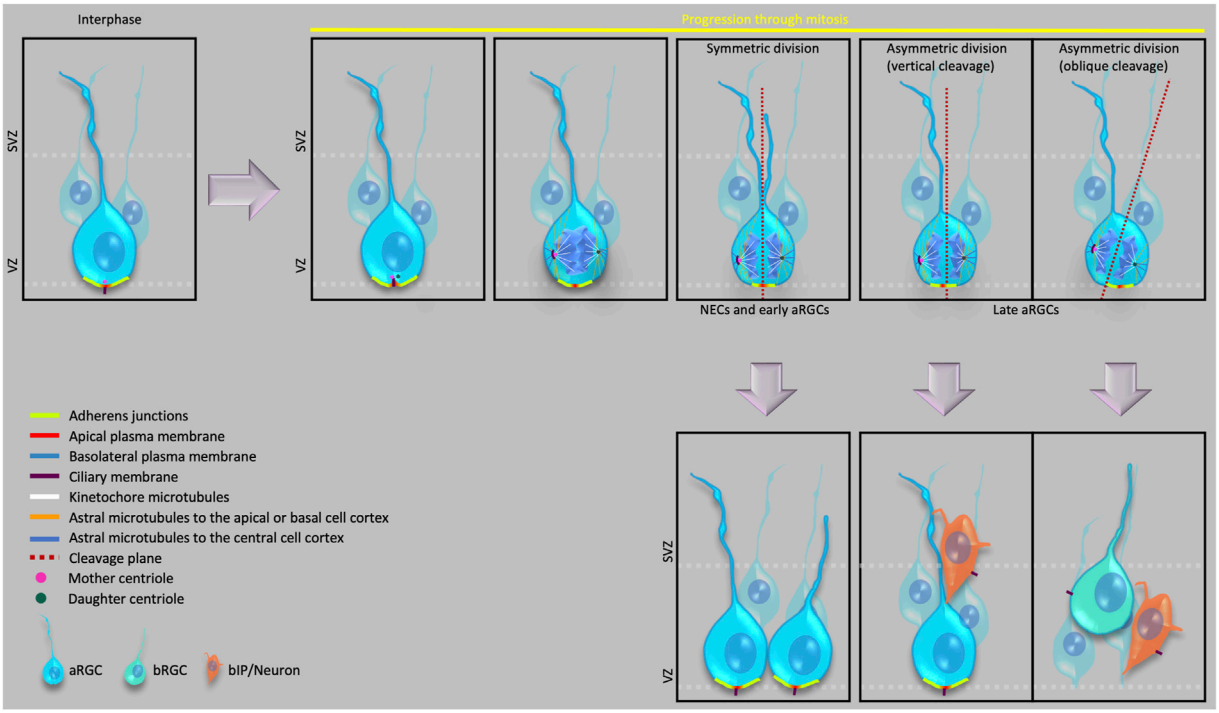

FIGURE 1 | Cell biological features of apical progenitors and their various modes of division.

Scratch 2, all members of the SNAG family (Vaid and Huttner, 2020). Insm1 has recently been shown to promote the expression of Robo2, a transmembrane receptor of the ROBO family, and to down-regulate the expression of Plekha7, an AJ belt-specific protein, causing the AJs to disassemble (Farkas et al., 2008; Tavano et al., 2018; Vaid and Huttner, 2020).

If a BP is born with apical plasma membrane, its delamination not only involves getting out of the AJ belt, but also getting rid of apical plasma membrane. This can be achieved either by an abscission of the apical endfoot, where the apical process is constricted in an actomyosin-dependent manner and gets pinched off, or by endocytosis of apical plasma membrane followed by its degradation (Das and Storey, 2014).

As discussed above, centrosome-associated proteins, by modulating microtubule and actin stability, also influence AJ stabilization and therefore regulate delamination (Camargo Ortega et al., 2019; Wang et al., 2020). Recently, the microtubule-associated protein Lzts1 has been shown to inhibit microtubule assembly and to activate the actomyosin system at the apical endfoot of newborn BPs, and hence functions in BP delamination by altering the organization of the apical AJ belt (Kawaue et al., 2019).

3.6 Cell Cycle Parameters

BPs generated during neurogenesis in the embryonic mouse neocortex have been shown to have a specific increase in the length of the G1 phase as compared to the aRGCs they are derived from (Calegari et al., 2005; Lange et al., 2009; Arai et al., 2011). In fact, increasing the length of the cell cycle of NECs is sufficient to increase the appearance of neuronally committed progenitors and to induce premature neurogenesis (Calegari and Huttner, 2003). Comparison of the cell cycle parameters of aRGCs undergoing symmetric proliferative divisions vs. aRGCs undergoing asymmetric BP-genic divisions revealed a substantially longer S-phase in the former aRGC subpopulation (Arai et al., 2011). This suggests that aRGCs undergoing divisions to expand their pool size invest more time into the quality control of the replicated DNA.

Not only cell cycle parameters of APs in interphase, but also of APs in mitosis have been observed to differ between proliferating and BP-genic APs. Specifically, it was found that prometaphase plus metaphase is longer in proliferating than BP-genic APs in embryonic mouse neocortex, with the other phases of mitosis (prophase, anaphase and telophase) showing no significant difference between these two AP subpopulations (Mora-Bermudez et al., 2016).

A comparison of M-phase length of APs in embryonic mouse neocortex, chimpanzee cerebral organoids and fetal human neocortex revealed that the length of AP M-phase increases from mouse to chimpanzee to human (Mora-Bermudez et al., 2016). Intriguingly, among the primates, the M-phase length difference reflected the specific lengthening by ≈50% of metaphase in human APs when compared to chimpanzee or orangutan; interestingly, this metaphase lengthening was only observed at an early stage of cortical development (Mora-Bermudez et al., 2016).

Taken together, these cell cycle parameter analyses show that although the BP-genic APs in embryonic mouse neocortex increase their cell cycle length, specifically the length of G1, compared to proliferating APs, they spend significantly less time in S-phase and in prometaphase-metaphase, the phases where quality control of DNA replication and the preparation for accurate chromosome segregation, respectively, take place. These findings imply that with regard to neurogenesis in the developing neocortex, the accuracy/fidelity of these processes is ensured at an early step, when aRGCs expand their pool size *via* symmetric proliferative divisions. Among the hominids, the specific increase in the metaphase length of mitotic APs in human compared to non-human great apes raises the intriguing possibility that the fidelity of chromosome segregation during the expansion phase of APs in the developing neocortex improved during human evolution.

4 CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this review, we have addressed cell biological features of the neural stem and progenitor cells in the developing neocortex. One focus has been how specific cell biological events regulate progenitor cell divisions and daughter cell fate. The canonical view of mitotic spindle and cleavage plane orientation being key determinants of aRGC daughter cell fate has evolved in light of studies showing that the relationship between mitotic spindle and cleavage orientation on the one hand and symmetric vs. asymmetric inheritance of apical and basal structures and daughter cell fate on the other hand is more complex than previously thought.

It is now well established that bRGCs, like the other type of BP, the bIPs, originate from aRGCs. However, in contrast to aRGCs, bRGCs show a high diversity in their morphotypes, which impacts their proliferative capacity (Kalebic et al., 2019). Since bRGCs have a key role in the evolutionary expansion of the neocortex, an understanding of the mechanism(s) underlying the generation of this high morphological diversity is very important. Understanding how these bRGC morphotypes evolved requires a more refined investigation of the dynamics of the radial processes in bRGCs and compare them to those in aRGCs.

Lastly, among the features that impact the expansion phase of aRGCs, changes in cell cycle parameters, specifically in the length of S-phase and of metaphase, are emerging as important determinants. This suggests that the underlying genomic changes allowing a tighter control over the quality of DNA replication and the fidelity of chromosome segregation provided advantages for neocortex expansion in the course of primate evolution.

AUTHOR CONTRIBUTIONS

SV and WBH wrote the manuscript. Both authors approved the submitted version.

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REVIEW
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The Symmetry of Neural Stem Cell and Progenitor Divisions in the Vertebrate Brain

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Robust brain development requires the tight coordination between tissue growth, neuronal differentiation and stem cell maintenance. To achieve this, neural stem cells need to balance symmetric proliferative and terminal divisions with asymmetric divisions. In recent years, the unequal distribution of certain cellular components in mitosis has emerged as a key mechanism to regulate the symmetry of division, and the determination of equal and unequal sister cell fates. Examples of such components include polarity proteins, signaling components, and cellular structures such as endosomes and centrosomes. In several types of neural stem cells, these factors show specific patterns of inheritance that correlate to specific cell fates, albeit the underlying mechanism and the potential causal relationship is not always understood. Here, we review these examples of cellular neural stem and progenitor cell asymmetries and will discuss how they fit into our current understanding of neural stem cell function in neurogenesis in developing and adult brains. We will focus mainly on the vertebrate brain, though we will incorporate relevant examples from invertebrate organisms as well. In particular, we will highlight recent advances in our understanding of the complexities related cellular asymmetries in determining division mode outcomes, and how these mechanisms are spatiotemporally regulated to match the different needs for proliferation and differentiation as the brain forms.

Keywords: Neurogenesis, neural stem cell, asymmetric division, brain development, cellular asymmetries, radial glial cells, symmetry-breaking

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1 INTRODUCTION

In central nervous system (CNS) development, pluripotent neural precursors derived from the ectoderm are responsible for the production of all types of neurons and macroglial cells, as well as adult progenitor cells. In vertebrates, neural stem and progenitor cells (collectively named neural progenitors, abbreviated as NPCs) arise from the neuroepithelium that lines the nascent neural tube. As typical epithelial cells, neural progenitors exhibit well-defined apicobasal polarity, with their apical side facing the internal lumen of the neural tube and their basal membrane contacting the pial surface. The neuroepithelium appears as a pseudostratified epithelium, with cell nuclei distributed along the entire apicobasal axis. NPCs exhibit interkinetic nuclear migration, a stereotyped movement of the nucleus towards the apical surface in the G_2 phase of the cell cycle, ensuring that NPC mitosis occurs at the ventricular surface.

At early stages prior to the onset of neurogenesis, self-renewing cell divisions expand the NPC pool. After the onset of neurogenesis, NPCs start producing neurons that migrate basally and start

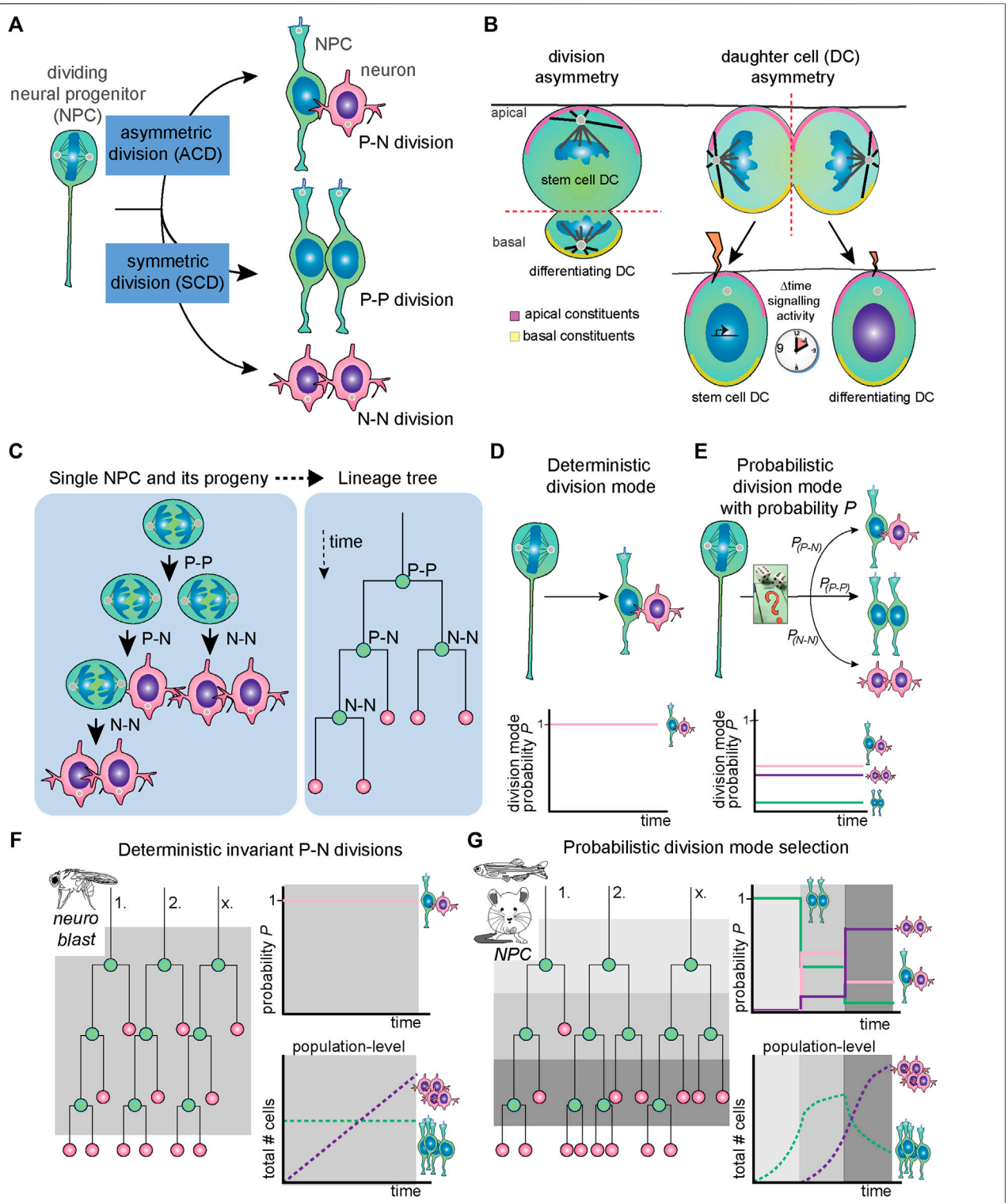

FIGURE 1 | General concepts regarding NPC division mode, mechanism of asymmetry and deterministic versus probabilistic division modes. **(A)** As any stem cell type, NPCs can undergo asymmetric division [typical example is a progenitor-neuron (P-N) division], symmetric proliferative division (P-P division) or symmetric differentiative (N-N) division. **(B)** In division asymmetry (left), asymmetrical daughter cell fates are induced by morphological asymmetries in terms of cell size, cleavage furrow orientation, apicobasal polarity and unequal distribution of fate-determinants. In daughter cell asymmetry (right), the division itself is morphologically symmetrical. However, small fluctuations in signaling states due to differential inheritance of signaling components and/or stochastic fluctuations in transcriptional activity lead to unequal and/or asynchronous signaling activity, which ultimately induces two unequal daughter cell fates. **(C)** The progeny of one single NPC and division modes

(*Continued*)

FIGURE 1 | of each round of cell division is shown (left) and translated into a lineage tree (right). **(D)** Deterministic division mode is defined as having a probability of a specific division mode (in this example, P-N division) of 1. **(E)** In probabilistic division mode, there are specific probabilities for each type of division mode named $P_{P\text{-}N}$, $P_{P\text{-}P}$ and $P_{N\text{-}N}$. **(F)** In systems with deterministic and invariant asymmetric divisions, such as the *Drosophila* neuroblast, the $P_{P\text{-}N}$ remains stable over time (bottom panel), whereas in probabilistic division, $P_{P\text{-}N}$, $P_{P\text{-}P}$ and $P_{N\text{-}N}$ can assume different values depending on the context. **(G)** In systems with probabilistic division mode selection, such as mouse and zebrafish NPCs, the probabilities $P_{P\text{-}N}$, $P_{P\text{-}P}$ and $P_{N\text{-}N}$ change over developmental time so that the predominant division mode in individual NPCs shifts. Thus, regulation of the spatiotemporal balance between proliferation and differentiation ensures robust development at the population level. DC, daughter cell; N, neuron; NPC, neural progenitor cell; P, progenitor, *P*, probability.

populating upper layers of the tissue. In mid-neurogenic stages, in some parts of the brain, especially in expanded regions such as the neocortex in mammals, a diverse range of specialized intermediate progenitors are generated from asymmetrically dividing NPCs termed radial glial cells (RGCs). Newborn basal progenitors (BPs) delaminate from the ventricular surface and migrate to a more basally located germinal zone, the subventricular zone. Depending on the species, these BPs have low or high self-renewing capacity and serve to increase neuronal production from one initial RGC (**Sections 5.3** and **5.4** for more details). At the end of embryonic neurogenesis, NPCs switch to gliogenesis and produce astrocytes, oligodendrocytes and ependymal cells.

As in any developing tissue, the timing of stem cell proliferation and differentiation needs to be tightly regulated in order to accommodate tissue growth and maturation in changing spatial constraints. Moreover, while the neural tube starts off as a relatively homogeneous structure, the mature CNS is composed of structurally and functionally distinct regions with different cellular composition. Therefore, the ratio between proliferation and differentiation needs to be regulated also at the local level to allow for these regional specializations. In this review, we will discuss the mechanisms that underlie the balanced ratio between self-renewal and differentiation in the context of division mode regulation.

2 NEURAL PROGENITOR CELL DIVISION MODE AND DIVISION OUTCOMES

A common biological strategy for mediating the balance between self-renewal and differentiation is the regulation of division modes (**Figure 1A**). In short, stem cell division can be proliferative, producing two new progenitor cells (a so-called P-P division); asymmetric, producing a new stem cell and a differentiating cell (P-N division); and terminal self-consuming, producing two cells initiating neuronal differentiation (N-N division). More generally, cell divisions can be considered asymmetric when they produce two different types of progenitors or two types of terminally differentiated cells.

Here, we can distinguish two concepts of mediating asymmetrically fated daughter cells, namely division asymmetry and daughter cell asymmetry (**Figure 1B**). In division asymmetry, asymmetries between daughter cells arise directly during cell division (**Figure 1B**, left panel). For instance, if division is such that during mitosis, subcellular structures are already asymmetrically partitioned between sister cells, or if sister cells have different sizes or display other morphological asymmetries. A classic example of division asymmetry is found in the *Drosophila* neuroblast, which has been extensively studied [**Figure 1B**, left; reviewed by (Loyer and Januschke, 2020)]. On the other hand, in daughter cell asymmetry, the mother cell splits in seemingly identical sister cells, and diverging fates arise sometime after division (**Figure 1B**, right panel). The cell division is symmetrical in the sense that morphologically, the cleavage of the mother cell is such that two equal sized and shaped daughter cells have emerged. However, through unequal exposure to signals, the daughter cells subsequently obtain different cell fates. Vertebrate NPC divisions that yield one progenitor and one differentiating daughter cell often portray this type of asymmetry. As we will see below in **Sections 3** and **4**, different mechanisms are used to result in asymmetric daughter cell fates arising from morphologically symmetric divisions.

The concepts of division asymmetry and daughter cell asymmetry overlap, as unequal segregation of intracellular parts leading to or biasing daughter cell fates could also be considered as division asymmetry. Moreover, it is likely that additional hidden asymmetries in segregation of subcellular content occur that contribute to daughter cell asymmetry despite having morphologically symmetrical divisions.

There are distinct molecular mechanisms by which asymmetric fates in sister cells can be introduced. These can be classified as intrinsic mechanisms, in which cell division results in two intrinsically different sister cells (e.g., asymmetric distribution of fate determinants in mitosis), or extrinsic, in which newborn sibling cells are virtually indistinguishable but lead to diverging fates by external influence (e.g., different exposure to extracellular signals). Some of these mechanisms appear to depend on initial small fluctuations due to stochastic processes such as transcription (see also below in **Section 3**). While many mechanisms of symmetry-breaking in neural stem cell division have been described, a *bona-fide* generally applicable fate determination mechanism or combination thereof does not seem to be the case, as we will discuss in the next section.

3 STOCHASTICITY VERSUS DETERMINISM IN DIVISION MODE SELECTION AND LINEAGE PROGRESSION

At the tissue level, NPC division mode progresses from symmetric proliferative to asymmetric and symmetric neurogenic divisions as brain development proceeds (**Figure 1C**). However, division mode progression at the single

NPC level seems to be more heterogeneous in vertebrates. Experimentally, the study of division mode selection is challenging because it requires long-term following of sister cells after cell division. However, lineage tracing and time-lapse imaging have provided insights in the pattern of division modes used by individual NPCs in different developing organisms.

In invertebrate organisms like *Drosophila*, neurogenesis results from NPCs divisions that follow a fixed pattern of subsequent divisions modes [**Figures 1D,F**; reviewed by (Loyer and Januschke, 2020)]. In this case, it could be noted that division mode and cell specification is invariant and underlying mechanisms deterministic in nature (**Figure 1D**; reviewed by Zechner et al., 2020). In contrast, studies of single NPC lineages in vertebrate systems show that individual clones follow a variety of trajectories and do not follow a strict pattern of division modes (**Figures 1E,G**). This was shown in the retina, where there is a stereotyped order of neuronal cell type birth. For instance, in the zebrafish and rat retina, tracking of individual clones through time-lapse imaging with cell fate markers shows high variability in the clonal size and composition of the lineages generated by individual NPCs (**Figure 1G**, Gomes et al., 2011; He et al., 2012). Similarly, live imaging of NPCs in the developing zebrafish telencephalon and hindbrain show heterogeneity in the NPC division modes present at neurogenic stages (Dong et al., 2012; Hevia et al., 2021). Intriguingly, in the zebrafish retina, the probabilities for retinal NPCs to undergo P-P, P-N or N-N divisions change over time (**Figure 1G**). These temporal changes ensure that at the tissue level, for each developmental stage the proper balance between proliferation and differentiation is achieved (**Figure 1G**).

Individual NPC division modes have also been investigated in developing mammalian brains. Here, it is difficult to track entire NPC lineages through live imaging. Instead, sparse labelling of individual NPCs and their progeny is achieved through low-titer retrovirus intraventricular injection and genetic tools, such as Mosaic Analysis with Double Markers (MADM). With these techniques, the lineages downstream of either both or one of the daughter cells arising from a division can be specifically traced (Gao et al., 2014; Llorca et al., 2019). Such single-clone tracing studies in the mouse cortex have reached somewhat contradicting results on individual NPC lineage generation (Gao et al., 2014; Llorca et al., 2019). Based on MADM tracing, the first study proposed that the neuronal output of individual NPCs in the cortex shows little heterogeneity and is quite predictable (Gao et al., 2014). Upon onset of neurogenesis, mouse NPCs were calculated to produce 8–9 neurons on average. Furthermore, about 1 in 6 NPCs were determined to proceed to gliogenesis upon finishing embryonic neurogenesis. In contrast, a more recent study showed higher diversity of clonal size and generated neuronal types per lineage (Llorca et al., 2019) similar to the earlier work in the vertebrate retina. In this study, sparse retroviral labelling, mosaic genetic Cre-lox based labelling as well as MADM were applied and results compared. A stochastic model with specific fixed probabilities for each division mode which that change over time fits the experimental observations well (Llorca et al., 2019). In this model, spatiotemporally regulated changes in probabilistic division mode and daughter cell fate selection by individual cells is key to building a reproducible pattern of neuronal layers and types in the mammalian forebrain (**Figure 1G**). While these different conclusions may seem difficult to reconcile at first glance, when technical restrictions such as the fact that MADM system only works in mitotic cells are considered, both studies are in agreement on the multipotency and average lineage sizes generated from the majority of mouse neocortex NPCs.

In this context, an important additional question is whether a subset of lineage-restricted NPCs, that is NPCs that are competent or biased to generate certain types of neurons, exists. Although data regarding this question is conflicting, taken together they suggest that at least in mammals, neurogenesis is mediated through multipotent NPCs as well as a small population of lineage-restricted NPCs that are biased to generate upper layer neurons (Llorca et al., 2019).

These findings suggest that non-determinism and apparent stochasticity (absence of predictableness) is an important factor in division mode selection in vertebrate brain development [more extensively reviewed by (Zechner et al., 2020)]. At the same time, many factors and processes have been described to influence cell division outcomes or to increase the probability of certain division outcomes (**Figure 1E**, see also below in **Section 4**). Biological processes such as transcription and molecular interactions between limited amounts of molecules are unpredictable and therefore stochastic by nature. Therefore, stochastic processes are proposed to contribute to the heterogeneity in division mode selection by individual NPCs [see also (Hiesinger and Hassan, 2018)]. However, mechanisms that are more deterministic and predictable are very relevant as well, as we will discuss in the next sections. Moreover, it is likely that specific factors or processes that result in division asymmetry, especially those that are technically challenging to visualize and measure, remain unknown and hidden. Taken together, it is probable that a weighted combination of deterministic factors and processes, stochastic fluctuations and biases, as well as still hidden asymmetries determine division mode used by individual NPCs. The relevant weight of each fate-determining factor and process, and exact combination used is likely to be stage-, species- and time-dependent.

4 WHICH NPC PROPERTIES ARE INVOLVED IN DIVISION MODE SELECTION?

In general, adoption of neuronal versus progenitor daughter fates is characterized by several aspects of their cell biology. First, newborn neurons typically need to lose their apical domain that tethers them to the ventricular surface in order to allow them to delaminate from the ventricular surface, initiate neuronal differentiation and move basally to their final position in the neuronal layer(s) [**Figure 2D**; reviewed by (Singh and Solecki, 2015)]. This loss of apical domain can occur through division asymmetry, in which the neuronal daughter either did not inherit

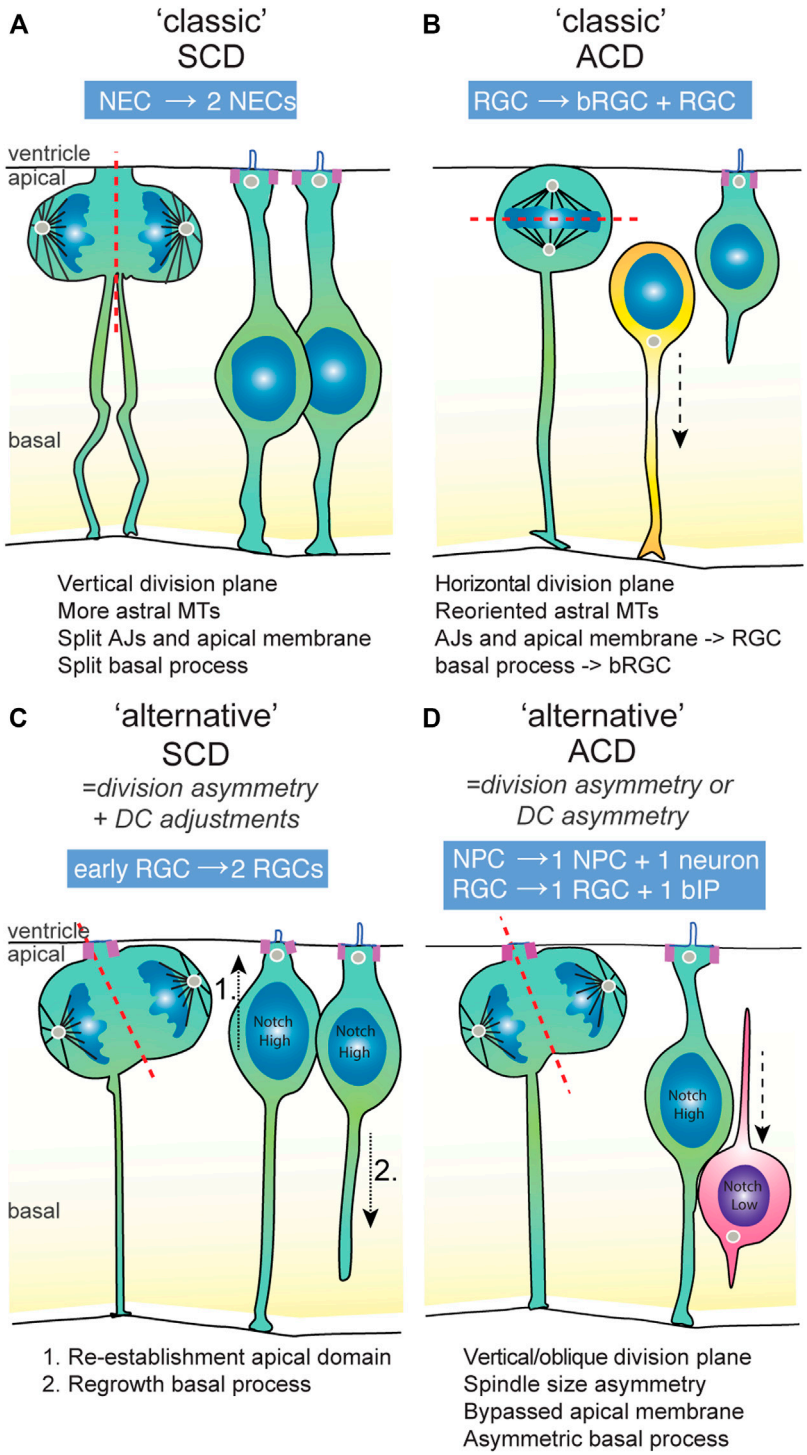

FIGURE 2 | The symmetric and asymmetric division modes used in vertebrate developing brains. **(A)** An example of a classical division symmetry, namely the neuro-epithelial cell (NEC) that undergoes P-P division prior to the onset of neurogenesis. The division plane is vertical, which splits the AJs, apical domain and basal process into equal parts that are inherited by either daughter cell. The presence of astral microtubules limit wobbling of the spindle, ensuring division symmetry. **(B)** An example of a classical division asymmetry in the mammalian developing brain, namely the radial glial cell (RGC) that generates basal RGC in an asymmetric division. The division plan is horizontal due to the re-oriented spindle towards the apical domain. This results in unequal segregation of the apical and basal constituents to the RGC and bRGC daughter cell, respectively. **(C)** An example of an "alternative" symmetric division in early stages of mammalian neurogenesis, in which an initial morphological division asymmetry is compensated for by adjustments in the daughter cells eading to symmetric Notch signalling. These adjustments constitute re-establishment of the apical and basal domain by the non-inheriting daughter cell, ensuring daughter cell fate symmetry. **(D)** An example of an "alternative" asymmetric division, which is typical for most NPC divisions in the vertebrate brain. The division plane is mainly vertical or slightly oblique. Therefore, the division is partially asymmetric, though some parts of the cell (like the apical domain) appear to be equally bisected. In general, the basal process is inherited by the NPC daughter cell.

(*Continued*)

FIGURE 2 | Because of asymmetric Notch signalling states between the daughter cells, the other daughter cell becomes an basal intermediate progenitor (IP) or neuron. ACD, asymmetric cell division; AJs, adherens junctions; bIP, basal intermediate progenitor; bRGC, basal radial glia; DC, daughter cell; NEC, neuroepithelial cell; NPC, neural progenitor cell; RGC, radial glial cell; SCD, symmetric cell division.

the apical membrane, or loses it through downregulation of apical adhesion complexes or abscission of apical membrane after division. In many vertebrate systems including mice, zebrafish and chick, after a neurogenic asymmetric division, the apically positioned daughter cell inherits (part of) the apical domain and induces neuronal differentiation (**Figure 2D**). In contrast, in general, the more basally positioned progenitor daughters either retain or re-establish an apical domain containing adhesion and polarity complexes (**Figures 2C,D**). Second, NPCs that maintain "stemness" usually also retain or regrow the basal process that spans the width of the neuroepithelium basally (**Figure 2C**). These general cell biological properties coupled to progenitor or neuronal fate often play key roles in mechanisms underlying (a)symmetric division modes.

In the next sections, we will provide an overview of currently known aspects of cell division and NPC properties that have been demonstrated to influence their division mode in development of the vertebrate nervous system. Many of these NPC properties are related to their unique morphology and cell biology. Moreover, as we will discuss below in this section and in **Section 5**, these properties are also often connected in some way to the regulation of, or are being regulated by, the activity of signaling pathways. Delta-Notch signaling and Sonic Hedgehog (Shh) are known to be of particular importance in NPC proliferation and differentiation (Garcia et al., 2018; Moore and Alexandre, 2020). Other pathways that play a prominent role are the FGF, Wnt and Hippo signaling pathways. Together, this supports a model where signaling pathways are intricately connected to generate particular division outcomes.

It is important to note that experimental evidence is naturally often limited to certain regions of the CNS or specific cellular subtypes, and thus the extent to which these are part of a universal model of vertebrate NPC regulation, and even if such a model exists, remains an open question. As we will discuss, many common players in division mode regulation seem to display specific behaviors that might differ in detail between vertebrate species, context or cell type. As introduced, there is also temporal progression of division modes in the CNS, and the mechanisms underlying this temporal regulation, when known, will also be discussed.

4.1 Apical Domain

NECs and RGCs possess a small apical domain that contains the primary cilium, which is nucleated from the mother centriole and protrudes into the brain ventricle to detect signalling molecules [reviewed by (Wilsch-Bräuninger and Huttner, 2021)]. The apical membrane is delineated by polarity and junctional complexes that tether the NPCs to the ventricular surface. These complexes are important to maintain neuroepithelial integrity and normal layering in the cortex [reviewed by (Veeraval et al., 2020)].

Upon cell division, the cleavage furrow is oriented towards the apical domain that is subsequently divided between the daughter cells. Initial studies indicated that asymmetric division of RGCs is accompanied by the cleavage furrow bypassing the apical membrane, dividing the daughter cells in apical domain-inheriting and non-inheriting cells (**Figure 2C**) (Kosodo et al., 2004). However, careful inspection of live imaging data in several studies has shown that bypassing of the apical membrane is not an absolute property of asymmetric division [(Shitamukai et al., 2011; Fujita et al., 2020)]. Instead, it appears that in asymmetric neurogenic divisions, the apical domain is often equally bisected, and the apical domain and junctions are disassembled later on in the differentiating daughter cell (**Figure 2D**).

While attachment to the ventricular surface is an important property of apical NPCs, there seem to be a clear distinction between the polarity and the junctional components of adherens junctions (AJs), as polarity proteins seem to have a role in division mode selection that has not been observed when junctional proteins such as N-cadherin are disrupted, despite its clear importance in keeping NPC in the proliferative niche near the ventricle (Miyamoto et al., 2015; Veeraval et al., 2020).

4.2 Polarity Proteins

Inheritance of cell cortex factors and polarity proteins is one of the best characterized mechanisms to introduce asymmetry in sister cells. As in all epithelia, NPCs are closely connected to each other through cell-cell contacts. These apical contacts are composed of junctional proteins such as cadherins and catenin and polarity complexes such as Par3-aPKC-Par6. Studies in the developing mouse cortex showed that cortical mPar3 can be symmetrically or asymmetrically inherited in sister cells, independently of cleavage plane orientation (Bultje et al., 2009). In oblique divisions, Par3 can be inherited towards the most apical cell or towards the more basal cell. This association between apical Par3 inheritance and maintenance of proliferative capacity was recently shown to occur in zebrafish forebrain NPCs as well (Zhao et al., 2021). This contrasts with previous reports from asymmetric divisions in the zebrafish spinal cord and the zebrafish hindbrain, where inheritance of apical Par3 was biased towards the neuronal daughter cell (Alexandre et al., 2010; Kressmann et al., 2015). The exact reason underlying these regional differences is unknown.

In the developing mouse cortex, there is a progressive downregulation of cadherin, Par3, Par6 and aPKC, indicating that their reduction is a key step during neurogenesis (Costa et al., 2008). Disruption of Par3 expression leads to an increase in symmetric divisions at the expense of asymmetric divisions. It seems that disruption of Par3 promotes either proliferative or differentiative divisions depending on the context (Costa et al., 2008; Bultje et al., 2009). Furthermore, disruption of Par3 can also lead to randomization of the spindle orientation (Liu et al., 2018). Overexpression of Par3 and Par6 leads to increased clonal size. In this context, Par3 functions in asymmetric stem cell division and

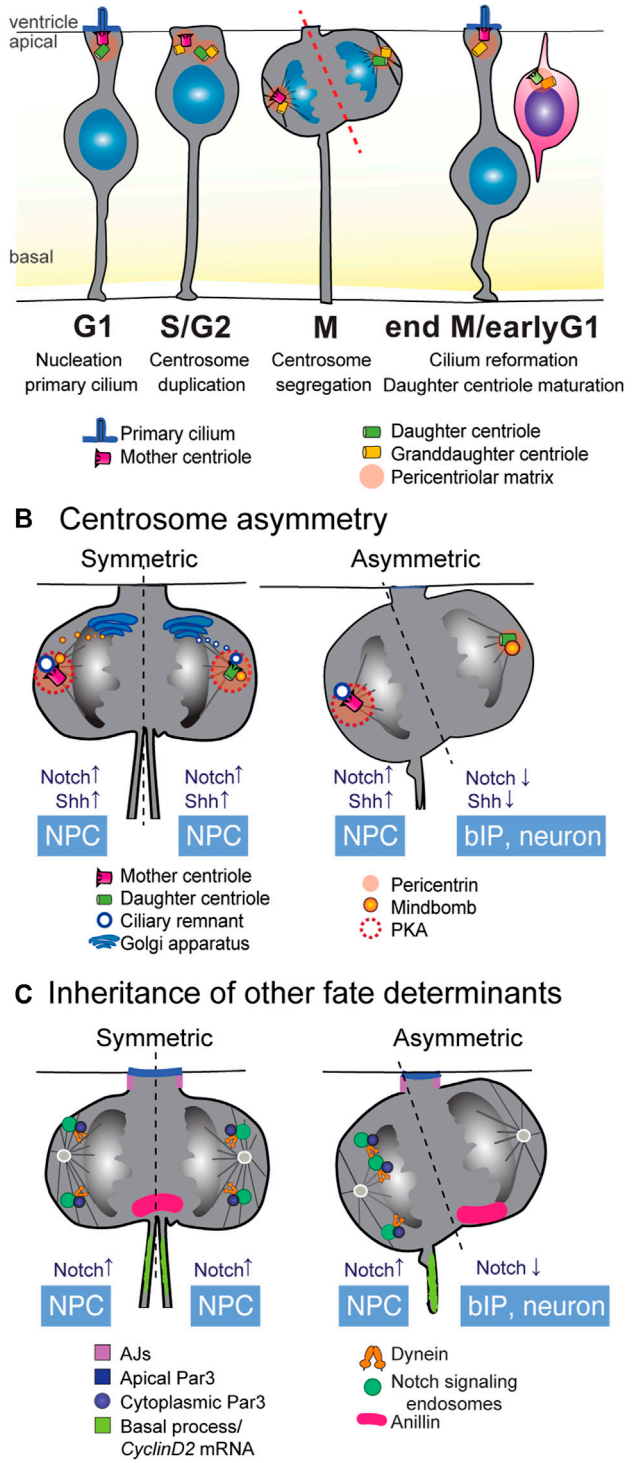

FIGURE 3 | Inheritance of cellular components as fate determinants. **(A)** Centriole duplication cycle in NPCs. In NPCs in interphase and G1, the mother centriole doubles as the basal body for anchoring of the primary cilium. As the cell cycle progresses through S and G2 phases, the centrioles within the centrosome disengage and the centrosome duplicates in a semi-conservative manner. In mitosis, one of the microtubule spindle poles is nucleated by the mother centrosome, containing the fully mature mother centriole, while the opposite spindle emanates from the immature daughter centriole. In P-N divisions, inheritance of the fully mature mother centriole is often correlated with acquisition of a P fate. **(B)** Several aspects of centrosome biology show asymmetry in P-N divisions (left), and there are active mechanisms by which these asymmetries are corrected in P-P divisions. Early P-P divisions show higher levels of pericentriolar matrix, which recruits PKA to the centrosomes and promotes Shh signaling. Inheritance of the ciliary membrane allows cells to quickly regrow a cilium after cytokinesis, promoting a P fate. In early P-P divisions, *de novo* ciliary membrane is proposed to derive from the Golgi and dock to the daughter spindle pole before cell division, equalizing the speed of cilium reformation. Notch modulator Mindbomb1 associates with the daughter centriole and its inheritance correlates with N fate in P-N divisions. In early P-P divisions, a pool of Mindbomb1 is released from the Golgi towards the mother centrosome spindle. **(C)** Many cellular components show specific patterns of behavior in P-P and P-N divisions. Inheritance of the basal process is known to be correlated with P fate, at least partly due to the presence of *CyclinD2* mRNA that can facilitate cell cycle re-entry. The apical polarity protein Par3 can be asymmetrically inherited in mitosis, albeit its correlation with specific fates is context-dependent. Inheritance of the midbody protein Anillin correlates with N fate. Asymmetric distribution of endosomes containing Notch signaling molecules occurs in mitosis and is dependent on dynein and Par3. It is suggested that differences in the abundance of spindle microtubules can underlie this asymmetric distribution. bIP, basal intermediate progenitor; NPC, neural progenitor cell.

(*Continued*)

acts upstream of Numb and Numb-like in regulating Notch signaling. Research indicates that Par3 can also regulate the activity of the pro-proliferative Hippo pathway in conjunction with Notch signaling (Liu et al., 2018). This shows that asymmetrical inheritance of polarity proteins influences cell fate through its effect on signaling activity, in particular upstream of Notch signaling.

Another unique property of NPCs that is connected to their apicobasal polarity is the presence of a basal extension.

4.3 Basal Process

The basal process is an extension of the cell body that connects NPCs to the pial surface through its basal endfoot (**Figure 2B**). In cortical development, it serves as scaffolding and support for neuronal migration. It is a dynamic structure. In mitosis, the basal process is not disassembled, but rather thins out and acquires a thread-like appearance. Subsequently, it can be split into two and inherited symmetrically by the two daughter cells (**Figure 2A**), or inherited by only one of the daughter cells (**Figures 2B–D**). However, even when inherited asymmetrically, in some cases, daughter cells can regrow a basal process after cell division and remain progenitors (**Figure 2C**; see also **Section 5.1**). As discussed above, retention of the basal domain is one of the determining factors in maintenance of self-renewing capacity and cortical expansion through increased divisions of basal RGCs (bRGCs) [reviewed by (Kalebic and Huttner, 2020)].

The basal process does not only have an architectural role, but it also serves in fate determination. In asymmetric divisions, the cell inheriting the basal process retains the stemness character. Research has shown that mRNAs can be specifically transported to the basal process (Pilaz et al., 2016; Tsunekawa et al., 2012). For instance, mRNA for the cell cycle factor *CyclinD2* is specifically localized in the basal process, and daughter cells that inherit the basal process containing *CyclinD2* mRNA remained progenitors

more often than not, showing that this promotes self-renewing capacity (Tsunekawa et al., 2012) (**Figure 3C**). The RNA-binding protein FMRP was recently shown to transport several mRNAs to the basal endfoot, which subsequently are locally translated (Pilaz et al., 2016). This suggests that the basal endfoot might be locally regulated through translation and interactions with the basal lamina and neighboring cells (Pilaz and Silver, 2017). It was also proposed that through the basal process, basal signals can be relayed to the NPC's soma. Indeed, maintenance of the basal process seems to be important for maintenance of proliferative potential, which is exemplified by the fact that the basally located bRGCs that retain some self-renewing capacity possesses a basal process, but no apical attachment (reviewed by Kalebic and Huttner, 2020).

Next to specific inheritance of apical and basal domains, the apicobasal polarity of NPCs is also connected to division mode regulation through changes in the orientation of the mitotic spindle and cleavage furrow.

4.4 Spindle Orientation

As briefly introduced above, most vertebrate NPCs divide at the apical surface with a vertical spindle orientation and their cleavage plane perpendicular to the apical surface (**Figures 2A,C,D**). Spindle orientation and the plane of division change throughout neurogenesis [reviewed by (Pietro et al., 2016)]. In early apical divisions, cells divide perpendicularly to the ventricular surface, thus parallel to the apicobasal axis (**Figure 2A**). In later neurogenic stages, oblique division planes become more frequent, albeit still in the minority [**Figure 2B** (Shitamukai et al., 2011)]. This temporal regulation is reminiscent of the division plane shift observed in *Drosophila* neuroblasts, where regulation of the mitotic plane of division is the central mechanism allowing for a switch between symmetric proliferative and asymmetric divisions (Pietro et al., 2016). Moreover, the molecular players regulating spindle orientation are conserved between *Drosophila* and vertebrates. However, in vertebrate development, the orientation of the spindle plane is not the main determinant of the symmetry of a NPC division, as asymmetric fates can and do arise from vertical divisions (Peyre et al., 2011).

The orientation of the mitotic spindle and cleavage plane is regulated by interactions between motor proteins, astral microtubules and the cell cortex. Astral microtubules interact with the cell cortex through microtubule-capture mediated by the LGN/NuMA (protein) complex. In mid-neurogenic stages in the developing mouse neocortex, expression of Inscuteable (Insc) leads to horizontal cleavage planes. Induction of spindle randomization through loss of LGN or overexpression of Insc is followed by increased neuron production and displacement of apical RGCs to a basal position in chick spinal cord (Das and Storey, 2012). This shows that spindle orientation regulation acts in positioning the NPCs within the neuroepithelium (**Section 5.3**).

Spindle orientation in metaphase is continually changing, and it is not stabilized until anaphase (Peyre et al., 2011; Roszko et al., 2006). Interestingly, the amplitude of variation is higher in neurogenic divisions than in proliferative NPC divisions, which might be at least partially explained by the downregulation of apical polarity and AJ proteins (**Figures 2A,D**). A specific population of astral microtubules—those reaching the apical and basal cell cortex—decreases in abundance in neurogenic progenitors when compared to symmetric proliferating progenitors (Mora-Bermúdez et al., 2014; Da Silva et al., 2021). The abundance of astral microtubules to reach the cell cortex is regulated by LGN (Mora-Bermúdez et al., 2014). In NPCs, the tight junction protein Occludin interacts with NuMA, indicating coupling between the junctional belt and the cortical machinery. Mice lacking a long isoform of Occludin show fewer astral microtubules, increased genomic instability and apoptosis (Bendriem et al., 2019), which authors suggest might be due to an elongation of the M-phase which is known to induce premature cell cycle exit and differentiation. Thus, defects in regulation of the cleavage plane can lead to an increase in neurogenesis through lengthening of the cell cycle as a side effect of mitotic challenges, rather than a direct effect on the mechanism of division modes.

Interestingly, many known mutations linked to microcephaly (smaller brains) in humans are related to centrosomal genes, which are implicated in spindle orientation regulation particularly in early symmetric expanding divisions (Marthiens and Basto, 2020). Moreover, mutations affecting spindle orientation can lead to an increase in asymmetric divisions (Konno et al., 2008; Fujita et al., 2020), possibly by inducing the asymmetric inheritance of fate determinants such as the apical membrane or the basal process. However, segregation of the apical membrane also seems to be independent from the cleavage plane (Kosodo et al., 2004), as, e.g., apical polarity components can be asymmetrically inherited even in perpendicular divisions, indicating that there are additional mechanisms regulating their inheritance.

4.5 Centrosome Asymmetry

The centrosome is the main microtubule organizing center of animal cells, and as such it has crucial functions in spindle formation, vesicle transport and cell signaling through the primary cilium. In agreement with its variety of cellular functions, the centrosome plays key roles in NPC biology. An indication of the central role of centrosomes in brain development is that many of the genes mutated in neurodevelopmental disorders such as microcephaly are centrosomal genes (reviewed by Marthiens and Basto, 2020). Some of those mutations cause microcephaly by affecting spindle pole orientation, leading to premature depletion of the progenitor pool as has been previously discussed. Some cause mitotic abnormalities which in turn activate progenitor cell apoptosis. The depletion of centrioles in a p53-null background leads to loss of the apical attachments and displacement of progenitors to a basal location in the mouse developing cortex (Insolera et al., 2014). Presence of supernumerary centrioles in the developing mouse neocortex and zebrafish developing brain leads to microcephaly due to increased multipolar spindles and apoptosis of NPCs (Marthiens et al., 2013; Hu et al., 2014;

Dzafic et al., 2015). Together this suggests that regulation of centriole number and functioning is important in maintenance of NPCs.

In the G_1-phase of the cell cycle, the centrosome is anchored at the ventricular surface where it serves as the basal body for the nucleation of a primary cilium that extends towards the signaling-rich environment of the ventricular lumen [**Figure 3A** (Wilsch-Bräuninger and Huttner, 2021)]. In G_2, the cilium retracts and centrioles duplicate in a semi-conservative manner: the former basal body templates a new centriole and becomes the mother centrosome, while the daughter centriole and the newly formed granddaughter centriole form the daughter centrosome [**Figure 3A**; see a recent review (Blanco-Ameijeiras et al., 2022)]. Notably, the mother and daughter centrosomes are not structurally and functionally equivalent, as the mother centrosome has already undergone the gradual process of centriole maturation and is decorated with appendage proteins important for nucleation of the primary cilium and for microtubule nucleation (Kumar and Reiter, 2021). The daughter centrosome will not fully mature until one and a half cell cycles later (**Figure 3A**). Each centrosome nucleates one half of the spindle pole and is segregated into the sister cell in mitosis. After cytokinesis, daughter cells can regrow a primary cilium, with the mother centriole-inheriting cells establishing a cilium that can respond to Shh signaling before its sister cell (Anderson and Stearns, 2009; Piotrowska-Nitsche and Caspary, 2012). In this way, the centriole duplication cycle leads to an asynchrony in centriole age, and thus, a functional asymmetry in maturation state between old mother centrioles and newly maturing (ex-daughter) centrioles (**Figures 3A,B**).

The inherent functional asymmetry of the mother and daughter centrosomes can be co-opted to introduce cellular asymmetries in daughter cells that can ultimately translate into fate asymmetries [reviewed by (Saade et al., 2018; Wilsch-Bräuninger and Huttner, 2021)]. This was first observed in the asymmetric division of *Drosophila* male germline cells, which divide in such a way that one of the daughter cells remains attached to the hub cells, becoming a new germline stem cell, whereas the other cell is born outside of the niche and becomes a gonioblast [reviewed by (Venkei and Yamashita, 2018)]. In these cells, the mother centriole is retained always in the new germline stem cell, purportedly due to the higher microtubule nucleation capacity of the mother centriole connecting it to the AJs. A stereotypical pattern of centrosome inheritance has since also been shown in the mouse neocortex, where the mother centriole is preferentially inherited by the daughter cell that will retain the progenitor potential, whereas the daughter centriole is inherited by the newborn neuron [**Figure 3A** (Wang et al., 2009; Paridaen et al., 2013)]. It is important to note that not in all cases of asymmetric stem cell division, the stem cell inherits the mother centriole. A good example are *Drosophila* neuroblasts, where the daughter centrosome is inherited by the prospective neuroblast. In the dividing cell, the mother centriole quickly loses MTOC functionality and the daughter centriole quickly acquires it. In vertebrates, random inheritance of centrioles was observed in cerebellum granule neuron precursors (Chatterjee et al., 2018). Together, these findings show an evolutionary conserved correlation between stereotypical centrosome inheritance, asymmetric cell division and cell fate specification in the developing brain.

Subsequent studies have shed light on what particular characteristics of the mother centriole might contribute to promoting stemness in vertebrates. For one, studies have shown that mutations in mother centriole proteins can lead to failure to maintain progenitor ability and induce premature differentiation, probably by promoting detachment from the apical surface (Jayaraman et al., 2016). Importantly, recent studies have demonstrated that mutations of the mother centriole protein Cep83 leads to macrocephaly and expansion of the progenitor pool by increasing apical membrane stiffness and activating the Hippo signaling pathway component YAP that promotes proliferation (Shao et al., 2020). Therefore, it appears that next to cilium nucleation and MTOC functioning, the mother centriole also acts in ensuring adequate apical attachment through controlling mechanical properties of the apical domain.

An additional mechanism whereby the mother centriole favors a progenitor fate is by retaining a fragment of the internalized ciliary membrane throughout mitosis (**Figure 3B**) (Paridaen et al., 2013). Cells that inherit the ciliary membrane remnant are faster in re-growing a cilium after division than its sister cell, and thus show active ciliary-mediated Shh signaling earlier than the sibling cell. Ciliary membrane inheritance is furthermore associated with retention of the stem cell character in asymmetric cell divisions. An interesting observation here is that the mother centriole and basal process are preferentially co-inherited during mitosis (**Figure 3B**), which might suggest an intracellular connection between the two through the cytoskeleton. Asymmetric ciliary remnant inheritance appears to be conserved in vertebrate neurogenesis as it has also been observed in the chick spinal cord (Saade et al., 2017). In the mouse neocortex, in cells destined to remain NPCs, the cilium reforms at the apical membrane, whereas in differentiating neurons, the cilium is established on the basolateral side (Wilsch-Bräuninger et al., 2012). These findings suggests that spatiotemporally controlled asymmetric ciliogenesis coupled to asynchronous Shh signaling is an evolutionary conserved mechanism in NPC divisions.

Taken together these finding, centrosome asymmetries have been found to determine NPC division mode and daughter cell fates through mechanisms that affect positioning within the tissue and synchrony of signaling states (**Figure 3B**). To which extent these mechanisms co-exist in all individual NPCs population or whether they are region-specific is still unclear. Moreover, centriole asymmetries might also be connected to asymmetries in other fate determinants, such as recycled signaling components. Recently, temporal changes to overcome centriole age asymmetry have been identified that are connected to signaling activities [reviewed by (Gonzalez, 2021)], which we will discuss in **Section 5.2**.

4.6 Endosomes

Endocytosis and recycling of ligands and receptors at the plasma membrane is a rapid and flexible mechanism to modulate cell

signaling. Asymmetric distribution of signaling endosomes during mitosis can impose signaling asymmetries in sister cells even before cytokinesis is fully complete [reviewed by (Daeden and Gonzalez-Gaitan, 2018)]. One the best characterized examples of endocytosis regulating symmetry is the regulation of Delta-Notch signaling in sensory organ precursors (SOP) cells in *Drosophila*, where a SOP undergoes an asymmetric division that produces two different precursors cells: pIIa (Notch ON) an pIIb (Notch OFF), that will go on to produce different types of cells (Daeden and Gonzalez-Gaitan, 2018). Establishment of this asymmetry in Notch signaling is determined by several concomitant mechanisms, and among those, endosomal compartments that harbour the ligand Delta and the receptor Notch feature prominently. In mitosis, Rab11+ recycling endosomes distribute symmetrically, but after cytokinesis, they accumulate around the centrosome in the pIIb cell, promoting recycling of Delta in pIIb and subsequent Notch activation in pIIa (Emery et al., 2005). Additionally, Sara endosomes—early endosomes that contain Notch and Delta and in which active Notch signaling can take place—are unequally distributed in cytokinesis and biased towards the pIIa cell (Coumailleau et al., 2009). Asymmetric distribution of Sara endosomes is reported to be caused by asymmetric microtubule density which directs more Sara endosomes towards the pIIa cell (Derivery et al., 2015).

Asymmetric dispatch of Sara endosomes has been shown in other asymmetric stem cell division systems, including asymmetric divisions of neural progenitors in the zebrafish spinal cord (Kressmann et al., 2015; Montagne and Gonzalez-Gaitan, 2014). In that study, authors investigated the partition of Sara endosomes in mitosis and found that the cell inheriting a higher amount of Sara endosomes was most of the time destined to become the progenitor cell [**Figure 3C**, right panel (Kressmann et al., 2015)]. It is important to note that asymmetry in Sara endosome segregation was not predictive of the symmetry or asymmetry of a division, as asymmetric Sara endosome dispatch occurred in symmetric proliferative divisions and asymmetric P-N divisions occurred even with low levels of Sara endosome asymmetry. Moreover, Sara mutants showed no difference in the number of P-P divisions, but an increase in differentiative N-N divisions at the expense of asymmetric P-N divisions, indicating that in P-N divisions, Sara is important for the acquisition of a progenitor fate. This contrasts with a recent study in Sara endosomes in asymmetric neural progenitor divisions in the zebrafish retina, where progenitors divide to produce an uncommitted progenitor (Notch High) and a neurogenic progenitor (Notch Low) (Nerli et al., 2020). Similarly to the spinal cord, Sara endosomes show asymmetric dispatch and its inheritance correlates with high Notch activity and pluripotency (**Figure 3C**). However, disruption of Sara led to an increase in Notch activity and in symmetric proliferative divisions. This indicates that most likely, the precise way in which Sara endosomes modulate Notch signaling is context-dependent, as several other possibly competing mechanisms influence Notch signaling simultaneously.

Other mechanisms in asymmetric cell division that we have mentioned earlier are also connected to Sara endosomes. In the spinal cord, Sara endosomes and apical Par3 segregate to opposite cells, partially corroborating previous results that apical Par3 correlates with neuronal fate in asymmetric P-N divisions (Kressmann et al., 2015). Moreover, the E3 ubiquitin ligase Mib1 that is required for Delta endocytosis, and DeltaD both were found associated with Sara endosomes, suggesting a cell-autonomous Notch activation. This appears to be different in the zebrafish forebrain: a recent study looked at endosomes containing internalized DeltaD and found them to segregate to the Notch-high cell independently and opposite to Mib1 (Zhao et al., 2021). Notably, here, apical Par3 also segregates to the Notch High cell. This study also offered some key insight into how asymmetric partition of internalized DeltaD endosomes is achieved, and found that similarly to Sara endosomes, internalized DeltaD endosomes localized at the center of the spindle in anaphase show asymmetric distribution in telophase. This asymmetric distribution is mediated by the dynein motor complex, and the authors found that a cytosolic pool of Par3 previously thought to be inert, is in fact responsible for engaging dynein in endosomal transport (**Figure 3C**). It is not known at the moment whether the asymmetry exists at the level of the Par3 cytosolic pool or, similarly to fly SOPs (Daeden and Gonzalez-Gaitan, 2018), at the level of microtubule density, leading to biased trafficking towards one pole.

Together, these studies indicate that intracellular asymmetries in distribution of endosomes containing Notch signaling components plays an important role in determining division outcomes (**Figure 3C**). Interestingly, the exact connection between inheritance of endosomes harboring different Notch components is not absolute and seems to be context-dependent, which could also be related to Notch signaling events occurring in *cis* (within a cell) versus in *trans* between neighboring cells (Baek et al., 2018; Nerli et al., 2020).

4.7 Midbody

In dividing NPCs, the cytokinetic furrow ingresses from the basal side toward the apical side, ending with the partition of apical membrane components that we discussed already. The midbody, a temporary structure that is formed in cytokinesis when the actomyosin cytoskeleton constricts around the microtubule bridge, can persist after cytokinesis and can be symmetrically or asymmetrically inherited [reviewed by (Dionne et al., 2015)]. Recent studies have suggested that midbodies can be internalised and can act as an intracellular signaling platform (Peterman et al., 2019). Interestingly, in cultured cells, midbodies were preferentially inherited by the daughter cell that also inherits the mother centriole (Kuo et al., 2011), suggesting that the cytoskeleton asymmetries connected to centriole age asymmetry might play a role in specific midbody inheritance.

In NPCs in mammalian cortical development, there is a bilateral abscission of the midbody remnant. Midbody remnants are much more abundant in early cortical progenitors than in late stage cortical progenitors, and maintenance of the midbody remnant is slightly correlated with symmetric proliferative divisions (McNeely and Dwyer, 2020). In the zebrafish retina, Anillin, an F-actin binding protein with important roles in the midbody, is inherited

asymmetrically in 60% of divisions (Paolini et al., 2015). In those divisions, the cell inheriting Anillin retracts its apical process and migrates basally, and asymmetric distribution of the apical protein Par3 is dependent on Anillin (**Figure 3C**). Hypomorphic Anillin mutants showed an increase in symmetric neurogenic division at the expense of proliferative divisions, and at the transcriptional level, the retinal neuronal marker Atoh5 downregulates Anillin.

Together, this suggests that midbodies indeed play a role in NPC proliferation, though more studies are necessary to elucidate the underlying mechanisms further.

4.8 Other Fate-Determining Factors

Taken together, the research results summarized above provide an overview of the currently known NPC biological features that play a role in division mode selection. In other stem cell systems, additional fate-determining factors have been identified that are asymmetrically segregated between daughter cells [see a recent review by (Sunchu and Cabernard, 2020)]. For example, specific segregation of old mitochondria to the differentiating daughter cells was observed in human mammary epithelial cells (Katajisto et al., 2015). Other cellular asymmetries that have been associated with division outcomes in other stem cell types such as *Drosophila* intestinal and germ stem cells are specific segregation of histones and sister chromatids (Ranjan et al., 2019; Wooten et al., 2020). Whether asymmetric segregations of these organelles and structures also plays a role in NSCs in vertebrates is currently unclear or controversial.

What is clear from recent findings is that asymmetric segregation of organelles and molecules is often interconnected. For example, the polarity protein Pard3 that localizes mainly near AJs also is localized near Sara endosomes and plays a role in their intracellular transport, which influences Notch signaling asymmetries (Zhao et al., 2021). Therefore, an intriguing open question is whether and how the different intracellular fate-determining factors and structures interact and depend on each other. If such fate determining factors act independently, what is the weight of each of them towards division mode selection? As we have discussed before, asymmetric segregation of fate determinants could be expected to act deterministically in selection of division mode. However, in reality, the occurrence of asymmetric segregation of fate determinants is not absolute. For instance, asymmetric retention and inheritance of the primary cilium remnant occurs in about 70%–80% of all mouse neocortical NPCs in early neurogenic stages (Paridaen et al., 2013; Jayaraman et al., 2016). This raises the exiting possibility that NPC subtypes [(Fischer and Morin, 2021; Ortiz-Álvarez and Spassky, 2021), see also **Section 3**] using different combinations of fate-determinant inheritance mechanisms might exist. Another explanation is that these mechanisms are influenced by stochastic processes, which could lead to higher heterogeneity in their prevalence in a population of more or less equipotent and similar NPCs. Future experiments will hopefully shed more insight into these open issues.

5 WHAT DETERMINES WHETHER A NPC DIVISION IS SYMMETRIC OR ASYMMETRIC?

Since individual neural progenitors use both symmetric and asymmetric division modes in their lineages, an obvious question is how the symmetry of the division is determined, and how inherent asymmetries connected to asymmetric division are overcome to allow symmetric division outcomes. In relatively more expanded areas of the brain such as the mammalian neocortex, a higher neuronal production per individual NSC is ensured through spindle orientation changes that underlie generation of specialized basally positioned intermediate progenitors (Uzquiano et al., 2018; Llinares-Benadero and Borrell, 2019; Kalebic and Huttner, 2020). Recent work has provided new insights into how progenitor properties change over time to ensure the proper balance of self-renewing and intermediate progenitors, and differentiating cells as the brain grows.

5.1 Switch From Pre-Neurogenic Symmetric Division to Neurogenic Asymmetric Divisions

The first obvious change in NPC division modes takes place when pre-neurogenic neuroepithelial cells (NECs) switch from their initial symmetric proliferative divisions (**Figure 2A**) to asymmetric neurogenic divisions (**Figures 2B–D**). During this switch, morphological and molecular changes in cell-cell junctions, cell shape and onset of glial cell markers occur (Taverna et al., 2014; Uzquiano et al., 2018). After this transition, these progenitors are commonly named radial glial cells (RGCs). NECs are wide and columnar in shape, whereas RGCs are more slender and elongated. Recent work showed that in primates, the NEC-to-RGC-transition is gradual and involves subtle cell shape changes induced by apical constriction and changes in polarity that is mediated by transient expression of the epithelial-mesenchymal transition (EMT) factor *ZEB2* during the transition, which induces apical constriction through regulation of the actin-regulator Shroom3 (Benito-Kwiecinski et al., 2021). Similarly, in the zebrafish embryonic retina, expansion of the apical domain surface by inhibition of Shroom3 or loss of Llgl1 led to increased Notch activity and diminished neurogenesis (Clark et al., 2012). This suggests that regulation of the apical domain through apical constriction can influence Notch signaling activity, which in turn affects cell fate. However, it is currently still unclear whether and how NEC to RGC transition morphological changes are directly coupled to onset of asymmetric divisions.

As mentioned already, several morphogens and signaling molecules play a role in regulation of NEC self-renewal and the onset of neurogenesis [reviewed by (Agirman et al., 2017)]. Some of these signaling molecules are expressed or secreted at specific locations within the developing brain, leading to anteroposterior and dorsoventral gradients of signaling activity. For instance, in the mammalian forebrain, the anterior neural ridge secretes several Fibroblast Growth

Factors (Fgfs) that influence NSC proliferation and division mode. For instance, Fgf2 shortens the cell cycle and promotes symmetric divisions *in vitro* (Ledesma-Terrón et al., 2020). Fgf10 is transiently expressed by cortical progenitors during NEC to RGC transition. Depletion of Fgf10 extends the pre-neurogenic symmetric proliferative period and delays expression of RGC markers and neurogenesis (Sahara and O'Leary, 2009). Delta-Notch signaling is involved in initiation of neurogenesis. Through salt-and-pepper patterns of proneural gene and Delta ligand expression, Notch signaling is activated in neural progenitors, which supports their proliferation [reviewed by (Kageyama et al., 2019)]. Subsequently, through lateral inhibition between neighboring cells and sister cells, asymmetric neuronal daughter fate is established [reviewed by (Moore and Alexandre, 2020]. Thus, timely onset of Fgf and Notch signaling are key to generating neurogenic radial glial cells that are able to undergo asymmetric divisions. At the same time, the NEC to RGC transition is gradual and in early neurogenic stages, symmetric divisions do still occur, albeit with lower prevalence (Gao et al., 2014).

Even though asymmetric segregation of polarized cell structures is an important mechanism to mediate asymmetric fates in early neurogenic stages, early RGCs appear to have some (latent) capacity to overcome these asymmetries. In mice and human samples, it was shown that early neurogenic stage-RGCs possess the capacity to regrow an apical process in divisions where the apical domain was inherited by the differentiating cells [**Figure 2C**, (Fujita et al., 2020; Shitamukai et al., 2011; Subramanian et al., 2017)]. This ability is dependent on Notch/Integrin-beta1 pathways and is linked to high levels of vesicle transport and recycling of junctional proteins and membranes in early-stage RGCs (Fujita et al., 2020). Similarly, splitting or regrowth of the basal process in the non-inheriting cell has been observed specifically in early neurogenic stages (Kosodo et al., 2008; Shitamukai et al., 2011). Similar to regrowth of the apical domain, re-establishment of the basal process is increased upon forced activation of Notch signaling [**Figure 2C** (Shitamukai et al., 2011; Fujita et al., 2020)]. In conclusion, at early stages when symmetric division predominates, NPCs are able to generate daughter cells that eventually are more symmetric in terms of apical and basal domains (through inheritance and fast re-growth) than their initial division asymmetry. NPCs appear to lose these abilities as embryonic neurogenesis proceeds towards mid- and late neurogenic stages.

Taken together, gradual changes in the morphology and ability to re-establish apical and basal domains appear to underlie the reduction of symmetric proliferative division modes as neurogenesis proceeds. A next question is how symmetric division modes can be reconciled with intrinsic cellular asymmetries.

5.2 Inherent Cellular Asymmetries and Symmetric Division Outcomes

Dynamic control of the asymmetric inheritance of cellular components in mitosis appears to be a fairly straightforward way to establish asymmetric daughter cell fates. The question arises however how inherent cellular asymmetries such as centriole age differences, are overcome in symmetric divisions. In theory, the centriole asymmetry could be compensated for by making centrioles more equal. This could be done in several ways. For instance, specific proteins and thereby functions of the mother centriole could be removed prior to or during mitosis [as observed in *Drosophila* neuroblasts (Marthiens and Basto, 2020; Gonzalez, 2021)] or maturation of the daughter centriole into a new mother centriole could be sped up (Blanco-Ameijeiras et al., 2022). Furthermore, regulation of the pericentriolar matrix (PCM) composition, which couples the centriole to the microtubule network, could also influence centriole symmetry (Kumar and Reiter, 2021). Such measures would enable functional symmetry through regulation of more synchronous primary cilium reformation, symmetric signaling states, and microtubule nucleation and anchoring, and transport of vesicles.

Recent studies have provided evidence that these mechanisms generating centriole symmetry indeed play a role in early pre-neurogenic symmetric divisions and that this involves signaling cascades [see also (Gonzalez, 2021) (**Figure 3B**)]. For example, Notch signaling components have been shown to associate specifically with centrosomes. In the chick neural tube, Mib1 that is inherited asymmetrically by the differentiating daughter cell, was found to associate specifically with the daughter centriole through interaction with the centriolar satellite proteins PCM1 and Azi1 (Tozer et al., 2017). In symmetric proliferative divisions, a pool of Mib1 emanating from the Golgi apparatus docks to the mother centrosome during division, leading to more symmetrical Notch signaling between sister cells (**Figure 3B**). Involvement of Golgi-derived trafficking in overcoming centriole asymmetries was also suggested during pre-neurogenic stages of mouse neocortex development. Here, higher occurrence of symmetrically localized primary cilium components as observed at both centrosomes, one of which presumably constitutes recycled ciliary membrane and the other *de novo* synthesized ciliary membrane (Paridaen et al., 2013) (**Figure 3B**). Together, this suggests that delivery of Golgi-derived vesicles can compensate for centriole asymmetry during symmetric divisions.

One remaining question is how these temporal centrosomal and Golgi dynamics are regulated at the transcriptional level. Another study in the chick spinal cord offers some clues on the transcriptional regulation underlying centrosome functional asymmetry. In chick spinal cord and mouse cerebellum, high levels of Shh signaling maintain symmetric proliferative divisions [**Figure 3B** (Merk et al., 2020; Saade et al., 2013)]. In the chick spinal cord, overactivation of Shh increases symmetric proliferative divisions at the expense of symmetric neurogenic divisions (Saade et al., 2013). A recent follow-up study showed that high Shh signaling activity in NECs increases pericentrin levels at both centrosomes (**Figure 3B**, left panel). This mediates symmetric sequestering of PKA at both centrosomes (Saade et al., 2017), which in turn enhances Shh signaling to promote symmetric proliferative divisions. In contrast, at later stages when asymmetric divisions occur, PKA distribution at centrosomes was unequal (**Figure 3B**, right panel). Crosstalk of Notch and Shh signaling was also observed in primary cilia that

are nucleated by the mother centriole. In the mouse spinal cord, Notch signaling restricts localization of the Shh receptor Patched and promotes accumulation of the Shh component Smo in primary cilia (Kong et al., 2015). In this way, Notch signaling controls the response of NPCs to Shh signals. Together, these findings suggest that signaling activities can induce differential recruitment of PCM proteins in order to compensate for centriole asymmetry to ensure symmetry of Shh and Notch signaling pathways in both daughter cells.

Whereas these findings suggest that mechanisms promoting daughter centriole function act in pre-neurogenic symmetric proliferative divisions, at later neurogenic stages, symmetric differentiative divisions occur in which both daughter cells initiate neuronal fate. Here, the question remains as to how centriole functional asymmetries are overcome in generating symmetric daughter cells. Intriguingly, loss of centrosomal-associated ciliary components is associated with later neurogenesis stages. In the mouse neocortex and chick spinal cord, the association of recycled ciliary membrane in mitotic progenitors is often lost, leading to slower and synchronous cilium reformation between sister cells (Paridaen et al., 2013; Saade et al., 2017). Furthermore, apical constriction through apical microtubule rearrangements and abscission of apical domain containing the ciliary membrane occurs prior to delamination of nascent neurons in chick spinal cord (Das and Storey, 2014; Kasioulis et al., 2017), suggesting that loss or later establishment of a functional primary cilium is important for delamination and differentiation of neurons. Microtubule re-organisation and apical constriction similar to epithelial-to-mesenchymal transition (EMT) through action of the mother-centriole specific protein Akna also plays a role in delamination of intermediate progenitors in the mammalian cortex (Camargo Ortega et al., 2019). This indicates that regulation of the attachments between mother centriole and primary cilium play a key role in neuronal cell fate specification.

Taken together, recent studies have provided some insights into how signaling activity can differentially affect the functional outcome of centriole age asymmetry through intracellular trafficking of centrosome satellite proteins. It would be interesting to learn more about how signaling activity levels are connected to processes such as fate determinant inheritance, intracellular trafficking, centriole composition and nucleation of primary cilia and microtubules by the mother centrioles at different developmental stages. An important open question in this context is what is the role of the timing of signaling activity relative to the cell cycle in determining individual NPC division modes. Though there is little information on this available, a study in chick spinal cord suggested that Notch activity prior to mitosis is connected to immediate activation of Notch activity in both daughter cells, whereas divisions with no prior active Notch signaling were linked to asynchronous and asymmetric Notch activity states between the daughter cells (Vilas-Boas et al., 2011). Another open question is whether localized signaling events can influence the orientation of the mitotic spindle through centriole age asymmetry, as has been demonstrated for Wnt3a signals in cultured embryonic stem cells (Habib et al., 2013).

5.3 Spatiotemporal Regulation of Spindle Orientation and NPC Diversity

As discussed earlier, regulation of spindle orientation is an important mechanism in division asymmetry (**Figures 1B**, **2B**). Interestingly, properties of the mitotic spindle and astral microtubules have been found to change as neurogenesis proceeds. For example, astral microtubules are more plentiful in early symmetric divisions and decrease in neurogenic RGCs (Mora-Bermúdez et al., 2014). This mechanism is proposed to restrict wobbling of the mitotic spindle, and thus, the chance of division asymmetry. In contrast, the density of spindle microtubules of neocortical RGCs increases from early to late-neurogenesis (Vargas-Hurtado et al., 2019). Loading of the microtubule nucleation factor Tpx2 to the spindle MT increases over time and appears to be linked to a higher fidelity of chromosome segregation during mitosis at later neurogenic stages. Asymmetries in spindle size within dividing NPCs has also been observed in mouse developing neocortex (**Figure 3C**). Here, the planar cell polarity regulators Wnt7a and Vangl2 promote asymmetric spindle size that peaks at mid-neurogenic stages. The larger spindle size is associated with neuronal cell fate and the smaller with RGC fate (Delaunay et al., 2014). Together, these findings indicate that astral and spindle microtubule properties are important determinants in restricting spindle orientation deviations and susceptibility to chromosome mis segregation, particularly in early neurogenic stages.

When the spindle orientation is randomized through experimental manipulations, in general this leads to an increase in NPCs that localize more basally away from the ventricle. For example, randomization of the mitotic spindle orientation can be induced through overexpression of Insc or depletion of LGN. Interestingly, the effect of such manipulations show regional and stage-dependent differences. Experiments in the mouse ventral and dorsal telencephalon using acute Insc or LGN manipulations show that these manipulations only affect the spindle of RGCs during mid- and late neurogenic stages, and not in early neurogenic, postnatal and adult stages (Falk et al., 2017). In the mouse ganglionic eminence (the ventral telencephalon), spindle randomization is linked to increased symmetric divisions generating another type of progenitor that lack basal processes, called apical intermediate (aIPs) or short neural progenitors (Falk et al., 2017). These aIPs have apical domains and remain anchored at the ventricular surface, but are not able to re-establish basal processes and typically only generate neurons (Falk et al., 2017; Ramos et al., 2020). These aIPs are also present in the dorsal telencephalon, where their generation depends on temporary expression of the non-canonical tubulin Tuba8 downstream of Fgf10 signaling (Ramos et al., 2020). In the dorsal telencephalon, spindle orientation change is key to the production of neocortical bRGCs in a classis type of asymmetric RGC division

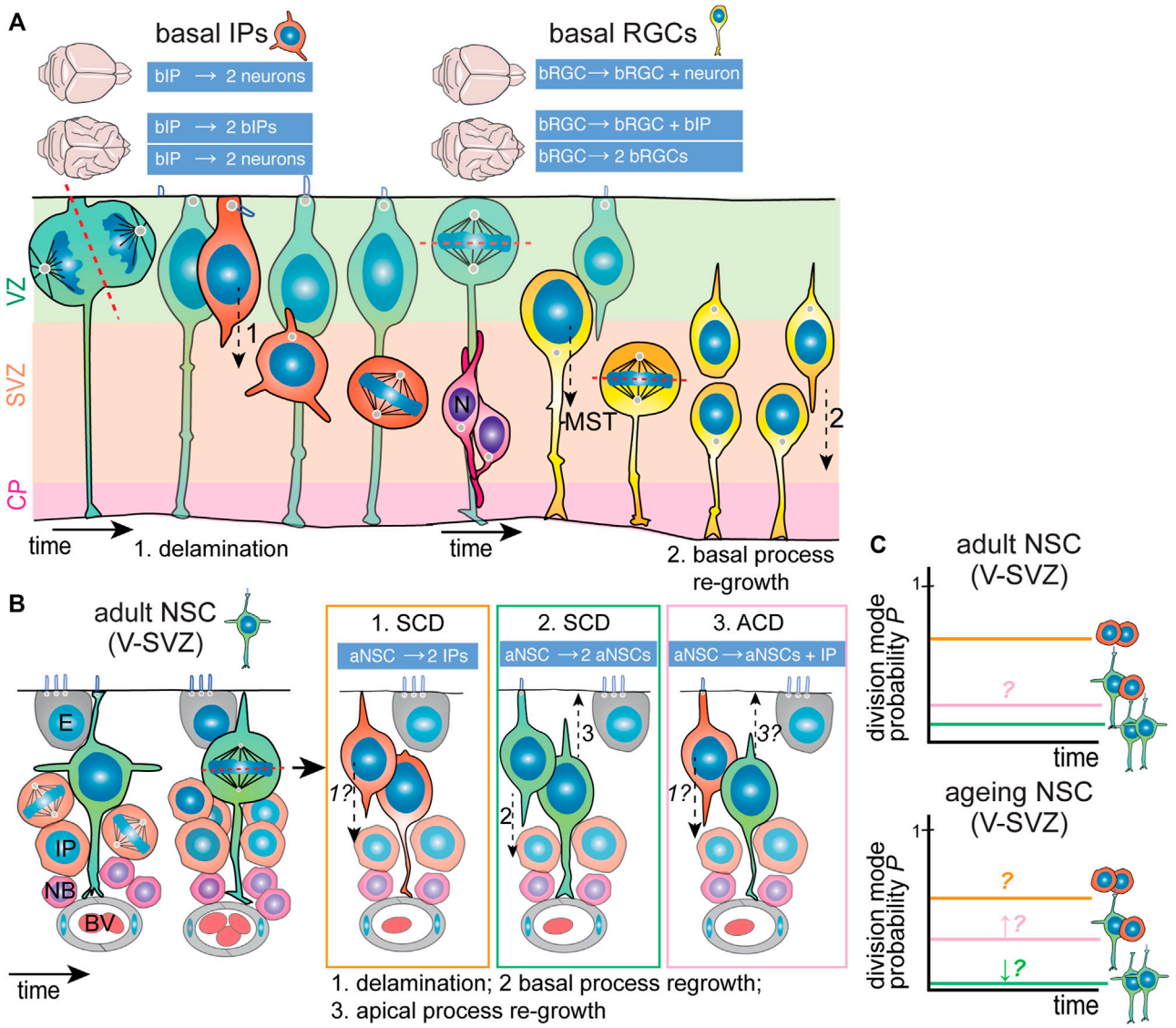

FIGURE 4 | Division modes used by neocortex embryonic IPs and adult V-SVZ NSCs. **(A)** Basal IPs (orange; left) are born from asymmetric RGC divisions. Once born, bIPs delaminate from the ventricular zone (VZ, light green) and migrate basally. Here, they are unpolarized and divide with random spindle orientation. Depending on the species and extent of lissencephaly/gyrencephaly, bIPs generate mainly neurons (magenta) directly (lissencephalic) or can also undergo self-renewing divisions (gyrencephalic brains). Basal RGCs (yellow; right) are born from horizontal RGC divisions. Once born, they relocate to the inner and outer subventricular zones (SVZ, light orange) where to undergo mainly horizontal divisions, preceded by mitotic somal translocation (MST). Depending on the species and extent of lissencephaly/gyrencephaly, they divide asymmetrically generating one self-renewing and one differentiating daughter cell (lissencephalic) that migrates to the cortical plate/neuronal layers (CP, light purple) or can also undergo self-renewing divisions (gyrencephalic brains). In the latter case, the apical-most daughter cell can re-grow a basal process. **(B)** Adult NSCs (green) in the ventricular-subventricular zone (V-SVZ) are positioned within a specialized niche. They are polarized and have a small apical domain neighboring the lateral ventricle. Once activated, they appear to undergo mainly horizontal divisions, and can generate two IPs (orange; option 1); expand through symmetric division (green; option 2) or (rarely) undergo asymmetric division (pink; option 3). In turn, IPs generate neuroblasts (magenta) that also amplify and migrate to the olfactory bulb. It appears that aNSC daughter cells possess the potential to re-grow apical (3) and basal (2) processes. Possibilities regarding delamination from the ventricular zone and process regrowth are indicated with italics and question marks. **(C)** The current models of division mode usage by aNSCs (top graph). Presumably, the main division mode is symmetric differentiative division (~70%–80%). The presence of asymmetric divisions is controversial due to conflicting data, though it has been proposed that asymmetric division increases during ageing (bottom graph). ACD, asymmetric cell division; aNSC, adult neural stem cell; bIP, basal intermediate progenitor; bRGC, basal radial glial cell; BV, blood vessel; E, ependymal cell; IP, intermediate progenitor/transient amplifying progenitor; N, neuron; NB, neuroblast; NSC, neural stem cell; SCD, symmetric cell division; V-SVZ, ventricular-subventricular zone.

(**Figure 2B**). bRGCs are born from horizontal divisions that start occurring at mid-neurogenic stages in the mammalian neocortex [reviewed by (Kawaguchi, 2021)]. The cleavage plane dissects the dividing cell into one daughter with apical constituents and one daughter cell inheriting the basal process which is important for their self-renewing capacity [**Figure 2B** (Shitamukai et al., 2011; LaMonica et al., 2013)].

A recent study showed that the time window restriction for bRGC generation to occur only from mid-neurogenic stages is due to a higher capacity of early aRGCs in the mouse neocortex to re-establish an apical domain upon spindle randomization [**Figure 2C** (Fujita et al., 2020)]. In this way, at early neurogenic stages, daughter cells without apical domains are able to re-establish their incorporation into the ventricular zone junctional belt. Taken together, these findings show that division asymmetry induced by spindle orientation (**Figure 2B**) coupled to the differential abilities of neural progenitors to re-establish apical or basal domains (**Figure 2C**) is an important factor in establishing the large diversity of morphological NPC subtypes in the developing mammalian brain.

5.4 Regulation of Cell Division in Basal Progenitor Cells

As mentioned in the introduction, basal progenitors (BPs) are the progeny of apical NPCs, and act as amplifying neurogenic progenitors. This strategy of indirect neurogenesis results in an increased neuronal output, and comparative studies in recent years have shown that BPs are in large part responsible for the increased relative neocortical size in humans compared to other primates [reviewed by (Penisson et al., 2019; Kalebic and Huttner, 2020)]. Comparative studies have proved to be especially useful in studying BPs. Here, small interspecies differences in BP biology underlie extremely important evolutionary changes, such as the extent of neocortical folding.

The shared biological feature of these BPs is their ability to divide away from the ventricular surface. Instead, BPs populate the outer sub-ventricular zone (OSVZ), where they establish a progenitor niche that self-expands and generates neurons. Notably, OSVZ relative thickness is much bigger in the neocortices of gyrencephalic species like the ferret than in those of lissencephalic species like mice (Martínez-Cerdeño et al., 2012). In the chicken embryonic dorsal pallium, the region analogous to the mammalian neocortex, a specific domain where non-apical mitosis are abundant has been described (Cárdenas et al., 2018). Interestingly, a very small number of non-apical progenitors have been identified even in the zebrafish embryonic brain and spinal cord (McIntosh et al., 2017). These progenitors share characteristics with mammalian BPs, such as loss of apical attachment and mitosis away from the ventricular surface. Whether these non-apical progenitors are evolutionarily related to mammalian BPs needs further investigation.

Generally, two types of BPs can be distinguished that differ in cell architecture and proliferative potential: 1) low-proliferative basal intermediate progenitors (bIPs), which are not tethered to the pial surface by a basal process and have a multipolar morphology (**Figure 4A**, left), and 2) highly-proliferative basal radial glia (bRGCs, also termed outer radial glia), which retain the basal process and radial architecture (**Figure 4A**, right). While the cause for difference in proliferative ability between these two subtypes is still an open question, it appears that the retention of the basal process, known to be of critical importance in apical NPC stemness, is a key factor. Though bRGCs constitute a small percentage of total BPs in the mouse neocortex, they are much more abundant in the embryonic brain of gyrencephalic species (Kalebic et al., 2019). It is now believed that bRGCs are indeed the cellular basis of neocortical folding. Efforts from different research teams have identified several ape-specific or human-specific genes that are able to induce the generation of bRGCs and tissue folding when expressed in mice embryonic brains (Florio et al., 2015; Ju et al., 2016).

bRGCs are born from apical NPC divisions, and thus can inherit an apical process that needs to be disassembled for their migration to the OSVZ (Tavano et al., 2018). Interestingly, research seems to indicate that the mechanisms underlying delamination of bRGCs and differentiating neurons are shared to an extent. For instance, the centrosomal protein Akna plays a role in delamination of both cell populations, purportedly by mobilizing microtubules away from junctional complexes (Camargo Ortega et al., 2019). Similarly, the microtubule-associated Lzts1 promotes bRGCs and neuron production by inducing apical constriction and inducing oblique divisions in apical NPCs of mice and ferrets (Kawaue et al., 2019). Recently, the gene LIS1, which codes for a dynein regulator and mutations in which cause lissencephaly, has also been shown to be important for bRGC production, as well as for neuronal migration (Penisson et al., 2022). These commonalities between differentiating neurons and delaminating bRGC further illustrate the intermediate character of bRGCs.

Still, bRGCs show certain distinct behaviors that distinguish them from bIPs. They typically divide with a horizontal division with the basal process being inherited by the basal-most daughter cell (**Figure 4A**). In mice, bRGCs divide mainly asymmetrically generating one bRGC daughter and a differentiating daughter cell. In contrast, in gyrencephalic brains, bRGCs have higher self-renewing capacity and can divide symmetrically into two bRGCs or asymmetrically into one bRGC and a bIP [**Figure 4A** (Wang et al., 2011; Betizeau et al., 2013; LaMonica et al., 2013; Gertz et al., 2014)]. The actin cytoskeleton is important for regulation of the length and direction of the basal processes, which in human bRGCs is regulated by the Rho GTPases Rac1 and Cdc42 activity downstream of mTor signaling (Andrews et al., 2020). Prior to mitosis, bRGCs undergo a rapid movement of their cell body to the OSVZ, which is termed Mitotic Somal Translocation (MTS). Similarly to interkinetic nuclear migration, MTS is coordinated in time with mitosis but functionally independent. Mechanistically, MTS is regulated by the actomyosin cytoskeleton (Ostrem et al., 2014; Kawaue et al., 2019; Andrews et al., 2020). The precise biological significance of MTS has still not been characterized, but authors have speculated that MTS might serve to reduce tissue crowding. Intriguingly, MTS is a cell-autonomous mechanism as it occurs even in dissociated bRGCs (Ostrem et al., 2014). Experiments have shown that inhibition of MTS does not directly affect cell fate, but whether it influences the long-term proliferative potential of individual bRGCs needs further investigation.

Collectively, the proliferative capacity of individual BPs is highly heterogeneous (Pfeiffer et al., 2016), yet the basis of this heterogeneity is not fully understood. Single-cell RNA-seq expression profiling has uncovered some previously unidentified subtypes of BPs in human embryonic neocortex (Pebworth et al.,

2021). Moreover, live imaging studies have revealed distinct characteristic morphotypes in gyrencephalic species (Betizeau et al., 2013; Kalebic et al., 2019; Pebworth et al., 2021). Strikingly, further research has shown that BP morphology has a strong influence on its proliferative ability (Betizeau et al., 2013; Kalebic et al., 2019). Aside from the basal process of bRGCs, both bRGCs and bIPs have small filiform protrusions of the cell body called lamellate expansions, and the number and length of these expansions positively correlate with proliferative potential. Moreover, these are present in human and ferret BPs, but not in the less proliferative mice BPs (Kalebic et al., 2019). A possible mechanistic basis underlying this correlation between cell body protrusions and proliferation might be that lamellate expansions mediate the reception of extracellular signals. For instance, integrin signaling through these protrusions has been demonstrated to support BP proliferation (Kalebic et al., 2019). In agreement with this, RNA-seq profiling of human BPs has also shown particular enrichment of extracellular matrix (ECM) related genes (Pollen et al., 2015). Lamellate expansions also mediate Notch signaling between BPs and apical RGCs (Nelson et al., 2013). Other signaling pathways known to influence BP proliferation are Sonic hedgehog, which promotes BP production both by stimulating apical RGC proliferation and BP self-renewal (Hou et al., 2021), and the Hippo pathway (Kostic et al., 2019). Conversely, Robo/Slit signaling promotes direct neurogenesis (Cárdenas et al., 2018). The identification of different BP subtypes open the possibility that signaling pathways influence specific subpopulations of BPs differently. Further efforts into characterizing the biological differences between BP subtypes and their relation to potential differences in proliferative ability will hopefully bring some answers in the near future.

As many advances as have been made in the knowledge of the morphological and transcriptional features that govern BP biology, the cell division symmetry or asymmetry mechanisms in these cells have not been uncovered. Furthermore, it is not known whether they show also show specific (spatiotemporally controlled) probability distribution of division modes. BPs lack signaling from the ventricle, apical membrane and classic apicobasal polarity and lateral junctions, all of which are important factors in establishing symmetry in apical NPCs divisions. It will be interesting to see to what extent these differences reflect on the mechanism of division mode selection.

6 HOW IS NEURAL PROGENITOR DIVISION MODE REGULATED IN THE ADULT NERVOUS SYSTEM?

Towards the end of embryonic mouse neurogenesis, a subset of RGCs slows their cell cycle and turns into adult NSCs (Fuentealba et al., 2015; Furutachi et al., 2015; Berg et al., 2019). Whereas mammalian species retain just a limited number of NSC niches where adult neurogenesis occurs, other vertebrates such as zebrafish show much more widespread neurogenesis (Labusch et al., 2020). However, adult NSCs (aNSCs) from different vertebrate species have in common that they are largely quiescent, and divide only rarely. When activated, the aNSCs generate intermediate progenitors that in turn undergo several divisions to increase neuronal output. The adult NSC zones in mammals are the ventricular-subventricular zone (V-SVZ, also called subependymal zone SEZ) of the lateral ventricle, and the subgranular zone (SGZ) in the hippocampal dentate gyrus in the hippocampus. The SEZ generates olfactory neurons and oligodendrocytes, with the long-term NSCs being mainly quiescent and activated NSCs producing transient amplifying progenitors and neuroblasts that are neurogenic. While aNSCs have certain morphological properties, such as apicobasal polarity, in common with embryonic progenitors, the adult NSC niche is an important regulator of NSC divisions and differentiation and the lineages produced are more restricted than that of embryonic NPCs [reviewed by (Obernier and Alvarez-Buylla, 2019)].

Evidence regarding aNSC division modes is sparse, as the tissue is less amenable to live imaging experiments and divisions of the *bona fide* stem cells are rare. Fortunately, elegant lineage tracing approaches and recent advances in microscopy have provided insights in aNSC division modes and their regulation (**Figure 4B**). Several studies using time-lapse imaging and lineage tracing methods have now shown that in the mammalian SEZ, asymmetric division of aNSCs hardly occurs. Instead, stochastic selection of either symmetric proliferative or differentiative divisions, with higher probability of the latter, have been observed [**Figures 1E**, **4B,C** (Basak et al., 2018; Obernier et al., 2018)]. From these studies, maintenance of quiescent NSCs (qNSCs) during the production of neurons was proposed to occur at the population level (population asymmetry), rather than invariant division asymmetry (**Figure 1F**) at the individual level. In this study, an important role was proposed for strict regulation and qNSC occupation in the niche similar to qNSC maintenance in the intestine. aNSCs were found to be able to return to quiescence to maintain the number of qNSCs per niche (Basak et al., 2018). Computational modeling based on lineage-tracing data comparing young and old mice showed that asymmetric division might actually be more prevalent than previously proposed (**Figure 4C**) (Bast et al., 2018). This modeling also predicted that over time, the probability of an aNSC to undergo asymmetric divisions (**Figure 4C**) increases and the frequency of qNSC activation and inactivation is decreased, leading to NSC progeny being less mature and with fewer activated NSC at each timepoint (**Figure 4C**) (Bast et al., 2018). These findings indicate that for the SEZ, data on the division modes used are contradicting and that next to population asymmetry, asymmetric divisions may also play a role.

In contrast, recent work on the SGZ in the adult hippocampus have indicated that the aNSC division modes are more diverse and are similar to that of embryonic NSCs (Pilz et al., 2018; Bottes et al., 2021). Time-lapse imaging and computational modeling showed that hippocampal aNSCs undergo division mode switches over time consisting of initial symmetric self-renewing divisions, followed by increasing probabilities for asymmetric and symmetric terminal subsequent divisions (Pilz et al., 2018; Bottes et al., 2021). Lineage-tracing in the adult zebrafish dorsal telencephalon demonstrated a subpopulation of deeply quiescent cells that divide asymmetrically, generating a more

active pool of NSCs that choose their division mode stochastically (Than-Trong et al., 2020). Taken together, studies from several vertebrate organisms indicate that adult NSCs show specific hierarchies, with possible presence of an asymmetrically dividing deeply quiescent reservoir NSCs and a more actively cycling pool of activated NSCs and progenitors. However, evidence is conflicting as to whether activated NSCs and progenitors show consistent population asymmetry through symmetric divisions or whether asymmetric division is also of significance (**Figure 4C**, top panel).

Even though we now have some insight into the division modes used by aNSCs, very little is known about the molecular mechanisms, and how these are similar or different from embryonic NSCs. Recent studies have provided some clues (**Figure 4B**, right panels). Time-lapse imaging of aNSCs in mouse SEZ slices were shown to retain the basal process during mitosis, with the non-inheriting daughter cell able to regrow a basal process (**Figure 4B**) (Obernier et al., 2018) similar to early symmetric embryonic NPCs (Shitamukai et al., 2011; Fujita et al., 2020). In contrast to its effect on embryonic NPCs, spindle randomization in the SEZ through acute overexpression of Insc does not affect the numbers of aNSCs and neuroblasts in mouse SEZ (Falk et al., 2017). Similar to embryonic NSC divisions, asymmetric segregation of signaling components and fate determinants could play a role in aNSC as well. For example, asymmetric segregation of Delta1 ligands has been observed in SEZ NSCs *in vitro* (Kawaguchi et al., 2013), but it is not yet clear whether this also happens *in vivo* and is related to specific daughter cell fates. A lateral endoplasmic reticulum (ER)-diffusion barrier has been demonstrated in both embryonic and adult hippocampal NSCs that mediates asymmetric segregation of damaged proteins into the differentiating daughter cell (Moore et al., 2015). Intriguingly, this barrier and asymmetric segregation of damaged proteins has been observed to weaken over age, which could contribute to the decreased functioning of aged aNSCs.

Similar to embryonic NPCs, epithelial properties such as apicobasal polarity is important for aNSCs. For example, recent work showed that the EMT factor Zeb1 is required to maintain hippocampal aNSC self-renewal and prevent premature differentiation. Here, Zeb1 maintains asymmetric division mode through regulation of the transcription factor Etv5, showing that specific TFs actively regulate division mode selection in the adult hippocampus (Gupta et al., 2021). The currently limited data suggest that segregation of apical/basal domains (**Figure 4B**) and asymmetric segregations of fate determining factors such as Notch ligands occur in aNSCs similar to embryonic NSCs (Obernier et al., 2018). However, seeing that aNSCs are very dependent on their niche and spend most of their time in quiescence, it is likely that the combination of mechanisms underlying the symmetry of their divisions is distinct from that in embryonic NPCs. Future work in species with higher levels of adult neurogenesis like the zebrafish, will hopefully provide more information on the specifics of division mode selection by aNSCs.

7 CONCLUSION AND OUTLOOK

In this review, we have summarized the current knowledge on how neural stem and progenitor division modes are determined, and how timely changes in division mode, and the proper balance between self-renewal and differentiation is key to brain development. Interestingly, recent studies regarding how cellular properties may be differentially regulated throughout development, show intriguing insights into the complexity of the molecular and cell biological mechanisms that underlie asymmetric and symmetric division. While individual mechanisms in asymmetric NSC division are reasonably well understood, an integrated view of the subtleties that go with gradual developmental changes in division mode outcomes is still far away. Moreover, we are just starting to understand how individual stem and progenitor cells determine their life path based on a combination of intrinsic and extrinsic input, including deterministic processes, such as fate determinant inheritance and stochastic processes such as gene expression levels that in turn influence cellular properties.

Based on the currently available information, a number of relevant outstanding questions can be identified. For example, how are transcriptional changes during development coupled to the gradual changes in NPC morphology, organelle inheritance and division modes? Despite our knowledge on the general role of signaling pathways in regulation of the proliferation versus differentiation balance, we know very little about the details at the individual cell level. For instance, what are the combinatorial effects of different signaling pathways affect division mode selection? What is the effect of cell cycle stage-specific activation states of signaling pathways on individual NPC division modes? Recent studies have identified intriguing links between centriole asymmetry and signaling in embryonic NPCs. It would be very interesting to explore this link further and to understand more on how the compensatory mechanisms to overcome centriole age differences are regulated over time transcriptionally. Furthermore, the role of centriole age asymmetries in aNSC lineage progression is completely unknown. Ultimately, NPC division mode outcomes depend on combinations of different mechanisms. If more hidden asymmetries and the weight of each deterministic factor is known, it is interesting to explore how well individual NPC division outcomes could be predicted using computational models.

As we have discussed, the mechanisms regulating division mode in adult NSCs are currently underexplored. Here, it would be of use to assess the differences and similarities in the mechanisms determining the symmetry of division in embryonic versus adult NSCs further. For instance, considering the prevalence of symmetric divisions in adult germinal zones such as the SEZ, is regrowth of apical and basal domains also more prevalent? How are embryonic NSC initially selected as adult NSCs and which mechanisms (for instance transcriptional and epigenetic changes) underlies their slowing of the cell cycle? Are individual quiescent aNSCs maintained through invariant asymmetric divisions, and which

mechanisms are involved in mediating such asymmetric fate outcomes? Furthermore, as in mammals, regeneration and repair of nervous system damage is limited, it would be valuable to explore further how neurogenesis in vertebrate species such as fishes with higher capacity for neuronal regeneration is regulated and how regulation of division modes is involved in regeneration.

To address these questions, specific challenges remain in connecting the individual fate-determining factors, asymmetries and processes and their interactions to find how the combination and weight of each deterministic and stochastic mechanism influences the division mode of each NPC type. Here, the recent advances in and increasing number of published reports on single-cell analysis [like single-cell genomics and proteomics, e.g., (Schier, 2020)] studies of NPCs in multiple life-stages and species, could play a key role to unravel those subtle hidden asymmetries. Moreover, mathematical modelling and simulations have proven extremely valuable in understanding the connection between specific fate-determining mechanisms and stem cell choices. In combination with more classic approaches such as lineage tracing and time-lapse microscopy (VanHorn and Morris, 2020), these approaches should lead us to integrated and robust models of NPC division outcomes in different life stages and distinct species.

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GC and JP conceptualized and wrote the manuscript, and prepared the figures.

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Forebrain Organoids to Model the Cell Biology of Basal Radial Glia in Neurodevelopmental Disorders and Brain Evolution

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The acquisition of higher intellectual abilities that distinguish humans from their closest relatives correlates greatly with the expansion of the cerebral cortex. This expansion is a consequence of an increase in neuronal cell production driven by the higher proliferative capacity of neural progenitor cells, in particular basal radial glia (bRG). Furthermore, when the proliferation of neural progenitor cells is impaired and the final neuronal output is altered, severe neurodevelopmental disorders can arise. To effectively study the cell biology of human bRG, genetically accessible human experimental models are needed. With the pioneering success to isolate and culture pluripotent stem cells *in vitro*, we can now routinely investigate the developing human cerebral cortex in a dish using three-dimensional multicellular structures called organoids. Here, we will review the molecular and cell biological features of bRG that have recently been elucidated using brain organoids. We will further focus on the application of this simple model system to study in a mechanistically actionable way the molecular and cellular events in bRG that can lead to the onset of various neurodevelopmental diseases.

Keywords: neural progenitor cells, neural stem cells, neurodevelopmental disease, brain evolution, cerebral organoid

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STUDY OF HUMAN DEVELOPMENT IN A DISH

The temporal series of events that leads to the acquisition of specific structural and functional features of different organs in the human body is a fascinating, yet not fully understood phenomenon known as organogenesis. Heart, brain, skin and liver are all very distinct and specific organs with their own distinct functions, yet they developmentally originate from a single cell. Unveiling the cascade of steps leading from such a simple disordered system to an ordered complexity is not only essential from a developmental biology perspective but also for establishing therapeutic approaches in the context of regenerative medicine.

Whereas the classical *in vivo* model systems, such as Drosophila, zebrafish and mouse provided fundamental insight into the basic animal, vertebrate and mammalian development respectively, certain aspects of the complexity observed in humans can only be studied in the human model system. Hence, 2D *in vitro* and *ex vivo* systems, such as organotypic cultures, have been valuable to reveal human-specific features of organ development and pathology (Shamir and Ewald, 2014).

Abbreviations: aRG, apical radial glia; bIP, basal intermediate progenitor; bRG, basal radial glia; GW, gestational week.

However, they lack either the spatial complexity of the tissue or the ability to study the development for prolonged time periods. This raised the need to establish a human model system that would mimic human organogenesis with a sufficient level of spatio-temporal complexity.

The first step towards this goal was provided by the pioneering work of somatic cells reprogramming into pluripotent stem cells (PSCs) (Takahashi and Yamanaka, 2006). The subsequent ability to grow PSCs enabled the exploitation of this technology to generate human stem cell derived cultures (Takahashi et al., 2007). Additionally, cultivation of PSCs in a 3D configuration enabled the cell-cell and cell-extracellular matrix (ECM) communication which would otherwise be absent in a 2D culture (Blau and Miki, 2019). In 2008 a remarkable work conducted by Eiraku and others established for the first time a 3D polarised cortical tissue from embryonic stem cells (ESCs) (Eiraku et al., 2008). This paved the road towards the use of organoids, as multicellular structures that exhibit the capacity to self-organise into a complex system, to study organogenesis in a dish. The term "organoid" was consolidated by Sato and others who established for the first time intestinal organoids from single adult stem cells (Sato et al., 2009). Together, these fundamental studies led to the widespread application of organoid technology in developmental biology (Kaluthantrige Don and Huch, 2021).

Organoids contain the genetic background along with the cell-cell and cell-ECM interactions similar to those *in vivo*, however, they are grown in a simpler and fairly controllable environment (Shamir and Ewald, 2014). On the one hand this is a limiting factor in recapitulating the physiological features of organogenesis. On the other hand, this is an opportunity to dissect in depth the biology of cells of interest within an environment that can be readily controlled. For instance, hepatocyte organoids, that recapitulate the spectacular ability of the liver to regenerate upon a partial resection, can be used as a magnifying glass to study the cell types underlying this regeneration (Hu et al., 2018; Peng et al., 2018). In the context of human pathologies, organoids hold potential to treat various diseases, such as acute kidney injury or diabetes (Lancaster and Huch, 2019). Recently, kidney organoids transplanted under the renal capsule of mice acquired *de novo* vascularisation and tubular maturation (van den Berg et al., 2018), allowing future applications for the treatment of renal failure. Furthermore, the use of pancreatic islet organoids as a source of β-cells *in vitro* may potentially be an alternative cell therapy for diabetes (Wang et al., 2020). Comparison between human fetal retina and retinal organoids showed considerable similarities, thus anticipating a potential role of retinal organoids as cell source for transplantation in cell therapy (Sridhar et al., 2020).

A striking example of a model system that successfully simplifies a highly complex organ, but however mimics the key aspects of the development, is a brain organoid (see section 1.3 for discussion on brain organoids) (Heide et al., 2018; Benito-Kwiecinski and Lancaster, 2020; Lopez-Tobon et al., 2020; Marton and Pașca, 2020; Velasco et al., 2020; Sidhaye and Knoblich, 2021). Untangling the functional dynamics of distinct brain cells using animal models is a very tedious process due to the remarkable complexity stemming from the interaction of the brain with the other organs and the environment. One fundamental question is to understand how this complexity arises during development.

BASAL RADIAL GLIA - A KEY CELL TYPE FOR HUMAN NEOCORTEX DEVELOPMENT

The cerebral cortex, and its evolutionary most recent part, the neocortex, arise from the forebrain region of the neural tube. It is arguably considered that the higher cognitive abilities of humans compared to other mammals are reflected by the size and the cytoarchitectural organisation of the human neocortex (Molnár et al., 2019; Rakic, 1988, 1995). Development of the neocortex initiates with the proliferation of neuroepithelial cells lining the neural tube. Transition from a proliferative state into a neurogenic state gives rise to apical radial glia (aRG), the chief parental progenitor cells that will initiate the neurogenesis, that is the series of events that lead to the production of neurons (Götz and Huttner, 2005; Taverna et al., 2014). The identity of the progenitors is defined based on the location of their mitosis (Taverna et al., 2014), which highlights the importance of the microenvironment for the cell fate specification. Indeed, proliferation of aRGs occurs on the ventricular (apical) surface and these cells form the apical-most neocortical histological layer, known as the ventricular zone (VZ) (Götz and Huttner, 2005). Moving along the apicobasal axis, asymmetrical divisions of aRGs give rise to basal progenitors that populate the second germinal layer, the subventricular zone (SVZ). In species with an expanded neocortex, the SVZ is divided into two distinct zones: the inner and the outer SVZ (ISVZ and OSVZ, respectively) (Smart, 2002; Dehay et al., 2015).

The neocortical expansion in mammals has been widely associated with a subpopulation of basal progenitors called basal or outer radial glia (bRG or oRG) (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011). The abundance of bRG and their proliferative capacity are strikingly increased in species with an expanded neocortex, such as human, macaque or ferret (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Betizeau et al., 2013; Kalebic et al., 2019), compared to species with a small brain, such as mouse (Wang et al., 2011; Wong et al., 2015). This results in an increased production of neurons, which in turn is associated with the expansion and folding of the neocortex. Hence, bRG are considered to be a key cell type underlying human neocortex development (Penisson et al., 2019; Kawaguchi, 2020; Pinson and Huttner, 2021; Del-Valle-Anton and Borrell, 2022). An additional layer of cellular complexity within the bRGs lies in their morphological heterogeneity (Kalebic and Huttner, 2020). We have identified that an increasing number of basal processes within the human bRGs coincides with an increase in their proliferative capacity (Kalebic et al., 2019). Interestingly, such bRGs complexity and proliferative capacity are absent in the mouse cortex, further corroborating the role of bRGs as chief cells underlying mammalian neocortical expansion.

As many studies have started to focus on this fascinating population of cells, multiple outstanding questions remain to be

TABLE 1 | Brain organoid protocols.

Organoid protocol	Method (Guided or unguided)	Cell line	EB generation	Matrigel	Bioreactor	Orbital shaker	Slicing
Kadoshima et al. (2013)	guided	ESCs	From single cells 96 WP	✓ From day 35 (Matrigel 1% vol/vol) From day 70 (Matrigel 2% vol/vol)	✗	✗	✗
Lancaster et al. (2013)	unguided	ESCs: H9 H1	From single cells 96 WP	✓ From day 11 in Matrigel droplets	✓	✗	✗
Giandomenico et al. (2021)	guided	ESCs H9 H1	From single cells 96 WP with microfilaments	✓	✓	✓	✓ Only once between day 45 and 60
Pasca et al. (2015)	guided	iPSC	From single cells 96 WP	✗	✗	✓	✗
Qian et al. (2020)	guided	iPSC	From whole colonies Use of collegenase to lift the colonies 6WP	✓ During the forebrain patterning day 7–14	✓ Optional during differentiation Day 14–72	✓ During differentiation and maturation Day 45–150	✓ Day 45 - Day 150 Once a month

Abbreviations: EB, embryoid body; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells.

addressed. For example, what is the molecular and cell biological heterogeneity of bRG across different species and what is their contribution to the onset of human intellectual disabilities. The use of a reductionist system containing abundant bRGs such as the organoids may elucidate the mechanisms that lead towards the complexity of the neocortex organogenesis and pathogenesis. This review will focus on the diverse modalities to generate brain organoids and how we can exploit this technology in the context of neocortex development and pathologies, specifically focusing on bRGs.

FOREBRAIN ORGANOIDS

During embryogenesis, the interplay of diverse signalling pathways leads to the differentiation into neuronal fate. Initial inhibition of the the bone morphogenic proteins (BMP) signalling is needed for the differentiation into the neuroectoderm which then invaginates to give rise to the neural tube (Muñoz-Sanjuán and Brivanlou, 2002; Sadler, 2005). Patterning of the neural tube into different regional identities is achieved through the regulated activity of WNT, fibroblast growth factor and retinoic acid pathways (Muñoz-Sanjuán and Brivanlou, 2002; Molotkova et al., 2005). To model such embryonic development *in vitro* and building on the earlier pioneering work, two studies reported the generation of brain organoids in 2013 (Kadoshima et al., 2013; Lancaster et al., 2013). Two distinct approaches have been applied for generating brain organoids: 1) the unguided method, which directs the generation of organoids with multiple regional identities; and 2) the guided method, which promotes the acquisition of specific regional identity through a step-wise time-dependent exogenous signalling (**Table 1**) (Lancaster and Knoblich, 2014; Di Lullo and Kriegstein, 2017). Each approach starts with the generation of 3D aggregates named embryoid bodies (EBs), which have the potential to differentiate into all three germ layers (Zhang et al., 2001). The first cerebral organoids were generated from EBs following the intrinsic program of neuroepithelial cells to differentiate into neural progenitor cells (Lancaster et al., 2013). Such unguided protocol results in a stochastic development of various and multiple regional identities (Ormel et al., 2018). Alternatively, the differentiation can be directed towards the acquisition of a specific regional identity, such as the dorsal forebrain. The latter can be achieved through the manipulation of the transforming growth factor-beta (TGF-β) signalling pathway and BMPs (Chambers et al., 2009; Kadoshima et al., 2013; Paşca et al., 2015; Qian et al., 2018, 2020; Sloan et al., 2018). Of note, the time dependent addition of small molecules in presence (Kadoshima et al., 2013; Lancaster et al., 2013; Qian et al., 2018, 2020; Sloan et al., 2018) or absence (Mariani et al., 2015) of Matrigel, a commercially available basement membrane matrix from mouse sarcoma (Li et al., 1987), shows a remarkable difference in the developmental timelines, prompting a question on how it might affect the progenitor biology (**Table 1**).

Although the timeline in the induction of neurogenesis is different, all protocols partially recapitulate the series of events known to occur in the developing human neocortex (**Figure 1**). Indeed, organoids readily contain aRGs marked by the expression of the transcription factors SOX2 and PAX6

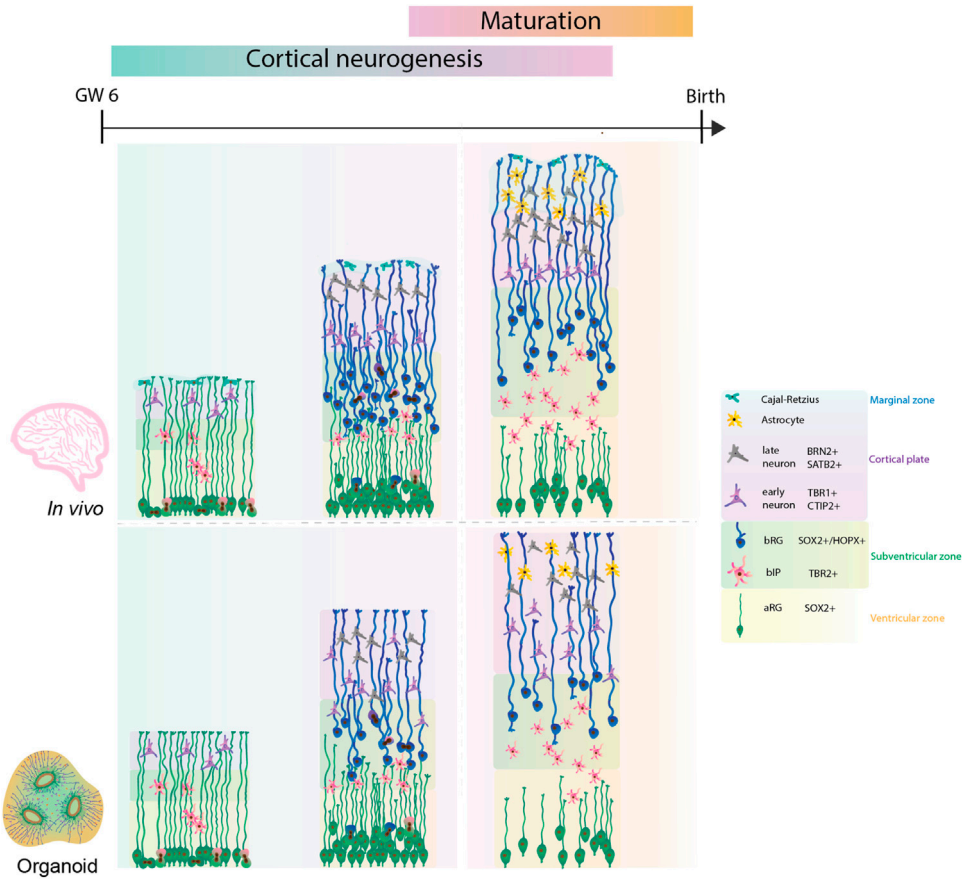

FIGURE 1 | Comparison of human cortical development between human fetal neocortex and human forebrain organoids. Cortical neurogenesis in human fetal development (upper panel) starts with proliferation of neural progenitor cells. In this process, bRG are highly abundant and give a key contribution to the final neuronal output. Consequently, the neocortex expands into specific cytoarchitectural layers with formation of cortical folding on the basal side. Following neurogenesis, functional maturation of neurons and glia takes place. Human forebrain organoids (lower panel) recapitulate the cell diversity and developmental lineages, however further improvements are required to achieve bRG abundance, improved cytoarchitectural organisation, neuronal and glial maturation, and cortical folding similar to the ones observed during the human fetal neocortex.

(Kadoshima et al., 2013; Lancaster et al., 2013; Qian et al., 2018, 2020). Both subtypes of basal progenitors, bRG and the intermediate progenitors can be observed in organoids. bRG are marked by the presence of HOPX, SOX2 and PAX6, along with the absence of TBR2 (Pollen et al., 2015; Qian et al., 2016, 2020). Moreover, organoids also display the presence of early born (CTIP2+ or TBR1+) and late born neurons (SATB2+ and/or BRN2+) (**Figure 1**) (Paşca et al., 2015; Lancaster et al., 2017; Sloan et al., 2018; Qian et al., 2020; Giandomenico et al., 2021). Transcriptomic analysis identified that mature brain organoids between days 250 and 300 correspond to postnatal stages of human brain development (Gordon et al., 2021). However, the timing of expression of different cell types and formation of specific cortical layers differs from protocol to protocol. For example, the organoids produced by the Lancaster protocol show the presence of neurons already at day 30 (Lancaster et al., 2013). This contrasts the organoids generated by the Kadoshima protocol, which start neurogenesis after 70 days (Kadoshima et al., 2013). Thus, the caveat of timing across protocols needs to be considered when comparing the fetal human development.

Most of the initial organoid protocols showed similar limitations as they poorly recapitulated the tissue architecture, notably cortical layering, the bRG abundance, the presence of all developmental lineages and neuronal maturation. To address these limitations and to improve the nutrient and oxygen exchange within the organoid, several improvements of the initial protocols were reported. For example, adding microfilaments and culturing organoids at air-liquid interfaces advanced the original Lancaster protocol (Lancaster et al., 2017; Giandomenico et al., 2021). Recently, the use of external magnetic nanoparticles or inclusion of signalling gradients showed enhanced local patterning of brain organoids (Cederquist et al., 2019; Fattah et al., 2022). It is tempting to speculate that such methods could direct an improved cytoarchitectural organisation of forebrain organoids (Cederquist et al., 2019; Fattah et al., 2022). Further, repeated slicing of Qian organoids facilitated an expansion of cortical layers and an increased

expression of HOPX + bRG cells at day 80, reminiscent of the OSVZ (Qian et al., 2020).

Another strategy to improve brain organoid maturation resulted in the fusion of phenotypically independent dorsal and ventral organoids, termed assembloid (Bagley et al., 2017; Sloan et al., 2018). The latter is particularly interesting because the ventral part of the forebrain is the principal origin of human interneurons, that subsequently migrate into the dorsal regions to integrate into the cortical circuits (Bandler et al., 2017; Hu et al., 2017; Lim et al., 2018). Interneurons are generated by the radial glia of the ventral forebrain, which appear to be more similar to the dorsal aRG than bRG (Velmeshev et al., 2021). Taken together, dorsal-ventral assembloids provide a model system to study generation, migration and integration of interneurons, which enables a more complete modelling of the human cortical development (Marton and Pașca, 2020).

Finally, several strategies have been adopted to improve vascularization. One approach consists in the co-culture of brain organoids with vascular cells such as human umbilical vascular endothelial cells (HUVECs). This resulted in a reduced hypoxic core and improved neuronal maturation (Shi et al., 2020). Additional strategy transplanted organoids into vascularised tissue of immunodeficient mice and showed functional blood circulation and improved organoid viability (Mansour et al., 2018). Implementation of vascularisation in forebrain organoids could enhance the viability and potentially promote neuronal maturation.

WHAT HAVE ORGANOIDS TOLD US ABOUT bRG?

As mentioned above, bRG are considered to be the key cell type underlying human neocortical development. Human bRG are highly proliferative, likely generate most of the human neurons and serve as the scaffold for the neuronal migration to the cortical plate (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; LaMonica et al., 2013). bRGs biology has been poorly assessed since the abundance and behaviour of this cell type is strikingly low in the key animal model, the mouse (Wang et al., 2011; Wong et al., 2015). Although the abundance of bRGs in cerebral organoids is still not comparable to the numbers present in fetal human tissue, organoids hold great promise to be a suitable *in vitro* system to study human bRGs.

Molecular Characterization of bRG

To understand the extent at which the organoid system recapitulates the bRG identity observed in human fetal tissue, initial studies examined the transcriptomic profiles of both model systems. This revealed similar lineage relationships between aRG and bRG in both systems (Camp et al., 2015; Pollen et al., 2015). Subsequent studies readily confirmed the existence of a cell population with a transcriptomic signature of bRG (Bershteyn et al., 2017; Giandomenico et al., 2019; Pollen et al., 2019; Velasco et al., 2019; Cheroni et al., 2022). A recent work aimed to understand the reproducibility of organoids, identified consistent generation of diverse cell types, including bRG, in multiple forebrain organoids (Velasco et al., 2019). Subsequent work combined the latter dataset with spatial transcriptomics and identified a spatial patterning of different cell types within the organoid, with bRG being superficially positioned with respect to aRG (Uzquiano et al., 2022).

One striking characteristic of brain organoids compared to other organoids, such as the liver, is their outstanding increase in size during maturation. However, this can result in poor oxygenation and nutrient exchange within the organoid core causing a systematic cellular stress (Bhaduri et al., 2020). A recent analysis, however, suggested that the cellular stress is a feature of a subpopulation of cells, which can be removed in subsequent computational analyses (Vértesy et al., 2022).

Additional transcriptomic studies extended the role of brain organoids not only as a promising tool to tackle human cortical development but also showed the valuable use of organoids in modelling human brain evolution (Heide et al., 2018; Muchnik et al., 2019). For example, *CTCL*, a fusion transcript and a Wnt signalling modulator, which is expressed in human but not mouse developing brain, has recently been shown to be implicated in the proliferative capacity of bRG in human organoids (Ou et al., 2021). Comparison between human and non-human primate brain organoids pointed at the increased activation of another key signalling pathway, the PI3K-AKT-mTOR, in human bRG (Pollen et al., 2019; Andrews et al., 2020). Moreover, mTOR signalling in human organoids was shown to regulate bRG morphology and behaviour (Pollen et al., 2019; Andrews et al., 2020). Building on earlier findings that identified the role of Notch signalling in promoting human bRGs proliferation (Hansen et al., 2010), recent work conducted in brain organoids, identified the role of a human-specific gene *NOTCH2NL* to enhance the activity of Notch signalling and to delay the neural differentiation of bRG (Fiddes et al., 2018). The second human-specific gene implicated in neocortical expansion and known to operate in bRG, ARHGAP11B (Florio et al., 2015; Kalebic et al., 2018; Heide et al., 2020), has been introduced to chimpanzee organoids where it promoted bRG proliferation (Fischer et al., 2020). Kanton and others performed a comprehensive cell-type specific analysis of gene expression in human, chimpanzee and macaque organoids and further revealed the molecular mechanisms underlying the differences in gene expression across these species (Kanton et al., 2019; Muchnik et al., 2019). Their ATAC-seq analysis showed divergence in chromatin accessibility between human and chimpanzee organoids, which could be associated with the human-specific gene expression (Kanton et al., 2019; Muchnik et al., 2019). Additionally, organoids also offer the possibility to compare the differences in brain development between modern humans and ancestral species such as Neandertals. For instance, Muotri and others modelled Neandertal brains in organoids by introducing an archaic variant gene called Neuro-oncological ventral antigen 1 (NOVA1) (Trujillo et al., 2021). These organoids exhibited changes in organoid

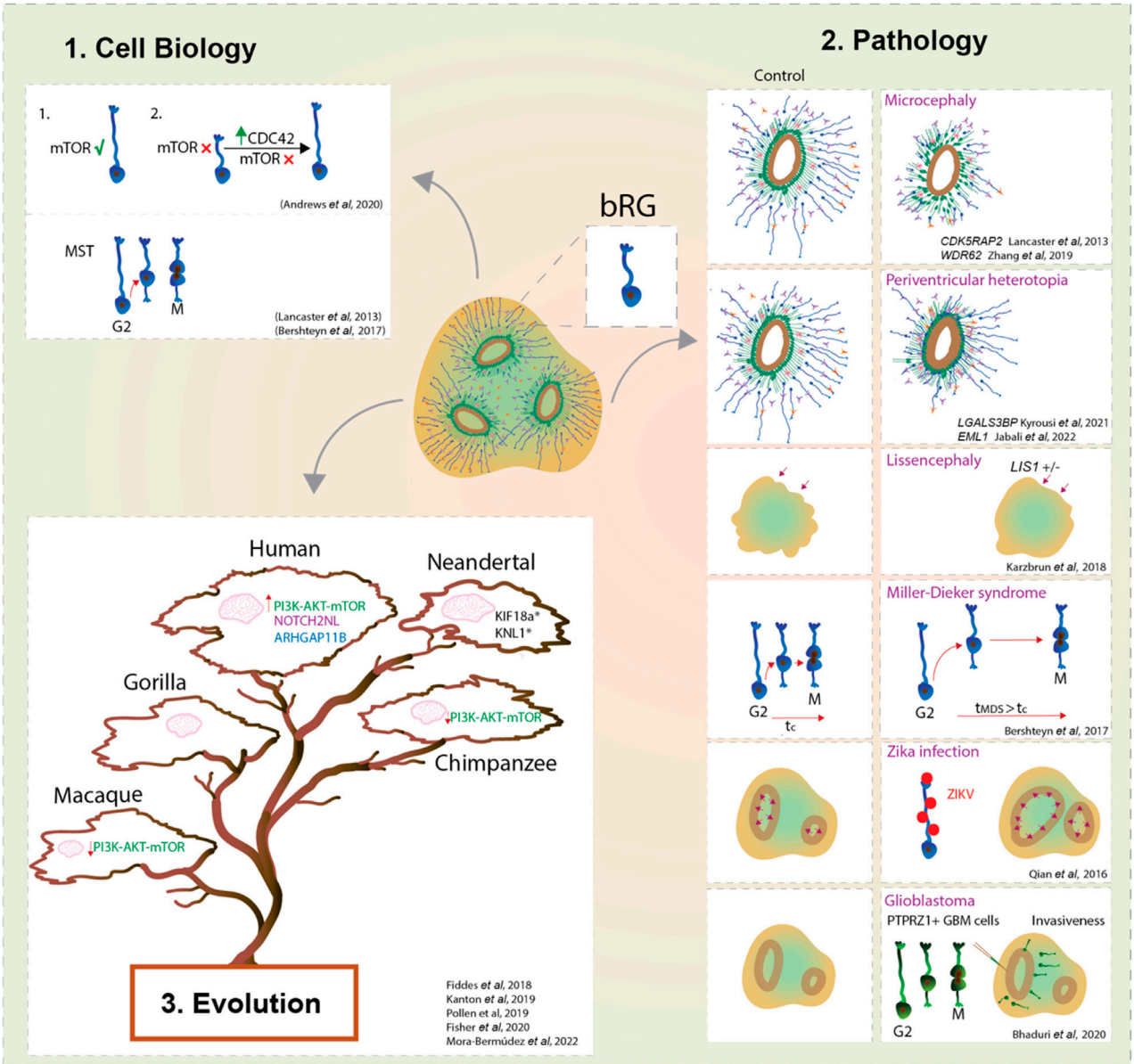

FIGURE 2 | bRG in development, evolution and pathology using forebrain organoids. Forebrain organoids enable us to study the cell biological features and cell behaviours that characterise bRG (1); their role in the onset of malformations of cortical development, brain cancers and viral diseases (2); and their contribution to the neocortex expansion of modern humans compared to ancestral human species and non-human primates (3). Overall, organoids provide a new window into bRG and their link with the expansion of the neocortex.

morphology and neuronal activity (Trujillo et al., 2021). Recently, Mora-Bermudez and others introduced specific ancestral variants involved in mitotic spindle and kinetochore function in organoids and showed shorter metaphase of apical progenitors compared to the longer metaphase of non-mutated modern human organoids (Mora-Bermúdez et al., 2022). It is interesting to speculate that such evolutionary differences between modern humans and ancestral human species might also be linked to bRG development and function.

Cell Biology of bRG

The transcriptomic studies described above have shown that organoids successfully recapitulate the diversity of cell types and their lineage relationships. Combined with the existence of the organoid polarity, it allows us to use this model system to also study the cell biological features of bRG (**Figure 2**). Previous identification on the role of mTOR signalling pathway (Nowakowski et al., 2017) in human bRG led to its deeper analysis using organoids (Pollen et al., 2019). Upon the pharmacological inhibition of mTOR signalling in organoids,

bRG exhibited a shorter basal process (Andrews et al., 2020). Interestingly, bRG morphology could be rescued by the activation of the Rho-GTPases CDC42/RAC1 in cortical tissue (**Figure 2**) (Andrews et al., 2020). CDC42 in particular has a very important role in radial glia morphology, as it has been found to affect polarisation, proliferation and migration of aRG (Cappello et al., 2006; Yokota et al., 2010). Another determinant of cell polarity involved in maintenance of the radial scaffold is GSK3 (Yokota et al., 2010). Recently, a pharmacological inhibition of GSK3 in organoids led to a reduction in the abundance of bRG and production of neurons, further emphasising the role of bRG polarity for normal neurogenesis (López-Tobón et al., 2019). Although bRG lack classical apicobasal polarity and the contact with the ventricular surface, they do possess a series of features that we have previously termed pseudo-apicobasal polarity (Kalebic and Namba, 2021). A key manifestation of such polarity is their morphology. Previous findings identified different bRG morphotypes in mouse, ferret, macaque and human developing cortex (Betizeau et al., 2013; Reillo et al., 2017; Kalebic et al., 2019), suggesting that an increased number of basal processes coincides with the proliferative capacity of bRG (Kalebic and Huttner, 2020). It would be interesting to identify these diverse bRG morphotypes in organoids and understand how morphology might be an important component for neurogenesis progression.

Another key advantage of the organoid system is that it enables the studies of bRG structural and temporal dynamics without the complexity inherent to the *in vivo* and *ex vivo* systems. Prior to mitosis, bRG exhibit a distinctive saltatory migrational behaviour named mitotic somal translocation (MST) (Hansen et al., 2010; LaMonica et al., 2013; Ostrem et al., 2014; Ostrem et al., 2017). Remarkably, organoid studies based on GFP-electroporated radial glia identified this unique feature in cells localised away from the VZ (Lancaster et al., 2013; Otani et al., 2016) The importance of MST for human cortical development is obvious when observing a form of human lissencephaly called Miller-Dieker syndrome (MSD). MSD brain organoids showed prolonged mitosis and longer MST distances (**Figure 2**) (Bershteyn et al., 2017), suggesting that defects in bRG mode of division could lead to premature neurogenesis in human lissencephaly.

bRG in Pathology

Organoids, especially when derived from patients' cells, are a par excellence platform to dissect the pathogenesis of neurodevelopmental diseases (**Figure 2**). One of the first examples comes from the work conducted by Lancaster and others who generated patient-derived microcephalic cerebral organoids carrying a mutation in the centrosomal protein CDK5RAP2 (Lancaster et al., 2013). They showed an increase of asymmetric cell divisions in neural progenitors which led to their premature differentiation (Lancaster et al., 2013). The second principal way to model neurodevelopmental disorders in organoids is to introduce disease-causing mutations *via* genome editing in PSCs. For example, deletion of *WDR62*, another key gene causing microcephaly, resulted in a reduction of bRG proliferation, which in turn led to reduced organoid size (Zhang et al., 2019).

In addition to microcephaly, organoids have been useful to model periventricular heterotopia (**Figure 2**). A recent study used both patient-derived and genome-edited PSCs to study EML1 deficiency in cortical organoids (Jabali et al., 2022). The analyses revealed defects in the primary cilium structure and mitotic spindle orientation of aRG, which led to an increase in aRG delamination and subsequent formation of ectopic neural progenitors and heterotopic neurons (Jabali et al., 2022). Interestingly, deeper characterization identified that the majority of these ectopic progenitor cells are bRG with an unusual morphology (Jabali et al., 2022), linking bRG morphology with neurogenesis. Phenotypes of periventricular heterotopia were successfully recapitulated in human brain organoids also by manipulation of the expression levels of *ECE2* and *PLEKHG6* (O'Neill et al., 2018; Buchsbaum et al., 2020). Another key gene that has been recently described to be enriched in human bRG (Pollen et al., 2015), while being linked to periventricular nodular heterotopia, is *LGALS3BP*. Studies using organoids showed that *LGALS3BP* expression is essential for proper positioning of bRG, whereas altered *LGALS3BP* expression resulted in neuronal heterotopia and defects in local gyrification, emphasising once again a potential role of bRG in disease (Kyrousi et al., 2021).

Further studies identified a role of bRG in the pathogenesis of Pretzel syndrome (polyhydramnios, megalencephaly, symptomatic epilepsy; PMSE) derived from mutations in the STRADA gene, part of the mTOR pathway. PMSE organoids showed an increase of HOPX + bRG which could be linked with the megalencephaly observed in PMSE individuals (Dang et al., 2021). This also further strengthens the role of the mTOR pathway in the regulation of bRG (Nowakowski et al., 2017; Andrews et al., 2020). Mutation of *CHD8* (chromodomain helicase DNA-binding 8) in cerebral organoids resulted in an increased proliferation of a population of radial glial cells which translated into altered neurodevelopmental trajectories (Villa et al., 2022).

Several studies modelled cortical folding using organoids (**Figure 2**). Activation of the PI3K-AKT signalling is known to be involved in increased proliferation of BPs (Kalebic et al., 2019) and its dysfunction is associated with brain overgrowth disorders (Hevner, 2015). Genetic ablation of *PTEN,* a regulator of PI3K, in human organoids showed an increase of HOPX + bRG with subsequent formation of cortical folding (Li et al., 2017). Interestingly, both *PTEN* mutant mice and human organoids showed an increase of brain or organoid volume, but only human organoids showed folding (Li et al., 2017). This suggests the importance of specific molecular and/or cellular features in humans, but not in mice, to direct cortical folding. Nevertheless, control human brain organoids lack the ability to achieve cortical folding, suggesting that they exhibit insufficient neuronal maturation and/or lack the mechanical signals from the microenvironment (Borrell, 2018; Kroenke and Bayly, 2018). Gyrification is important for the development of the

neocortex as it maximises the surface to pack neurons relative to the brain size. Karzbrun et al. reported an induction of folding in organoids by physically constraining brain organoids using a chip (Karzbrun et al., 2018). Together with the mechanical forces from the cytoskeleton contraction and cell migration this induced wrinkles in organoids that are reminiscent of cortical folding (Karzbrun et al., 2018). In contrast, lissencephalic organoids (*LIS-1* mutant) showed changes in the cytoskeleton and ECM that resulted in a reduced organoid wrinkling (Karzbrun et al., 2018). It would be interesting to apply the chip device to organoids whose age corresponds to the onset of bRG neurogenesis and examine a link between bRG and the mechanisms of cortical folding. Interestingly, in lissencephalic organoids modelling Miller-Dieker syndrome, bRG showed mitotic defects, suggesting a role of bRG in pathogenesis of lissencephaly (Bershteyn et al., 2017).

Apart from neurodevelopmental disorders, the use of brain organoids was beneficial to elucidate a role of bRG-like cells in malignant brain tumours such as glioblastoma (**Figure 2**). Live imaging on primary resected tumours displayed a population of bRG-like cells undergoing MST (Bhaduri et al., 2020). Upon transplantation into cortical organoids, these cells exhibited typical invasiveness and expansion of tumour-like cells (Bhaduri et al., 2020). This highlights an important role of bRG biology not only during brain development but also in the context of cancer progression.

Finally, brain organoids have a potential to mimic viral infectious diseases (**Figure 2**) (Harschnitz and Studer, 2021). An outstanding example was given in response to the outbreak of Zika virus (ZIKAV), in which ZIKAV induced microcephalic organoids were generated (Qian et al., 2016; Krenn et al., 2021). These organoids exhibited increased apoptosis, reduced proliferation with subsequent reduction of organoid size. Interestingly, the authors showed that bRG were readily infected by the Zika virus (Qian et al., 2016), hence indicating the advantage of using organoids to understand the contribution of different cell types, such as bRG, in the disease aetiology (**Figure 2**).

Brain organoids have hence provided invaluable insight into the role of bRG for human neurodevelopmental pathologies. Since rodent models poorly recapitulate features of human bRG, ferrets and primates are typical species of choice for *in vivo* exploration of the bRG role in neurodevelopmental disorders (Feng et al., 2020; Gilardi and Kalebic, 2021). Although they recapitulate well the key aspects of bRG biology, these models require substantial time and resources in addition to important ethical considerations for disease modelling. Hence the application of organoids, and particularly patient-derived organoids, has been instrumental for the advancement of knowledge regarding neurodevelopmental diseases and role of bRG in this context.

CONCLUSION

The ability to recapitulate organogenesis outside the embryo makes the organoid system a fascinating and useful technology. Although brain organoids differ from the brain *in vivo*, their ability to reproduce the diverse cell types and lineage trajectories comparable to human fetal cortex, makes the organoids a promising tool to address fundamental questions in neocortical development and pathologies. This is particularly relevant for bRG, a key progenitor cell type underlying human brain development. Future research will likely focus on further cell biological characterization of bRG in organoids and will better dissect the steps along the developmental trajectories examining the contribution of bRG for neocortical development. Given that organoids are becoming a key model system to study differences in brain development between modern humans and ancestral species, it is likely that further efforts in this direction will elucidate the contribution of specific genetic changes between these species for the biology of basal progenitors. Finally, disease modelling has been one of the principal directions of organoid-based research. Future efforts in this domain are expected to further develop in the direction of an ever-more personalised medicine combining patient-derived organoids with genetic and pharmacological screens. An elegant example of a genetic screen has been performed by Esk and others who tested 173 microcephaly-related genes in human brain organoids using CRISPR/Cas9-mediated genome editing (Esk et al., 2020). Future approaches can be used to study candidate genes of other neurodevelopmental pathologies, genes that have more subtle differences in expression level between control and pathological development and, finally, genes whose phenotype is likely to be pertinent to the later stages of organoid development, when bRG become more dominant. Hence, although brain organoids still do not recapitulate all the features of human cortical development, further advancement of the technology and/or combination with xenografting into animal models, are likely to pave the way for an ever-increasing use of this model system to study neurodevelopmental pathologies and human brain evolution.

AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript, approved it for publication and agreed to be accountable for the content of the work.

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Evolution of astrocytes: From invertebrates to vertebrates

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The central nervous system (CNS) shows incredible diversity across evolution at the anatomical, cellular, molecular, and functional levels. Over the past decades, neuronal cell number and heterogeneity, together with differences in the number and types of neuro-active substances, axonal conduction, velocity, and modes of synaptic transmission, have been rigorously investigated in comparative neuroscience studies. However, astrocytes, a specific type of glial cell in the CNS, play pivotal roles in regulating these features and thus are crucial for the brain's development and evolution. While special attention has been paid to mammalian astrocytes, we still do not have a clear definition of what an astrocyte is from a broader evolutionary perspective, and there are very few studies on astroglia-like structures across all vertebrates. Here, I elucidate what we know thus far about astrocytes and astrocyte-like cells across vertebrates. This information expands our understanding of how astrocytes evolved to become more complex and extremely specialized cells in mammals and how they are relevant to the structure and function of the vertebrate brain.

KEYWORDS

astrocyte, evolution, central nervous system, glia, vertebrates, astrocytes

Introduction

The central nervous system (CNS) shows incredible diversity across evolution at the anatomical, cellular, molecular, and functional levels. Over the past decades, the number and heterogeneity of neuronal cells, together with differences in the number and types of neuro-active substances, axonal conduction, velocity, and modes of synaptic transmission, have been among the most investigated characteristics in comparative studies of the brain.

Astrocytes, a specific type of glial cell in the CNS, play pivotal roles in regulating all of these features and thus are crucial for the development and evolution of the CNS. However, they have received less attention in comparative studies. Though special attention has been paid to mammalian astrocytes, we still do not have a clear definition of what an astrocyte is from a broader evolutionary perspective, and very little research has been done on astroglia-like cells across different non-mammalian species. The purpose of this review is to report what we do know about astrocytes and astrocyte-like elements across vertebrates and about primitive astroglia-like structures in invertebrates (see Figure 1 for the species included in this review). This analysis will help us understand how astrocytes evolved to become more complex and extremely specialized

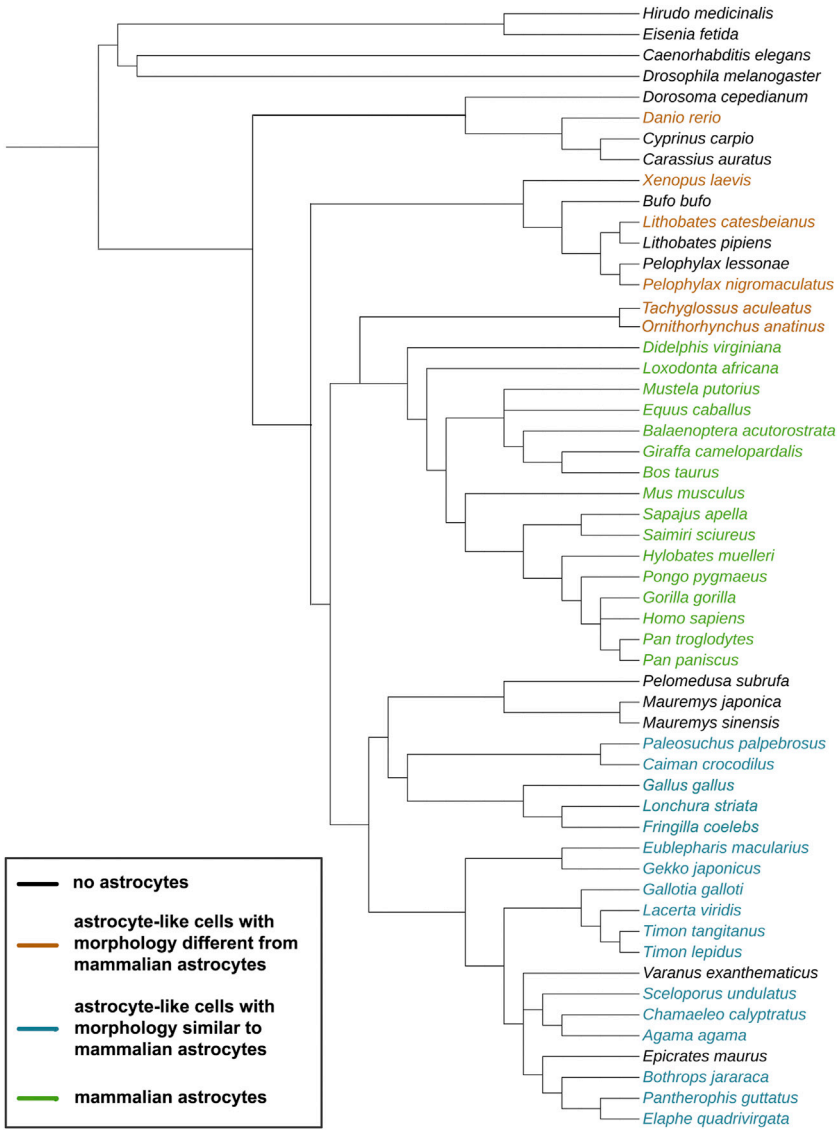

FIGURE 1
Evolutionary tree of the species discussed in this study. The cladogram was created on https://phylot.biobyte.de/, based on the NCBI taxonomy database. Color code: black = no astrocytes; orange = species showing astrocyte-like cells with morphology different from mammalian astrocytes; blue = species showing astrocyte-like cells with morphology similar to mammalian astrocytes; green = species showing mammalian astrocyte morphology.

cells in mammals and how they are relevant to the structure and function of the brain in vertebrates.

Astrocyte identity and the origin of neuroglia

The term "astrocytes" was used for the first time by Lenhossek in 1895 (Lenhossék M. 1895) with the intent to replace the term "glia" (from the Greek for "glue"), which did not represent the many functions of these cells. Lenhossek based his term on the cell's morphology and, specifically, on the presence of multiple cellular processes resembling those of a star. The first nomenclature by Del Río Hortega (Del Rio-Hortega P. 1932; Hortega 1942) distinguished two types of astrocytes in mammals—protoplasmic and fibrous astrocytes—based on their morphology and position in the CNS. These are still considered the main types of astrocytes; however, already in 1965, Duncan (Duncan D. 1965) noted that this classification was an oversimplification of astrocyte heterogeneity.

Astrocytes are indeed a highly heterogeneous cell population, with different morphologies, molecular profiles, distributions in different

anatomical regions, physiology, and functions across different species. The fact that there is still no unique marker to label all astrocytes and not other brain cell types makes it challenging to define criteria for classifying astrocytes across different species. However, several attempts have been made to describe astroglia across nervous systems of different species and to identify primitive astrocyte forms in more ancient vertebrate and invertebrate species.

Astrocytes have been broadly described in mammals, where they have reached their highest specialization. Currently, astrocytes are known to play crucial roles in the CNS, such as regulating water and ion homeostasis and exchanging nutrients across the blood-brain barrier. They are also pivotal players in the development and regulation of connectivity, such as in synapse formation and pruning, and in synaptic function and plasticity across development and in the adult. Moreover, astrocytes are able to react to injury or stress with a series of processes called reactive astrogliosis, which results in scar formation or glial borders (Sofroniew 2009; Sofroniew and Vinters 2010; Sofroniew 2015; 2015b) and thus exerts both a protective and neurotoxic effect (Escartin et al., 2021). Astrocytes likely co-evolved with neurons and became more and more specialized in mammals compared to other vertebrates due to mammals' higher CNS complexity and higher energy demands. Understanding how astrocytes evolved across species is crucial to understanding their contribution to CNS complexity and functions in human and non-human primates.

When we look at the most ancient nervous systems in invertebrates, only neurons—which evolved from epithelial cells—are present, and they are organized in a diffuse network. The nervous system showed its first organization in neuronal ganglia in Cnidaria, then became a proper centralized CNS in Bilateria (e.g., flatworms), subsequently became more complex in protostomes (e.g., insects and crustaceans), and finally showed higher degrees of complexity in vertebrates. The appearance of a CNS necessitated supportive cells, i.e., glial cells (Verkhratsky, Ho, and Parpura 2019). The specific phylogenetic relationships of glial cells between invertebrates and vertebrates are still debated. In fact, different studies have shown that different genes are involved in glial differentiation in invertebrates vs vertebrates (e.g., gcm drives glial differentiation in *Drosophila*, while the mammalian homologue Gcm has not retained that function) and that there are highly conserved pathways between the two groups (e.g., BMP FGF) (Yang and Jackson 2019).

The first glial cells are visible in Acoelomorpha, although they became more complex (e.g., sheath glia) in *Caenorhabditis elegans* and Anellida, with anatomical and functional features of both mammalian astrocytes and oligodendrocytes. In Deuterostomes, radial glia (RG) cells are seen for the first time, then in early Chordata, they became the predominant type of glial cells and are present throughout life (Figure 2B). RG cells show a characteristic radial morphology with an elongated shape of the cell bodies, long radial processes directed into the parenchyma, and the typical expression of intermediate filaments in the cytoplasm. RG cells represent the main type of glia in the adult CNS of many early vertebrates, where they exert functions similar to those of mammalian astrocytes. For example, the well-developed RG cells in zebrafish send processes travelling from the ventricles to the pial surface, show robust glial fibrillary acidic protein (GFAP) expression, and are involved in glutamate and water homeostasis (thanks to *Gs* and *Aqp4* expression). However, RG cells are considered purely neural stem cells in the mammalian CNS, where they are abundant during embryonic development but largely disappear after birth. Across evolution, we witness an increase in the complexity of the CNS, accompanied by a need for the specialization and diversification of neuroglia, and more specifically, of astrocytes. Astrocyte complexity and heterogeneity is especially noticeable in primates (Verkhratsky, Ho, and Parpura 2019).

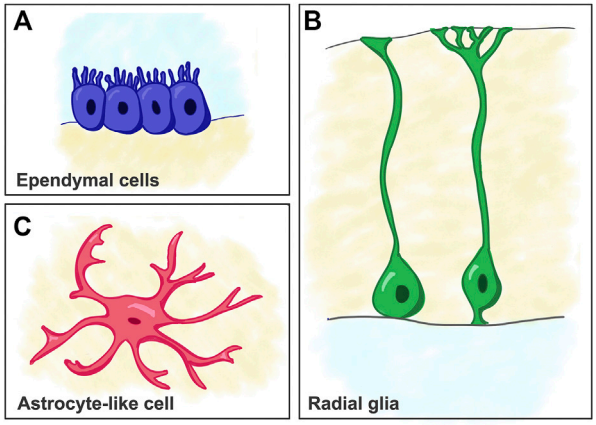

FIGURE 2
Schematic of cell types considered as predecessors of mammalian astrocytes. **(A)** Ependymal cells. **(B)** Radial glia cells. **(C)** Astrocyte-like cells (e.g. as seen in reptiles).

Methods for conducting comparative studies of astrocytes

Due to the lack a comprehensive description of what an astrocyte is across species and due to astrocytes' tremendous heterogeneity, to identify their presence, we often rely on the astrocytic features that we find in mammals, the group in which these cells have been most frequently investigated. However, astrocytes or astrocyte-like cells can be studied in different species comparatively with regard to morphology, gene expression, and functions under normal conditions and in reaction to injury or stress.

Some pivotal studies comparing the presence of astrocytes across different species are very old and are mostly based on

morphology; nevertheless, they still convey fundamental information (some of these pivotal morphological studies are reviewed in (King 1966). However, those studies need to be followed up with research that employs more modern technologies. For example, most evidence of astrocytes' presence across different vertebrates relies on immunohistochemistry against GFAP, a type III intermediate filament protein. Morphological observations include cell shape and position; shape; number and direction of processes; contact with blood vessels, neurons, and synapses; and cellular distribution in different regions of the CNS. The GFAP gene has been shown to be highly conserved in vertebrates and has been considered for decades as the main marker for astrocytes; however, it has been recognized to label only a subpopulation of astrocytes and to label also other cell types such as RG cells. Therefore, though GFAP expression, when combined with anatomical and structural observations, is still valid for identifying astrocyte-like cells across many different species, it may be insufficient to comprehensively define the features and heterogeneity of the whole astrocyte population. For example, *tiling* (a process for which astrocytes cover discrete, non-overlapping anatomical territories in the parenchyma) has never been tested in all astrocytes due to the use of only GFAP and S100b markers (O'Leary and Mechawar 2021). In the future, the use of multiple astrocyte markers will be fundamental for analyzing specific astrocyte features.

Criteria for identifying astrocytes and astrocyte-like cells across evolution should, however, not be limited to the study of their morphology, but also of their physiological properties and their molecular profiles. More recent studies have taken advantage of the RNA expression of genes involved in astrocyte differentiation and function across species belonging to vertebrate and invertebrate species (Chen et al., 2020; Li et al., 2021). However, such studies are still few and do not include broad collections of species across evolution. The most relevant functional studies of astrocytes across species involve the comparison of calcium (Ca^{2+}) signaling (intracellular Ca^{2+} elevations, with specific spatial and temporal properties, involved in the regulation of synapses, circuits, and ultimately, behavior) and gliotransmission (Guerra-Gomes et al., 2018). A few studies have also looked at how astrocytes react after injury in different species, with a special focus on astrocytes' reactivity after traumatic brain or spinal cord injury (Gu et al., 2015; Du et al., 2021; Perez, Gerber, and Perrin 2021).

Here, I will review both old and recent findings of astrocytes and astrocyte-like cells across evolution.

Astroglia in invertebrates

Among the most relevant challenges in comparative studies of glia between vertebrates and invertebrates is the almost total absence of markers conserved between the two groups. Early invertebrates present primitive neuroglia that likely represent the predecessor of vertebrate astroglia. Flatworms are the first Bilateria presenting a centralized nervous system: they show glia-like mesenchymal cells exhibiting long processes that contact nervous cells. Earthworms have slightly more morphologically complex neuroglia that show, for the first time, a certain degree of specialization. In fact, earthworm (*Eisenia fetida*) neuroglia include different types of cells: neurilemmal, subneurilemmal, supporting-nutrifying, and periaxonal sheath-forming cells. Among these, the supporting-nutrifying neuroglia are GFAP-immunoreactive and play roles similar to the nutritive roles of mammalian astrocytes (Coles 2009; Csoknya, Dénes, and Wilhelm 2012; Verkhratsky, Ho, and Parpura 2019). The nematode *C. elegans* presents what have been defined as "proto-astrocytes"—a type of neuroglia involved in several functions similar to those of mammalian astrocytes, but mostly associated with sensory systems. Among the different types of neuroglia in *C. elegans*, the cephalic sheath cells in the nerve ring control ion homeostasis in perisynaptic regions and are involved in regulating neuronal development and morphogenesis and in suppressing locomotion during sleep (Bacaj et al., 2008; Oikonomou and Shaham 2011; Stout Jr; Katz et al., 2018; Verkhratsky et al., 2018). However, both glial and neuronal cells are different from the neuroglia of other species in terms of morphology and physiology. For example, due to the lack of a circulatory system in *C. elegans*, such proto-astrocytes do not form the glia limitans barrier that we see in mammals nor do they express mammalian glia markers. Members of the phylum Anellida have *homeostatic* proto-astrocytes. The leech (*Hirudinea medicinalis*)'s glial cells are interconnected due to the presence of gap junctions. In this species, *packet glial cells* are able to buffer extracellular K^+, and *giant glial cells* exhibit processes that contact neuronal dendrites (Nicholls and Kuffler 1964; Munsch and Deitmer 1992; Saubermann, Castiglia, and Foster 1992). In insects—and more specifically in the common fruit fly (*Drosophila melanogaster*), a model organism studied extensively in neuroscience—proto-astrocytes are present; they not only display a higher degree of specialization (compared to other invertebrates) but also are analogous to typical astrocytes in terms of physiology and function. The *Drosophila surface glia* are in charge of building a brain-hemolymph barrier, resembling what would later evolve into the blood-brain barrier in vertebrates. The *cortex glia* have processes contacting neuronal cell somata, with each glial cell contacting multiple neurons, and display calcium oscillations similar to those occurring in mammalian astrocytes. The *neuropil glia* include ensheathing/fibrous and astrocyte-like glia. Like mammalian astrocytes, the *Drosophila* astrocyte-like glial cells in the neuropil wrap axons and synapses (i.e., one astrocyte-like cell contacts many neuronal synapses) and are implicated in synaptogenesis and synaptic transmission. Interestingly, such astrocyte-like cells show many arborizations in their processes for the first time, thus they are

quite similar to vertebrate astrocytes from a morphological perspective (Freeman and Doherty 2006; Parker and Auld 2006; Edwards and Meinertzhagen 2010; Hartenstein 2011) (Figure 2C). In addition, these cells also employ Ca^{2+}-dependent signaling mechanisms, they are electrically coupled by gap junctions, and they show tiling, in which astrocytes cover discrete, non-overlapping anatomical territories in the parenchyma. Genomic studies in *Drosophila* have revealed genes important in glial differentiation and functions. *Glial-cell missing* (*gcm*) is one of the most important genes regulating glial lineage differentiation in the fly, but those same functions in glial development have not been preserved in its homologue in mammals (*Gcm1*). However, comparative studies between fly, mouse, and human gene transcription have shown that there is a certain degree of gene conservation that points to a common evolutionary origin of glial cells among invertebrates and vertebrates. Among the 900 genes that are orthologues among these species, there are transcription factors, ion channels, and transporters important in mammalian astrocyte physiology (Yang and Jackson 2019). For example, *Drosophila* astrocyte-like glia express GABA transaminase (*Gat-1*), the dEAAT1 glutamate transporter, and glutamate synthase 2 (*Gs2*) (Yang and Jackson 2019). Glial cells in the fly are also critical in regulating circadian rhythms, a function that is conserved in astrocytes of mouse suprachiasmatic nuclei (Brancaccio et al., 2017, 2019; Yang and Jackson 2019).

Astroglia in fish

A series of morphological studies have been conducted in both model and non-model organisms of teleosts, which comprise the largest number of fish species, widely distributed in different habitats. In one carp species (*Cyprinus auratus*), mostly ependymal glia were found, with processes not contacting blood vessels (Achúcarro N. 1915; King 1966) (Figure 2A). In another species of carp, *Cyprinus carpio*, GFAP expression has been studied in depth (Onteniente, Kimura, and Maeda 1983). The authors found dense GFAP immunoreactivity within thick and straight bundles of processes around ventricles in the adult, both in sub-ependymal areas and in strict association with blood vessels. Strong GFAP expression has also been documented in white matter (especially in the optic tract and in the fasciculus longitudinalis medialis), while it has not been found in grey matter, with the exception of the optic tectum and mesencephalon, where rare radial processes end on the pial surface with small endfeet. Again, no proper astrocytes have been observed in this species of carp (Onteniente, Kimura, and Maeda 1983).

In the American gizzard shad (*Dorosoma cepedianum*), ependymal cells are still the predominant type of glia; however, they show some similarities with astrocytes in terms of process endings. In fact, their processes contact neurons and blood vessels and thicken beneath the pial surface. Moreover, there are both bipolar and tripolar non-ependymal cells found close to large neurons, without typical perivascular endfeet. It has been suggested that these types of non-neuronal cells could be predecessors of astrocytes and oligodendrocytes as we know them in mammals (King 1966).

Astroglia-like cells have been investigated in depth in the well-studied animal model zebrafish (*Danio rerio*) in a pivotal paper recently published by (Chen et al., 2020). While previous studies proposed that RG would functionally substitute for astrocytes in the adult zebrafish nervous system, Chen et al. showed that zebrafish spinal cord RG differentiate into cells that share several similarities with mammalian astrocytes. They found that such spinal cord RG cells express mammalian astrocyte marker genes such as Glt-1, Glast, and Gat-3 across development. With elegant genetic manipulations and *in vivo* imaging, they were able to document the transformation from RG to astrocyte-like cells—a transformation that revealed dynamic cellular process elaboration and arborization early in development (between two and 4 days post fertilization). These astrocyte-like cells express glutamine synthetase in somata and processes, and their processes are closely associated with synapses (labeled with the synaptic vesicle glycoprotein 2A, SV2). Interestingly, these cells are able to establish individual cell territories with minimum overlap with each another, similar to what mammalian astrocytes do with their tiling. This work is also among the few to show similarities in electrophysiological kinetics between zebrafish astrocyte-like cells and mouse astrocytes. Zebrafish astrocyte-like cells present spontaneous microdomain Ca^{2+} transients in their fine processes, and, more specifically, respond to norepinephrine activation, thus displaying calcium dynamics similar to awake behaving mice. Finally, this *bona fide* astrocytic cell population shows a conservation of factors involved in astrocyte morphogenesis, such as Fgfr3 and Fgfr4. These findings are not only important from an evolutionary perspective but also point to the zebrafish as a valuable model for investigating the molecular mechanisms that govern astrocyte functions (Chen et al., 2020).

Astroglia in amphibians

Very few studies on glial cell identity and functions have been conducted in amphibians. In the frog *Rana esculenta*, an RG morphology similar to that of fish has been observed, though with thicker processes contacting blood vessels (similar to mammalian RG and astrocyte endfeet). However, both in the frog *Pelophylax esculentus* and in the toad *Bufo vulgaria*, ependymal cells remain the predominant type of glial cell, forming a sparse glial network across the CNS (Achúcarro N. 1915; Bairati and Maccagnani 1950; King 1966). Anatomical studies done specifically in the frog *Lithobates pipiens* reported two densely packed rows of ependymal cells in the primitive

hippocampus and striatum, sending out processes that travel through the superficial neuronal layers and course very close to the neuron somata, sometimes contacting them. Moreover, very few non-ependymal cells with processes in close association with neurons have been observed in the same frog species (King 1966). In the frog species *Lithobates catesbeianus* and *Pelophylax nigromaculatus*, astrocyte-like cells have been observed, but, like in other amphibians, no typical stellate astrocytes have been detected (Onteniente, Kimura, and Maeda 1983). In the frog animal model *Xenopus laevis*, astrocyte-like glial cells express Blbp, a well-known marker for RG in mammals, thus they show a molecular expression profile that more closely resembles that of immature mammalian astrocytes or RG (Mills et al., 2015).

Astroglia in reptiles

Reptiles represent a key group in the phylogenetic evolution of astroglial cells because they are the first to that show cells with clear astrocyte morphology (Bodega et al., 1990). Several studies have investigated the presence of astrocytes in reptiles and have shed light on the heterogeneity of their distribution and on putative astrocyte predecessor cells in multiple brain regions. In general, in the reptile forebrain, the predominant cells are unipolar ependymal cells and bipolar RG-like cells with processes directed to the parenchyma, with sparse astrocytes close to neurons and almost always intermingled within RG fibers (Bairati and Tripoli 1954; King 1966). Drs. Lõrincz and Kálmán reviewed the most relevant findings in Squamata, the largest order of reptiles comprising lizards and snakes (Lõrincz and Kálmán 2020). In the telencephalon and anterior hypothalamus of the leopard gecko (*Eublepharis macularius*), GFAP$^+$ RG cells are organized in a layered structure with a lighter middle zone less densely packed with GFAP-immunoreactive fibers and dense with neurons. In the septum, lateral pallium, and dorsal ventricular ridge (DVR), the glial structure is more complex, but is almost devoid of GFAP expression. In the same species, GFAP is instead evenly distributed in the diencephalon. Very few astrocyte-like cells have been observed in the ventricular surface of the mesencephalon, and, more specifically, in the torus semicircularis (Lõrincz and Kálmán 2020). In the monitor lizard (*Varanus exanthematicus*), the telencephalon shows strong GFAP expression, with a trilaminar structure like in the gecko, while the DVR shows little to no GFAP immunoreactivity (Lõrincz and Kálmán 2020). In agama (*Agama*), the telencephalon is low in GFAP expression, with GFAP being detected mostly in the mediodorsal pallium, septum, striatum, and amygdala. Few cells with typical astrocyte morphology are visible in the septum and nucleus accumbens, and they are intermingled among RG fibers (Lõrincz and Kálmán 2020) (Figure 3A). In the agama diencephalon, GFAP has a variable distribution, with RG fibers penetrating the thalamus and hypothalamus (Lõrincz and Kálmán 2020). Similarly, in the chameleon (*Chamaeleo calyptratus*), GFAP$^+$ cells are present in the medial pallium and the septum, with the striatum penetrated by arching RG processes, while they are mostly absent in the diencephalon and the DVR. GFAP$^+$ stellate astrocytes are present in both the septum, the preoptic hypothalamus, and, in small groups, in the tegmentum. In the agama optic tract and spinal cord, astrocytes are intermingled within RG fibers (Lõrincz and Kálmán 2020) (Figures 3B,E,F). In the lacertid Moroccan eyed lizard (*Timon tangitanus*), there is an intermediate distribution of GFAP immunoreactivity compared to the lizard species discussed above. Most of the DVR is deficient in GFAP$^+$ cells or fibers. In the telencephalon, glial processes terminate on vessels with wide, round endfeet, similar to mammalian astrocytes. Here, the diencephalon presents a similar structure to other lizards, with the optic tract showing a zone dense with astrocytes (Lõrincz and Kálmán 2020) (Figure 3D). The Moroccan eyed lizard is the only lizard to have astrocytes in the mesencephalon (Lõrincz and Kálmán 2020). In the European green lizard (*Lacerta viridis*), ependymal cells show pial ending, while non-ependymal neuroglia are present in the basal ganglia and the septal nucleus (Achúcarro N. 1915; King 1966). In the eastern fence lizard (*Sceloporus undulatus*), ependymal cells with single elongated processes are detectable in the primitive hippocampus and striatum, while non-ependymal glial cells show a small, bipolar morphology or a large, oval, multipolar shape similar to that of mammalian astrocytes (King 1966). In the Western Canaries lizard (*Gallotia galloti*), at embryonic day 35 (E35), astroblasts and immature astrocytes have been identified in the midbrain by their structural properties, such as the presence of gliofilaments and dense glycogen granules. Astrocytes have been also found in the white matter of this lizard species and in grey matter in small numbers (Monzon-Mayor et al., 1990). At E35, Vimentin$^+$ RG cells line the ventricles and send fibers that run radially into the cortex and ventral striatum, and throughout the basal nuclei. The endfeet of these fibers contact blood vessels and are intensively stained with Vimentin at this stage, while they are weakly immunoreactive for GFAP; GFAP immunoreactivity grows in intensity at E40. In the adult, an uneven expression of GFAP is retained in the RG fibers in the basal nuclei, cortex, and walls of lateral vessels. However, there are no stellate astrocytes in the cortex or hippocampus, and very few are present in the optic tectum together with predominant RG processes (Yanes et al., 1990). In the jewelled lizard (*Timon Lepidus*), the same three types of glia are present: ependymal cells, RG, and free astrocytes. In the spinal cord, RG surround the ependymal layer, while in white matter, astrocyte morphology is more developed in the ventral vs the dorsal portions. Transitional elements with an intermediate morphology between RG and astrocytes have been documented in both white and grey matter (Bodega et al., 1990), with a simpler morphology than that of mammalian astrocytes. In grey matter, cells similar to mammalian protoplasmic astrocytes are visible, with variable number and orientation of the processes depending on the region

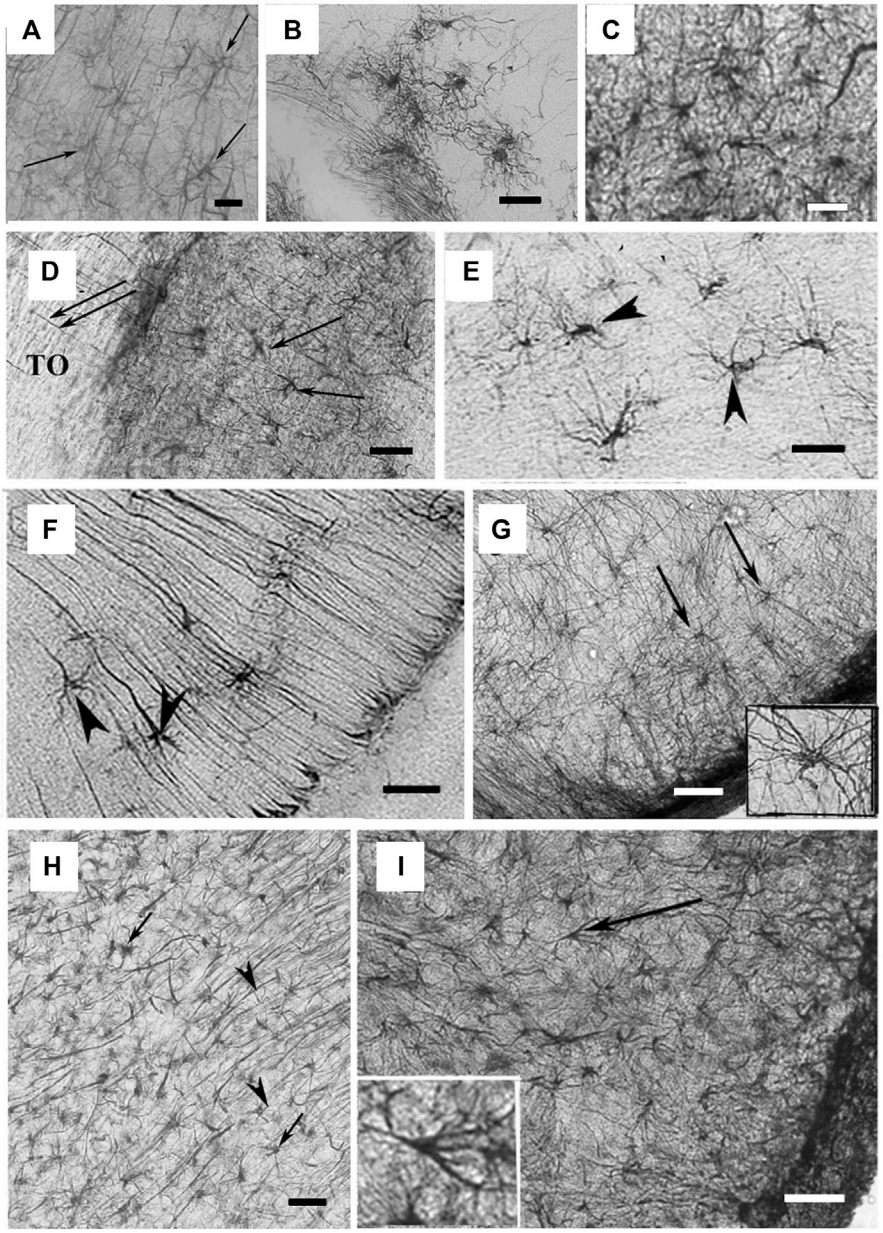

FIGURE 3
Astrocyte-like cells in reptile brains. **(A)** Adapted from Lõrincz and Kálmán, 2020, from Figure 4H. GFAP-immunopositive elements in agama telencephalon; arrows point to astrocytes intermingled within RG processes in the nucleus accumbens. Scale bar: 20 μm. **(B)** Adapted from Lõrincz and Kálmán, 2020, from Figure 5F. Astrocytes from the chameleon septum. Scale bar: 20 μm. **(C)** Adapted from Lõrincz and Kálmán, 2020, from Figure 5G. Astrocytes from the chameleon hypothalamus. Scale bar: 20 μm. **(D)** Adapted from Lõrincz and Kálmán, 2020, from Figure 8C. Arrows point to astrocytes within the optic tract (TO) of Moroccan eyed lizard diencephalon. Scale bar: 80 μm. **(E)** Adapted from Lõrincz and Kálmán, 2020, from Figure 9J. Arrowheads point to astrocytes in the chameleon brain. Scale bar: 20 μm. **(F)** Arrowheads point to astrocytes soma in the chameleon brain. Scale bar: 20 μm. **(G)** Adapted from Lõrincz and Kálmán, 2020, from Figure 13J. Arrows point to astrocytes with long processes in the brain stem of the python. Scale bar: 50 μm. **(H)** Adapted from Lõrincz and Kálmán, 2020, from Figure 10G. Astrocytes in the corn snake brain; arrows point to astrocytes, arrowheads point to RG processes. Scale bar: 40 μm. **(I)** Adapted from Lõrincz and Kálmán, 2020, from Figure 13L. Astrocytes in the ventrolateral part of the brain stem in the corn snake; arrow points to a cell enlarged in the inset. Scale bar: 50 μm.

(few/short dorsally and numerous/more complex ventrally). Elements similar to fibrous astrocytes with long and numerous processes have also been documented in white matter. In general, perineuronal astrocytes are more abundant than in other lizard species and are often associated with blood vessels (Bodega et al., 1990).

In the Japanese striped snake (*Elaphe quadrivirgata*), there are several GFAP$^+$ RG with radial processes and a very small number of stellate-shaped astrocytes only in the optic tract and molecular layer of the gyrus dentatus in the hippocampus (Onteniente, Kimura, and Maeda 1983). In the boa (*Epicrates maurus*) and python (*Python regius*), GFAP expression is null in the most rostral telencephalon and the DVR and is high in the caudo-medial pallium, septum, striatum, and preoptic hypothalamus (where the radial pattern becomes absent). Free stellate astrocytes are visible in the spinal cord of the python (Lõrincz and Kálmán 2020) (Figure 3G). The corn snake (*Pantherophis guttatus*) shows cells with clear astrocyte morphology in the telencephalon (i.e., in the septum), in the mesencephalon (i.e., in the tecum, tegmentum, and isthmus), and in the spinal cord. In this species, GFAP expression is detectable even in more rostral brain parts (Lõrincz and Kálmán 2020) (Figure 3H,I). In the pit viper (*Bothrops jararaca*), ependymal cells are present in a single layer around ventricles, the cerebral aqueduct, and the central canal of the spinal cord, with cilia arising from basal bodies. Astrocytes represent a homogeneous cell population in terms of density, but one that is more irregular in terms of cell body shape, cytoplasmic organelle distribution, and nuclei shape. Lastly, there are no distinguishable protoplasmic or fibrous astrocytes in snakes (Bondan et al., 2015).

All turtle species investigated in the literature thus far present an abundance of RG cells but no stellate astrocytes. In particular, in the adult turtle (*Mauremys japonica, Mauremys sinensis,* and *Pelomedusa subrufa*) GFAP$^+$ RG processes travel from the ventricle to the pia, with few branches and strict contact to blood vessels (Onteniente, Kimura, and Maeda 1983). Here, the GFAP expression distribution is quite homogeneous, without lighter areas like those found in lizards (Lõrincz and Kálmán 2020).

Interestingly, the reptiles belonging to the clade of Archosaurs—a group that includes crocodilians and birds—show the highest density and degree of regional adaptation for stellate astrocytes among reptiles, although ependymoglia and RG are still the predominant types of GFAP$^+$ glia. In the common caiman (*Caiman crocodilus*), the presence of GFAP$^+$ astrocytes is highly heterogeneous and does not correlate with brain wall thickness. Here, the astrocytes are more numerous than in other reptiles, but less numerous than in birds. Astrocytes are visible in the middle and posterior parts of the telencephalon and in the striatum. They are also present in the diencephalon, in the mesencephalon, and in the spinal cord, where they are intermingled with RG fibers. In the caiman's cerebellum, GFAP$^+$ RG fibers are numerous and so are the astrocytes between them, similar to the Bergmann glia of mammals and birds (Kálmán and Pritz 2001). In the Cuvier's dwarf caiman (*Paleosuchus palpebrosus*), RG processes are intermingled with non-radial process like in the common caiman; however, thick radial astroglial processes are not present in this species (Kálmán and Pritz 2001).

From a functional perspective, comparative studies have been done on astrocyte activation during wound healing in the gecko (*Gekko japonicus*) vs the rat. The gecko, like other reptiles, is an interesting animal to study in terms of regeneration and wound healing. Astrocyte activation is attenuated in gecko vs rats, resulting in a more efficient wound healing process. This attenuation may be due to different secreted factors, by comparing RNA sequencing (RNA-seq) data from adult gecko, adult rat, and embryonic rat (E18) astrocytes in wound healing models. The different astrocyte responses in different species may have an endogenous origin. Interestingly, RNA-seq data revealed that adult gecko astrocytes express genes similar to those of E18 rat astrocytes (i.e., genes involved in migration and proliferation), pointing to a conservation of astrocyte response to injury between reptiles and mammals (Gu et al., 2015). Moreover, gecko adult astrocytes retain an immature phenotype, resembling rat embryonic astrocytes, because of sustained Vav1 expression (Du et al., 2021).

Astroglia in birds

Astrocyte morphology and functions in birds are similar to those of mammals. In the chaffinch (*Fringilla coelebs*), few primitive ependymal cells and a network of both protoplasmic and fibrous astrocytes have been observed. Astrocytes send processes that contact blood vessels with typical mammalian-like vascular endfeet, and they wrap neuronal synapses, suggesting a role in synapse formation and functions (Achúcarro N. 1915). In the chicken (*Gallus domesticus*), astroglial cells with 4–8 processes and a round/oval soma had already been found in both the dorsolateral and the ventromedial portion of the pallium in 1966, with processes encompassing the neuronal surface and in close association with blood vessels (Bairati and Maccagnani 1950; King 1966). Finally, in the finch (*Lonchura striata*), a variable number of GFAP$^+$ astrocytes have been observed in white and grey matter, with few perivascular astrocytes close to the ependymal layers (Bairati and Maccagnani 1950). However, to the best of my knowledge, no studies are available to show the distribution of astrocyte populations different from the GFAP$^+$ population in birds.

Astroglia in mammals

Astrocytes as we know them today have been mostly investigated in mammals, and more specifically in the mouse, in humans, and in non-human primates. Although scarce, studies in different mammalian species point to great astrocyte heterogeneity across mammals, with specific astrocyte morphology, density, and functions in primates (Oberheim et al., 2009; Verkhratsky et al., 2018; Falcone et al., 2019; 2021).

A compelling electron microscopy study on monotremes (the most ancient mammals) showed that there are strong differences between the monotremes and therian mammals in terms of glial structure and function (Lambeth and Blunt 1975). The authors showed clear differences between neuronal and glial cells, with the latter being smaller size and having denser cytoplasmic and nucleoplasmic matrices. The microtubules contained in such glial cells are neither arranged circumferentially (as in therian oligodendrocytes) nor associated with filaments (as in therian astrocytes). Only one type of macroglia was found in the platypus (*Ornithorhynchus anatinus*) and in the echidna (*Tachyglossus aculeatus*) immature brain, with a lighter and a darker variant in the echidna (based on electron microscopy observations). However, this glial cell type morphologically resembles neither the astrocytes nor the oligodendrocytes of therian mammals, yet has the potential to exert the functions of both therian astrocytes and oligodendrocytes (Lambeth and Blunt 1975). Unfortunately, no recent studies are available to offer more details about glial cells in monotremes.

Among marsupials, the opossum (*Didelphis virginiana*) presents astrocytes and transitional glia elements, while it lacks adult ependymal cells with processes projecting from the ventricle, as we observe in other non-mammalian species. Opossum astrocytes have round, oval, polygonal cell bodies with 4–12 fine and thicker processes that often contact blood vessels or end around neurons or on other glial cells. They are present in the hippocampus, often with processes running parallel to the apical dendrites of the pyramidal cells. In the neocortex, astrocytes sparsely populate all cortical layers and are denser than in the archipallium. Cortical astrocytes' main processes are thick, with secondary branches, and their endfeet contact blood vessels in the parenchyma and close to the pial surface. In caudate and lentiform nuclei, there are glial elements similar to those of the cell types found in the neopallium, with some thin processes contacting neuronal fibers (King 1966).

In cattle (*Bos taurus*) and horse (*Equus caballus*), a wide network of protoplasmic astrocytes and perivascular astrocytes have been observed (Bairati and Tripoli 1954). In the hippocampus, astrocyte processes contact pyramidal neurons, and in the cortex, astrocyte endfeet on the vasculature are present. Older studies also reported transitional forms of astrocytes, with a morphology intermediate between protoplasmic and fibrous astrocytes, and between protoplasmic astrocytes and oligodendrocytes (Contu P. 1954).

Astrocyte spatial organization has been studied in depth in the ferret (*Mustela putorius*) visual cortex. These astrocytes are twice as large as mouse/rat cortical and hippocampal astrocytes, but smaller than those in humans. Intriguingly, the classical astrocyte tiling model may not apply to the ferret visual cortex, where there is a large overlap of the processes of neighboring astrocytes (López-Hidalgo, Hoover, and Schummers 2016).

Interestingly, Bergmann glia, a specialized type of unipolar astrocytes derived from RG and intimately associated with Purkinje cells in the cerebellum, can be found in all mammals.

Astrocyte heterogeneity from a morphological, spatial, molecular, and functional perspective has been thoroughly investigated in the mouse (*Mus musculus*), thanks to state-of-the art technologies (Matyash and Kettenmann 2010; Bayraktar et al., 2020; Westergard and Rothstein 2020). In terms of regional specialization, protoplasmic astrocytes in the cortex and hippocampus possess more branches than those in the hypothalamus and in other subcortical regions, which allows their anatomical domain size to be larger. Moreover, a transcriptomic analysis revealed region-specific and cortical layer-specific gene expression in astrocytes too, similar to what happens for neurons (Bayraktar et al., 2020). Finally, physiological Ca^{2+} activity is also region-dependent in the mouse (Yang and Jackson 2019). While this high degree of specialization and heterogeneity has been confirmed in humans, further molecular studies will be necessary to investigate astrocyte heterogeneity in a larger cohort of different mammalian orders.

Astroglia in human and non-human primates

Recently, increasing attention has been paid to astrocytes in the brains of human and non-human primates not only due to astrocytes' relevance in human pathology research but also due to their extraordinary complexity and heterogeneity, as well as their potential role in the evolution of advanced cognitive functions. An increase in astrocyte morphological complexity in primates vs mouse was already evident in old comparative studies of astrocytes. King et al. demonstrated that Rhesus macaque astrocytes are increased in number, length, and thickness of the processes, with an stronger glia-vascular and glia-neuronal relationship (King 1966).

While protoplasmic and fibrous astrocytes can be found in all mammalian species (Figures 4A,B), there are two types of astrocytes that reach a high degree of specialization in the primate cerebral cortex: interlaminar astrocytes (ILAs) and varicose-projection astrocytes (VP-As).

ILAs have their cell body in cortical layer I, very close to the pia, and have long, $GFAP^+$ interlaminar processes that travel perpendicular to the pia toward deeper cortical layers, reaching layer IV in humans (Figure 4C). *Pial* ILAs are strictly associated with the pial surface, while subpial ILAs have their somata in upper layer I but are not attached to the pia. ILAs were considered a primate-specific cell type for long time (Jorge A. Colombo and Reisin 2004). They were first described by Andriezen, who initially named them *caudate neuroglial fiber cells* in 1893 (Andriezen 1893). Ramón y Cajal and Retzius included ILAs in their drawings of the human cerebral cortex.

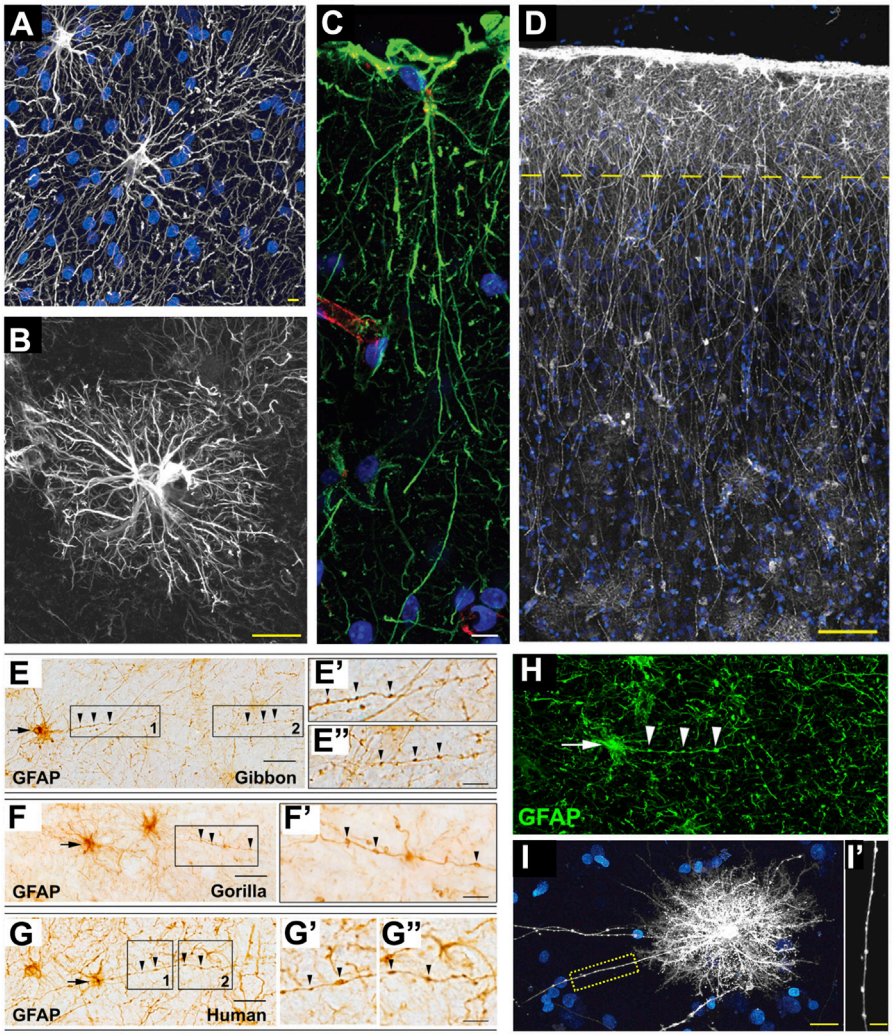

FIGURE 4
Astrocytes in hominid brains. **(A)** Adapted from Oberheim et al., 2009, Figure 4B. Typical human protoplasmic astrocyte. White: GFAP; blue: DAPI. Scale bar: 20 μm. **(B)** Adapted from Oberheim et al., 2009, Figure 7B. Human fibrous astrocytes in white matter. Grey: GFAP. Scale bar: 10 μm. **(C)** Example of ILA in the rhesus macaque dorsofrontal cortex. Green: GFAP; Red: Lectin; Blue: DAPI. Scale bar: 20 μm. **(D)** Adapted from Oberheim et al., 2009, Figure 3A, showing ILA palisade. Pial surface and layers one to two of human cortex. Dashed yellow line indicates border between layer 1 and 2. White: GFAP; Blue: DAPI. Scale bar, 100 μm. **(E–E")** Adapted from Falcone et al., 2021, Figure 2A. GFAP + VP-A in frontal cortex of a gibbon. Scale bar: 30 μm. **(E',E∋)** Higher magnification of 1 and 2 in E, respectively. Scale bar = 10 μm. **(F,F')** Adapted from Falcone et al., 2021, Figure 2C. GFAP + VP-A in frontal cortex of a gorilla. Scale bar = 30 μm. **(F')** Higher magnification of squared area in **(F)**. Scale bar: 10 μm. **(G–G∋)** Adapted from Falcone et al., 2021, Figure 2D. GFAP$^+$ VP-A in the human frontal cortex. Scale bar = 30 μm. **(G',G∋)** Higher magnification of 1 and 2 in G, respectively. Arrows point to cell somata, arrowheads point to varicosities on VP-A processes. Scale bar: 10 μm. **(H)** Adapted from Falcone et al., 2021, Figure 3E. VP-A in human frontal cortex. Green = GFAP; Blue = DAPI. Scale bar = 20 μm. Arrows point to cell somata, arrowheads point to varicosities on the VP-A processes. **(I,I')** Adapted from Oberheim et al., 2009, Figure 2B. Diolistic labeling (white) of a VP-A whose long process terminates in the neuropil. Blue = sytox. Scale bar: 20 μm. **(I')** High-power image of the yellow box in I highlighting the varicosities seen along the processes. Scale bar, 10 μm.

Nearly 100 years after, Colombo thoroughly described these unique cells in the brains of several species of primates (i.e., *Sapajus apella* and *Saimiri sciureus*, among others), including human (J. A. Colombo et al., 1995; J. A. Colombo 1996; J. A. Colombo et al., 1997; 2000; Jorge A. Colombo and Reisin 2004). He was the first to use the term *interlaminar astroglia* for this astrocyte subtype. With these studies, his group suggested that the long ILA processes represent a predominant feature of the postnatal primate cerebral cortex and form "palisades" due to their abundance and densely-packed distribution. Colombo's group hypothesized a potential role for ILAs in the columnar organization of the primate cerebral cortex; however; ILA functions are still largely unknown (Jorge A. Colombo and Reisin 2004). ILAs, with diverse morphologies,

have more recently been found to be present in all mammals, suggesting a very ancient origin for ILAs during mammalian evolution. In fact, in a study of 46 mammalian species (22 of which were primates), some mammals were found to have a rudimentary form of ILAs (*rudimentary ILAs,* observed in marsupials, Xenarthra, rodents, Scandentia, Chiroptera, Carnivora, and Artiodactyla), with processes not crossing the layer I–II boundary, while others displayed the typical form of ILAs (*typical ILAs*, observed in primates, Hyracoidea, and Proboscidea), with proper inter-layer processes (Falcone et al., 2019). However, ILAs have specific features in primates that are not present in non-primates: higher density, higher morphological complexity (i.e., ILA processes are more numerous, longer, and more branched), a specific developmental trajectory, and specific molecular markers (i.e., S100b, HOPX, and CRYAB, together with the astrocyte markers Vimentin, Glast, and Aqp4) (Falcone et al., 2019; 2020). Interestingly, among primates, the ILAs of the great apes have greater morphological complexity than other primates. ILAs appear during prenatal development, putatively originate from local RG cells, proliferate at their final destination close to the pia, but reach their final maturation and morphological complexity only after birth in both macaque and human (Falcone et al., 2020). Primate- and great ape-specific ILA features point to specific functions that ILAs might exert in primates' brain complexity and cognition; however, further studies are necessary to elucidate those functions.

VP-As are another fascinating type of GFAP$^+$ astrocyte present in deeper layers of the cerebral cortex and in white matter; they are characterized by bushy short processes and one to five long processes—spanning all directions—with prominent, evenly spaced varicosities (Figures 4D–H). They were first described by Dr. Needergard's group, which showed their morphology and presence in several human cortex specimens and one chimpanzee cortex specimen (Oberheim et al., 2006; 2009). VP-As have since been referred to as "human-specific" astrocytes (Verkhratsky et al., 2018); however, a more recent study has described their presence in five different species of great apes, as well (i.e., gorilla, *Gorilla*; bonobo, *Pan paniscus*; chimpanzee, *Pan troglodytes*; orangutan, *Pongo pygmaeus*; and gibbon, *Hylobates muelleri*) (Falcone et al., 2021), with comparable morphology in terms of soma shape and size, process length, and density of varicosities. The authors also screened several other species of primates from Old and New World monkeys, as well as prosimians, but they did not detect VP-As in any of these non-hominoid species. Furthermore, VP-As were not present in other mammals with large brains either (i.e., African elephant, *Loxodonta africana*; minke whale, *Balaenoptera acutorostrata*; or giraffe, *Giraffa camelopardalis*), indicating that they are not a feature of larger brains per se, but seem to occur only in human and apes, probably due to mechanisms that evolved in a common hominoid ancestor. Intriguingly, VP-As were not present in all human and ape individuals analyzed, leading to the hypothesis that they might represent a modified form of astrocytes undergoing a morphological change in response to specific brain conditions (Falcone et al., 2021). However, more studies are needed to identify the specific functions of VP-As.

Thanks to state-of-the-art single-cell RNA-seq technology, recent findings have shed light on the species-specific molecular profile of astrocytes. Human protoplasmic and fibrous astrocytes not only are morphologically different from their mouse counterparts (i.e., they are bigger and more complex) but also show a species-dependent transcriptomic profile that is intrinsically programmed (Li et al., 2021). In fact, human astrocytes display a certain degree of conserved gene expression compared to mouse, but also thousands of differentially expressed genes. In particular, human astrocytes show higher expression of genes involved in defense response to stress and genes linked with the extracellular space and secreted factors, while mouse astrocytes exhibit higher expression in genes involved in the regulation of metabolism and mitochondrial respiration (Li et al., 2021). Moreover, an RNA-seq analysis of induced pluripotent stem cell-derived astrocytes from human and chimpanzee showed inter-species differential gene expression in astrocytes, with notable differences in cellular respiration, glucose and lactate transmembrane transport, and pyruvate utilization, suggesting higher metabolic capabilities of human vs chimpanzee astrocytes (Zintel et al., 2020).

Discussion

Astrocytes are highly heterogeneous across different CNS regions, developmental stages, and species (Oberheim et al., 2009; Matyash and Kettenmann 2010; Oberheim, Goldman, and Nedergaard 2012; Verkhratsky et al., 2018; Yang and Jackson 2019; Bayraktar et al., 2020; Westergard and Rothstein 2020; Falcone et al., 2021). Findings related to the distribution and presence of astrocyte and astrocyte-like cells are scattered across several species of invertebrates and vertebrates (Supplementary Table S1), and, most of the times, relie on investigating their morphology, which is often revealed by immunohistochemistry against the GFAP marker. Ependymal cells and RG are currently hypothesized to be the predecessors of astrocytes in non-mammalian species. GFAP$^+$ ependymal cells are abundant in fish and amphibians, have variable distribution in reptiles, and are very scarce in birds and mammals. RG cells are present in all vertebrates, but with variable distribution: they are still the predominant type of astroglia-like cells in fish and amphibians, and in reptiles, they are retained in the adult and exert functions similar to those of mammalian astrocytes. Proper stellate astrocytes were historically first identified in mammals (where fibrous and protoplasmic astrocytes were first distinguished), but have been also observed in birds and reptiles, where they contact blood vessels and synapses and

begin to show features typical of astrocytes, like those we see in mammals (Suárez et al., 1995). Interestingly, in reptiles, intermediate forms between RG cells and astrocytes can be found; however, in adult mammals, most RG cells are replaced by astrocytes that retain some plasticity and their ability to proliferate under specific conditions. GFAP$^+$ astrocytes are found in lizards, snakes, and caimans, often intermingled within RG fibers, while being absent in turtles (Onteniente, Kimura, and Maeda 1983). The appearance of astrocytes in reptiles is concomitant with the reduction of ependymal cells and RG. This phenomenon points to reptiles being a key group in the phylogenetic evolution of astrocytes. Moreover, GFAP-free areas and the presence and diversity of astrocytes increased during evolution (Lőrincz and Kálmán 2020). The mixed presence of RG and astrocytes in reptiles suggests that the appearance of astrocytes anticipated and maybe contributed to increases in brain size and complexity. Astrocytes, in contrast to RG cells, have the advantage of forming a dynamic network that can provide local adaptability. In general, findings regarding astrocytes across evolution show a trend of the progressive regional adaptation of the glial structure, resulting in extraordinary astrocyte heterogeneity in primates and more specifically in humans (Kálmán and Pritz 2001).

However, the appearance of astrocytes in evolution has been proposed to be an apomorphic trait, especially when comparing caimans and turtles to phylogenetically related birds (i.e., chicken). For example, the presence of RG in several brain regions, often with an arched course of RG fibers, is a feature shared by caiman, turtles, and chicken. In contrast, GFAP expression is present everywhere in caiman (and astrocytes are intermingled with RG fibers and never predominant), while that expression is absent in turtle and predominant in birds.

We still have a long road ahead in understanding where astrocyte diversity comes from. To go farther down that road, a few new steps must be taken. First, to counteract the limitations linked to the use of GFAP to detect the presence of astrocytes, there is an urgent need to compare the expression of other astrocyte markers and specific GFAP isoforms (e.g. GFAP-α, GFAP-δ) across vertebrates. Moreover, more modern techniques, such as bulk and single-cell RNA profiling and spatial transcriptomics, will tell us more about astrocyte heterogeneity and origin across evolution (Yang and Jackson 2019). Finally, due to the high variability of astrocyte distribution, it is imperative that we investigate not only model organisms but also non-model organisms, so that important details about astrocyte evolution can be reconstructed.

Understanding how astrocytes evolved across vertebrates, and more specifically in mammals, is important information not only for its own sake but also because it can offer insights into primate-specific astrocytic pathologies, which are currently difficult to model in rodents.

Author contributions

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.931311/full#supplementary-material

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