Regulation of gene expression by growth differentiation factor 15 in the developing mouse and human neocortex

by

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Abstract

The evolution of the neocortex, specifically its expansion, is the key advancement that made higher cognitive abilities possible. Humans today have a neocortex that constitutes up to two-thirds of their overall brain mass. This expansion reflects increased and prolonged activity of neural progenitor cells (NPCs), which give birth to neurons during fetal cortical development. Growth differentiation factor 15 (GDF15) is a cell-extrinsic signal that has previously been found to promote basal progenitor proliferation, one of the main classes of NPCs critical to human brain development and evolution. Previously, the Xing Lab discovered several potential downstream targets that could be regulated by GDF15 in the fetal human neocortex, including Dachshund Family Transcription Factor 1 (*DACH1*), Glypican 6 (*GPC6*), Semaphorin 5A (*SEMA5A*), and Shroom Family Member 3 (*SHROOM3*). All of which have been reported to have various roles in neurodevelopment. In this study, we manipulated GDF15 levels in the developing mouse and human neocortex to understand its regulatory effect on the potential downstream targets. My range-finding experiment indicated a concentration-dependent increase in mRNA levels of the downstream targets, with the most increase observed in fetal human neocortical tissue incubated with 100ng/ml recombinant human GDF15 protein. Knockout of the GDF15 gene in embryonic mice revealed decreased mRNA levels of all the downstream targets. Furthermore, adult GDF15 knockout transgenic mice exhibited a decrease in interneuron and deep-layer neuron numbers. My findings are consistent with the notion that GDF15 regulates NPC proliferation through these downstream targets, contributing to long-term effects on neuron abundance in the adult brain.

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I would like to sincerely thank Dr. Lei Xing for his invaluable guidance, support, and encouragement throughout the course of this project. I am also grateful to honours thesis committee member Dr. Jae-Hyeok Lee and external member Dr. Margaret Docker for serving on my thesis committee and for their expertise and insightful feedback. I would also like to thank the members of the lab – Erica Wong, Drishti Sandhu, Ho In Shin, and Kalan Azaransky – for their support and encouragement, which made my learning experience and studies in the Xing lab both meaningful and enjoyable. Finally, I want to thank my friends and family for their patience and unwavering support, which has been the foundation of everything I have accomplished.

Table of Contents

List of Tables

List of Figures

Introduction

The evolution of the neocortex, the outer covering of the cerebral cortex, is the hallmark structure of mammals and the key advancement that made higher cognitive abilities possible (Florio & Huttner, 2014; Namba *et al*., 2020). Since its emergence in the mammalian ancestor around 220 million years ago, the neocortex has significantly expanded in several mammalian lineages, particularly in *Homo sapiens* (modern human) (Lewitus *et al*., 2013; Meredith *et al*., 2011; Rakic, 2009). Modern humans' brains are often considered the most capable of higher cognitive function compared to other mammals (Azevedo *et al*., 2009). Modern humans have a neocortex that constitutes up to two-thirds of their overall brain mass and contains approximately 16 billion neurons in the mature human brain (Tynianskaia & Heide, 2024). This expansion reflects increased and prolonged proliferative activity of neural progenitor cells (NPCs), the cells from which neurons are generated during fetal development (Wilsch-Bräuninger *et al*., 2016).

There are two main classes of NPCs: apical progenitors (APs) and basal progenitors (BPs) (Wilsch-Bräuninger *et al*., 2016). Primary NPCs, known as neuroepithelial cells, make up the population of APs; with the onset of neurogenesis, the process by which neurons are generated, neuroepithelial cells transform into apical radial glia (aRG) at approximately embryonic day (E) 10.5 in mice development (Florio & Huttner, 2014; Haubensak *et al*., 2004; Wilsch-Bräuninger *et al*., 2016; Xing & Huttner, 2020). aRG serve as a scaffold for radial migration of neurons and are also thought to have highly proliferative behaviour, serving as a self-replicator and as a source to give rise to BPs (Malatesta *et al*., 2008; Nonaka-Kinoshita *et al*., 2013; Rakic, 1995; Xing & Huttner, 2020). Two types of BPs can be found in the mammalian neocortex: basal

intermediate progenitors (bIPs) and basal radial glia (bRG) (Lewitus *et al*., 2013; Wilsch-Bräuninger *et al*., 2016). The primary sources of neuron production in the developing human neocortex are bIPs and bRGs, they undergo mitosis to give rise to cortical neurons, which migrate to various cortical layers to form the neocortex (Malatesta *et al*., 2008). Proliferation of BPs begins around E12.5 in mice and peaks at E14.5 (Yeh *et al*., 2014). Subsequently, neurogenesis will slow down, allowing glial cell development to become more predominant around E18.5 (Penit & Vasseur, 1989; Yeh *et al*., 2014). These proliferative stages in mice correspond to post-conceptional weeks (PCW) 9 and 11 in humans (Chan *et al*., 2002). This evolutionary increase in NPC abundance is considered a critical developmental event that increases the number of neocortical neurons, playing a key role in shaping the highly complex human brain (Xing *et al*., 2024).

Various cell-extrinsic signals, such as hormones, neurotransmitters, and growth factors, may regulate NPC proliferation (Xing *et al*., 2020). In previous unpublished work, the Xing Lab analyzed single-cell RNA sequencing datasets from human placental tissue across various developmental stages to identify placental factors relevant to neocortical development and NPC proliferation. This analysis revealed that growth differentiation factor 15 (GDF15) was enriched during placental developmental stages that coincide with peak proliferation of fetal NPCs, suggesting a potential role for GDF15 in modulating fetal neurogenesis (unpublished data). Notably, GDF15 is a multifunctional cytokine and a divergent member of the transforming growth factor β superfamily, a family of proteins involved in regulating a variety of cellular processes, including inflammation, cell proliferation, and repair (Jiang *et al*., 2021; Wischhusen *et al*., 2020). GDF15 is initially synthesized as a precursor protein, pro-GDF15, which undergoes a series of post-translational modifications (Baek & Eling, 2019). The pro-GDF15

monomer dimerizes via disulfide linkages in the endoplasmic reticulum; the dimer is cleaved by a protease, forming a mature C-terminal dimeric protein and a pro-peptide (Baek & Eling, 2019). The mature form of GDF15 protein is secreted into the bloodstream to exert its biological effects through various pathways, while the pro-peptide is localized in the nucleus and contributes to transcriptional regulation in GDF15 synthesizing cells (Bauskin *et al*., 2005; Jiang *et al*., 2021; Min *et al*., 2016).

The mature GDF15 protein can be detected in various tissues at a low concentration under normal physiological conditions, with exceptionally high concentrations detected in the placenta (Assadi *et al*., 2020). The placenta is a critical interface between the fetus and the mother, facilitating nutrient and waste exchange as well as playing a role in endocrine signalling, all of which are essential for fetal development (Moore *et al*., 2000). During pregnancy, circulating levels of GDF15 in maternal blood increase significantly, peaking in the third trimester (Andersson-Hall *et al*., 2021). These elevated levels have been suggested to play a role in maintaining pregnancy and influencing fetal growth, notably neocortical development (Assadi *et al*., 2020; Moore *et al*., 2000; Rosenfeld, 2015). For instance, in the developing mouse hippocampus, a brain region integral to learning, memory, and behaviour regulation, GDF15 has been shown to influence NPC proliferation and migration (Carrillo-García *et al*., 2014). Research by Carrillo-Garcia *et al*. (2014) demonstrated that reduced levels of GDF15 in the mouse hippocampus or the deletion of the *Gdf15* gene results in decreased cell proliferation and migration in hippocampal regions – cornu ammonis and dentate gyrus – via the epidermal growth factor signalling pathway in mice. This reduction in neurogenesis adversely affects brain development (Carrillo-García *et al*., 2014). The mouse choroid plexus, a group of capillaries in the ventricles that produce cerebrospinal

fluid, also synthesize GDF15 (Schober *et al*., 2001). Here, GDF15 is thought to play a neuroprotective role, aiding in the survival and differentiation of neural cells (Maetzler *et al*., 2016). However, much is still unknown about the molecular mechanisms underlying the action of GDF15 in regulating NPC proliferation in the developing neocortex, especially on the molecular targets that GDF15 influences. Understanding the genes and mechanisms regulated by GDF15 provides critical insights into healthy brain development and the potential causes of neurodevelopmental disorders.

Preliminary findings

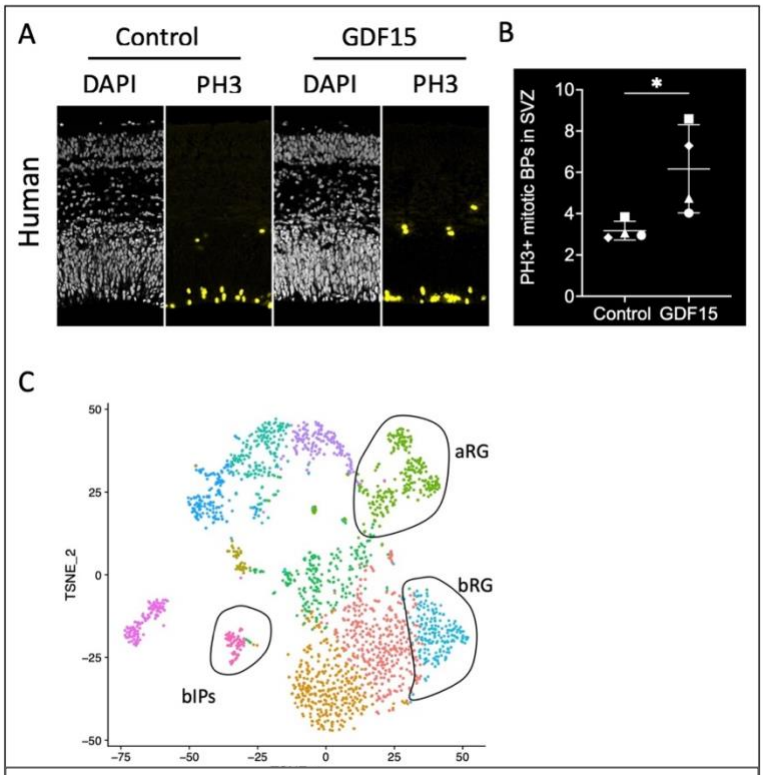

Figure 1. GDF15 increases basal progenitor abundance in fetal human neocortex.
A) Immunofluorescence of PH3 combined with DAPI staining of fetal human neocortical tissue incubated with and without recombinant GDF15 protein.
B) Quantification of mitotic basal progenitor abundance.
C) TSNE plot of sequenced nuclei showing the cell clusters and annotated cell types in fetal human neocortex.

Previous studies in the Xing Lab have shown that 100ng/ml recombinant human GDF15 protein increases the level of mitotic BPs in the cultured fetal human neocortical tissue. Primary antibody against the mitotic marker phosphohistone H3 (PH3) was used for immunostaining to label all mitotic NPCs in fetal human neocortical tissue incubated with (GDF15) or without (control) recombinant human GDF15 protein (Fig. 1A;

unpublished data). Quantifications of PH3+ BPs showed an increase in the GDF15 group compared to the control group (Fig. 1B; unpublished data). To understand the molecular mechanisms underlying the action of GDF15 increasing BP abundance in the fetal human neocortex, cultured human neocortical tissue incubated with or without GDF15 was submitted for single-nucleus RNA sequencing and differential gene expression analysis.

Data obtained from single-nucleus RNA sequencing and analysis revealed several potential downstream targets of GDF15 within aRG, bRG and bIPs of the fetal human neocortex (Fig. 1C; unpublished data). Among the most regulated genes by GDF15 are Dachshund Family Transcription Factor 1 (*DACH1*), Glypican 6 (*GPC6*), Semaphorin 5A (*SEMA5A*), and Shroom Family Member 3 (*SHROOM3*).

The Dach1 gene has been found to encode a transcription factor involved in developing the central nervous system (CNS), neural crest, eyes, and limb buds in mice (Castiglioni *et al*., 2019). *DACH1* expression was also detected in the early human neocortex during CNS development (Straccia *et al*., 2015). Further research has found that, in the human forebrain, *DACH1* is expressed in proliferating NPC populations in the human neocortex before significantly decreasing after cell proliferative stages (Castiglioni *et al*., 2019).

The *Gpc6* gene belongs to the Glypican family, a group of proteoglycans that anchor to the cell membrane of the developing mammalian brain and aid in synaptogenesis and organization (Allen *et al*., 2012; Filmus, 2022). *Gpc6* is readily expressed in NPCs from the anterior to posterior of the embryonic mouse brain (Luxardi *et al*., 2007). Further along in mouse development, *Gpc6* has also been found to be secreted by astrocytes, specialized glial cells, to induce synapses between neurons (Allen *et al*., 2012).

The *SEMA5A* gene belongs to the semaphorin family and is highly expressed in the fetal human hippocampus within the dentate gyrus (Zhu *et al*., 2014). The *SEMA5A* protein functions as an axon-regulating molecule and plays a significant role in neural and vascular development (Sadanandam *et al*., 2010). The *SEMA5A* protein also contributes to axonal guidance, neurogenesis, neuron and glial migration, and brain and spinal cord axonal midline crossing, meaning the axons connect to the right and left side of the CNS; this process is important for the establishment of neural circuits (Izzi & Charron, 2011; Limoni & Niquille, 2021; Zhu *et al*., 2014).

Lastly, *Shroom3* belongs to the Shroom family and is expressed in the neuroepithelium of mice (Hildebrand & Soriano, 1999). The Shroom3 protein is known to be a key player in epithelial apical construction during vertebrate neurodevelopment (Baldwin *et al*., 2022; Nishimura & Takeichi, 2008). Apical construction is an important cellular process during early neurodevelopment, driving neuroepithelial sheet bending and subsequent neural tube closure (McGreevy *et al*., 2015; Nishimura & Takeichi, 2008). Downregulation or mutation of *Shroom3* is associated with failed closure of the neural plates and results in neural tube defects, such as spina bifida, a condition in which the spine and spinal cord have not formed properly, in embryonic mice (Hildebrand & Soriano, 1999).

Each potential downstream target mentioned plays an essential role in neural development, and each target contributes to distinct aspects of this process. However, we do not know if GDF15 indeed regulates these targets and is thus involved in NPC behaviour and embryonic neurogenesis. Therefore, understanding how a cell-extrinsic signal, such as GDF15, can impact these targets is essential for dissecting the mechanisms underlying the neurogenesis process and brain development. This project is a continuation

of the previous research to narrow down the exact targets that GDF15 regulates in the developing mouse and human neocortex. I aim to investigate the mechanisms by which GDF15 influences NPC proliferation in the developing brain and validate the downstream targets of GDF15 that may play roles in NPC proliferation. Moreover, I seek to examine the long-term effects of GDF15 deficiency on adult brain anatomy and neuron abundance.

Materials & Methods

Mice

All animal protocols conformed to the standards of the University of Manitoba Animal Care Committee in accordance with the guidelines outlined by the Canadian Council for Animal Care and the University of Manitoba (Protocol F23-018 ID #AC11846). Wildtype (WT) and GDF15 knockout (KO) C57BL/6N mice were used for this project. The GDF15 KO mice were imported from Unsiker lab in Germany, which were generated and genotyped as previously described (Strelau *et al*., 2009). The WT and homozygous GDF15 KO embryos were obtained from heterozygous GDF15 KO mating.

Human tissue

All protocols related to the use of human tissue conformed to the standards of the University of Manitoba in accordance with the guidelines outlined by the Canadian Office of Human Research Ethics and the University of Manitoba (Protocol H2024:D41 ID #HS26312). Fetal human neocortical tissue at PCW9-15 was provided by the Human Development Biology Resource (HDBR). Upon arrival, fresh neocortical tissue from three fetuses was dissected and incubated with different concentrations of recombinant human GDF15 protein (0, 10, 100, 1000 ng/ml) using a free-floating tissue culture

system, previously in the Xing lab, for 48 hours. Incubated tissue and other fresh human neocortical tissue received from HDBR were flash-frozen using liquid nitrogen and stored at -80°C until further experimentation.

RNA extraction and cDNA synthesis

Embryonic mouse neocortex and fetal human neocortical tissue were subjected to total RNA extraction using the RNeasy Plus Mini kit from Qiagen (Cat. No. 74134) according to the manufacturer's instructions. The Maxima first-strand cDNA synthesis kit (Cat. No. K1642) from Thermo Fisher Scientific was used to synthesize cDNA for real-time quantitative polymerase chain reaction (RT-qPCR) according to the manufacturer's instructions.

RT-qPCR

The two-dye tracking system PowerTrack™ SYBR™ Green Master Mix (Cat. No. A46109) from Thermo Fisher Scientific, along various primers, was used to perform RT-qPCR following the 10µl reaction recipe shown in Table 1. The primers were designed previously in the Xing lab. The sequences for the primers used for analysis are shown in Table 2.

Table 1. PowerTrack™ SYBR™ Green Master Mix Recipe.

Component	Stock concentration	Final concentration	Volume for 10–µL reaction (µl)	Number of reactions	Overage (20%)	Total volume for all samples (µl)
PowerTrack ™ SYBR™ Green Master Mix	2X	1X	5	21	1.2x	126

Forward primer	8 µM	400nM	0.25	21	1.2x	6.3
Reverse primer	8 µM	400nM	0.25	21	1.2x	6.3
Ultrapure water	-	-	3.5	21	1.2x	88.2

Table 2. Primers used for RT-qPCR.

Human	*GAPDH*	Forward	AAGGTGAAGGTCGGAGTCAA
		Reverse	AATGAAGGGGTCATTGATGG
	ACTB	Forward	CGGGACCTGACAGACTACCTC
		Reverse	GGTGGTGAAGCTGTAGCCACG
	GPC6	Forward	TCTTGGTGGCAGAGCGACT
		Reverse	TGGAGCAGGTTTGGGCTGAC
	SEMA5A	Forward	AGGCTGCAATGTGGAGTACC
		Reverse	CGGGCTTTGCATGTGTATCG
	SHROOM3	Forward	GCTTTTGGGGCAAGACAGTGG
		Reverse	AACGAGGGGTGAGATGGTGGA
	DACH1	Forward	GGCAATGAGCCAGATGAACCA
		Reverse	CTGCTGCGATGTGATGATGGG
Mouse	*Gapdh*	Forward	TGAAGCAGGCATCTGAGGG
		Reverse	CGAAGGTGGAAGAGTGGGAG
	Actb	Forward	CGGGACCTGACAGACTACCTC
		Reverse	GGTGGTGAAGCTGTAGCCACG
	Gpc6	Forward	CAACGAGGAGGAGTGCTGGA
		Reverse	GCTGTCTGATGAAAGTGTCTGG
	Sema5a	Forward	CCCTGGTTACGGGAGTTCAGAG
		Reverse	GCCTTCTTGGTGGCTTCGTCA
	Shroom3	Forward	TGAATTCCAGCGGGTGTAGTG
		Reverse	GGTCAAGTCGGCTTCGGTTT
	Dach1	Forward	GCGTTTAGCCTTCCAGTTAGCC
		Reverse	AATGAGAGGGTGGGGCATCA

Quantstudio™ 3 Real-Time PCR system (Cat. No. A28567) instrument was used to amplify and quantify the cDNA. Design and Analysis 2 software was used to create the thermal profile to analyze the amount of DNA in samples.

Image analysis

The mouse neocortex of WT and GDF15 KO mice at postnatal day (P) 56 was dissected and embedded in liquid 3% low-melt agarose in embedding moulds. Then, it was cut out in blocks after solidification with a razor blade, previously in the Xing Lab.

Sections of 100µm thickness were cut and transferred with a soft brush onto a slide. Sections were then stained for different cell types using immunofluorescence with various antibodies and fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI) to stain cell nuclei. Staining was carried out based on the immunofluorescence protocol in previous experiments by Xing *et al*. (2020).

All stained sections were imaged using Zeiss LSM 880 Airy upright confocal microscope and Zeiss Plan-Apochromat 20× 0.8 or Zeiss Plan-Apochromat 40× 1.2 water objectives previously in the Xing Lab. Multi-tile images had 5% overlap and were stitched together using Zen. All cell counting was performed using Fiji image analysis software in standardized microscopic fields. The boundaries between cortical layers were deduced from the orientation and density of nuclei as observed by DAPI.

Experimental Design

Experiment 1. Temporal profile of molecular targets of GDF15 in developing mouse and human neocortex at different developmental stages.

Using primers of the downstream targets (Table 2), I first analyzed the temporal profile of the downstream targets of GDF15 in developing mouse and human neocortical tissue at E10.5-18.5 and PCW9-15, respectively. Fifteen samples of WT embryonic mouse neocortex tissue at E10.5-E18.5 and nine samples of fetal human neocortical tissue

at PCW9-15 were collected. RNA was extracted and cDNA was synthesized previously in the Xing lab. There were six developing human cDNA samples (one per developmental stage) and 15 WT embryonic mouse cDNA samples (three per developmental stage). For RT-qPCR, I diluted cDNA using Ultra-Pure Distilled H_2O from Thermo Fisher Scientific (Cat. No. 10977015). I used the diluted cDNA (1:50) as a template with forward and reverse primers for the molecular targets and PowerTrack™ SYBR™ Green Master Mix in the recipe listed in Table 1. I ran each biological sample in the Quantstudio™ 3 Real-Time PCR system in triplicates to amplify and quantify gene expression. I used housekeeping genes, Glyceraldehyde-3-Phosphate Dehydrogenase (*Gapdh*) and β-Actin (*Actb*), as internal controls for RT-qPCR normalization. I used Design and Analysis 2 software to analyze the RT-qPCR data.

Experiment 2. Regulation of gene expression by GDF15 in fetal human neocortex.

Fifteen fetal human neocortical tissue samples, collectively, from three fetuses aged PCW12 and 13 were collected and treated with 0, 1, 10, 100, or 1000ng/ml recombinant human GDF15 protein in free-floating culture previously in the Xing lab. I extracted RNA from the tissue and synthesized cDNA. I used the cDNA as the template, with forward and reverse primers for the genes of interest and the PowerTrack™ SYBR™ Green Master Mix recipe, as seen in Table 1. I ran the resulting reaction solution in the Quantstudio™ 3 Real-Time PCR system in triplicates to amplify and quantify gene expression. I used housekeeping genes *GAPDH* and *ACTB* as internal controls for RT-qPCR normalization. I used Design and Analysis 2 software to analyze the RT-qPCR data.

Experiment 3. Gene expression alteration in the embryonic GDF15 knockout mouse neocortex.

Six embryonic mouse neocortical tissue samples from E14.5 WT and ten GDF15 KO embryonic mice samples were collected previously in the Xing Lab and were used for this experiment. I extracted RNA from the tissue and synthesized the cDNA. I used cDNA as the template, with forward and reverse primers of the molecular targets and PowerTrack™ SYBR™ Green Master Mix following the recipe in Table 1. I ran the resulting reaction solution in the Quantstudio™ 3 Real-Time PCR system in triplicates to amplify and quantify gene expression. I used housekeeping genes *Gapdh* and *Actb* as internal controls for RT-qPCR normalization. I used Design and Analysis 2 software to analyze the RT-qPCR data.

Experiment 4. Neuron abundance analysis in adult wildtype and GDF15 knockout mouse neocortex.

I carried out quantifications of neuron abundance using Fiji image analysis software. After selecting comparable sections using the anatomical position from images sourced from the brain of seven WT and six GDF15 KO adult P56 mice, I measured the perimeter of the cortical hemisphere using the length from the middle cerebral artery along the pia mater as previously described (Xing *et al*., 2021) (see blue line, Fig. 6). I measured cortical thickness using a line from the lateral-most point of a ventricle radially through the cortex towards the pia mater as previously described (Xing *et al*., 2021) (see red line, Fig. 6). I used the perimeter and thickness as parameters to evaluate the neocortical size. I also manually counted cell type-specifically labelled cells on one

optical layer of the columnar multichannel images (see orange box, Fig. 6) using the CellCounter plug-in in Fiji.

Statistical analysis

I used Excel (Microsoft) for data processing and Prism (GraphPad Software) for data calculation and illustration. I tested data for normality using the Kolmogorov–Smirnov and the Shapiro–Wilk tests, and equality of variance was confirmed using the F-test. If the data was normally distributed, I compared the means between two groups using a two-tailed unpaired Student's t-test; means among multiple groups (more than two groups) were compared using one-way analysis of variance (ANOVA), followed by post hoc Tukey's or Bonferroni's multiple comparison tests. I used the Mann-Whitney or Kruskal-Wallis test if the data was not normally distributed. Error bars indicate the standard error of the mean (SEM).

Results

Molecular targets are highly expressed at cell proliferative developmental stages

I first determined the temporal expression patterns of the molecular targets of interest in WT embryonic mouse neocortex and fetal human neocortical tissue at various developmental stages (E10.5-18.5 and PCW9-15, respectively) (See 'Material & Methods' and 'Experimental Design' for details). I calculated the mean and SEM of three technical triplicates for three biological samples of embryonic mouse neocortical tissue and one biological sample of fetal human neocortical tissue at each developmental stage. I compiled the calculated data to construct the temporal profile accordingly. Consequently,

the expression patterns of *Gpc6, Dach1*, *Sema5a,* and *Shroom3* in the embryonic mouse neocortex showed that *Gpc6* exhibited the highest mRNA levels at E12.5 relative to the other developmental stages indicated (Fig. 2A). A similar pattern was observed for *Dach1* and *Shroom3,* with high expression at E12.5 followed by a dramatic decrease in mRNA levels (Fig. 2B, D). A peak in expression was observed for *Sema5a* at E16.5, with a smaller peak at E12.5 relative to the other developmental stages (Fig. 2C).

Similarly, the expression patterns of *GPC6*, *DACH1*, *SEMA5A*, *SHROOM3* in fetal human neocortical tissue showed that *GPC6* exhibited its highest peak in mRNA levels at PCW15 and an earlier smaller peak at PCW9 relative to the other developmental stages (Fig. 3A). A high level of expression was displayed for *DACH1* at PCW9 and 11, before dramatically decreasing at PCW12 and remaining at a low expression level during the later developmental stages (Fig. 3B). A similar pattern was exhibited with *SEMA5A,* showing a high level of expression at PCW9 and 11 before dramatically declining at PCW12 (Fig. 3C). The highest level of expression for *SHROOM3* was observed at PCW9, followed by a slow decrease throughout the subsequent developmental stages and later exhibiting increased expression at PCW14 (Fig. 3D). Note that the cell proliferative stages in embryonic mice are E12.5 and 14.5 which correspond to PCW9 and 11 in humans (Chan *et al*., 2002; Penit & Vasseur, 1989). Thus, these data show that the molecular targets are, in fact, highly expressed during the cell proliferative stages and lay the groundwork for further investigation into how GDF15 may promote cell proliferation through the regulation of *GPC6*, *DACH1*, *SEMA5A*, and *SHROOM3*.

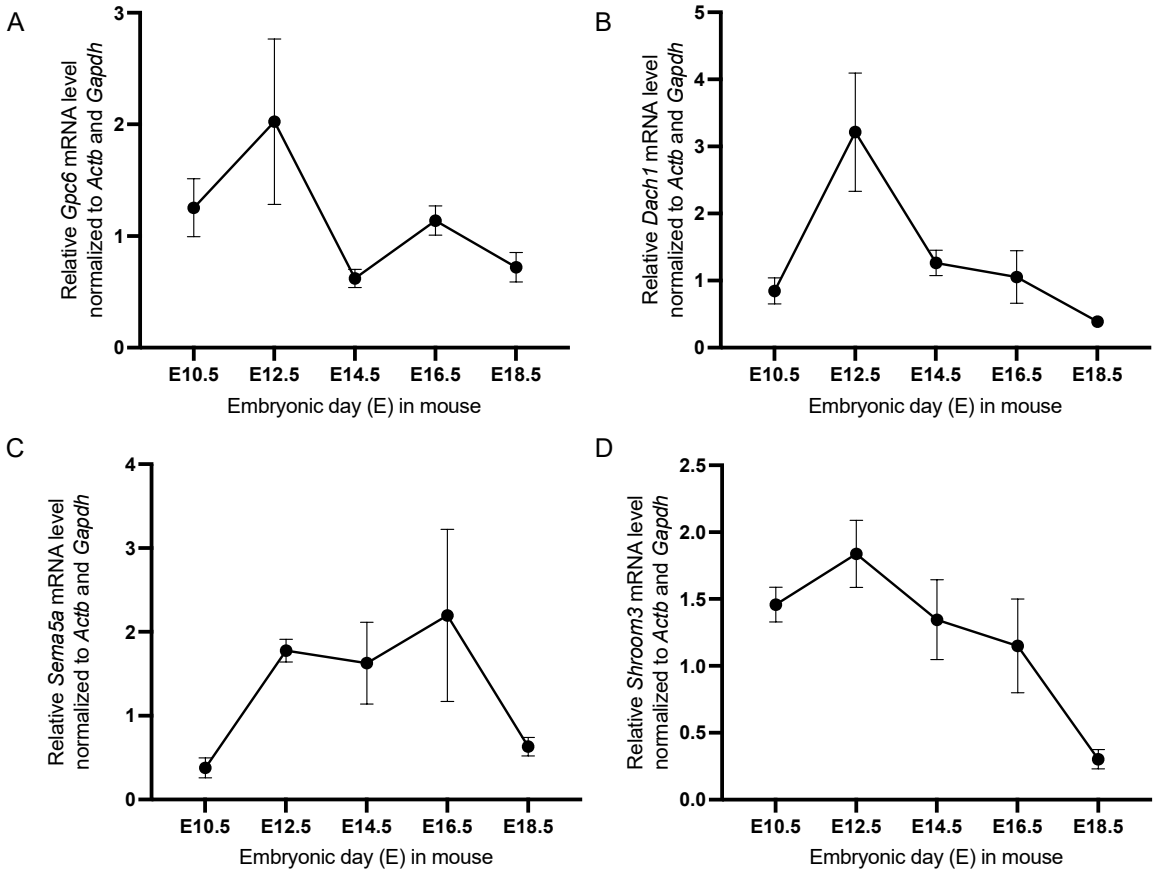

Figure 2. Temporal profile of the mRNA level of molecular targets in WT mouse neocortex (n = 3) revealed by single-nucleus RNA sequencing. Data were normalized to *Gapdh* and *Actb.* Error bars, SEM.

A) RT-qPCR analysis of relative *Gpc6* mRNA levels in WT mouse neocortex at the indicated developmental stages.

B) RT-qPCR analysis of relative *Dach1* mRNA levels in WT mouse neocortex at the indicated developmental stages.

C) RT-qPCR analysis of relative *Sema5a* mRNA levels in WT mouse neocortex at the indicated developmental stages.

D) RT-qPCR analysis of relative *Shroom3* mRNA levels in WT mouse neocortex at the indicated developmental stages.

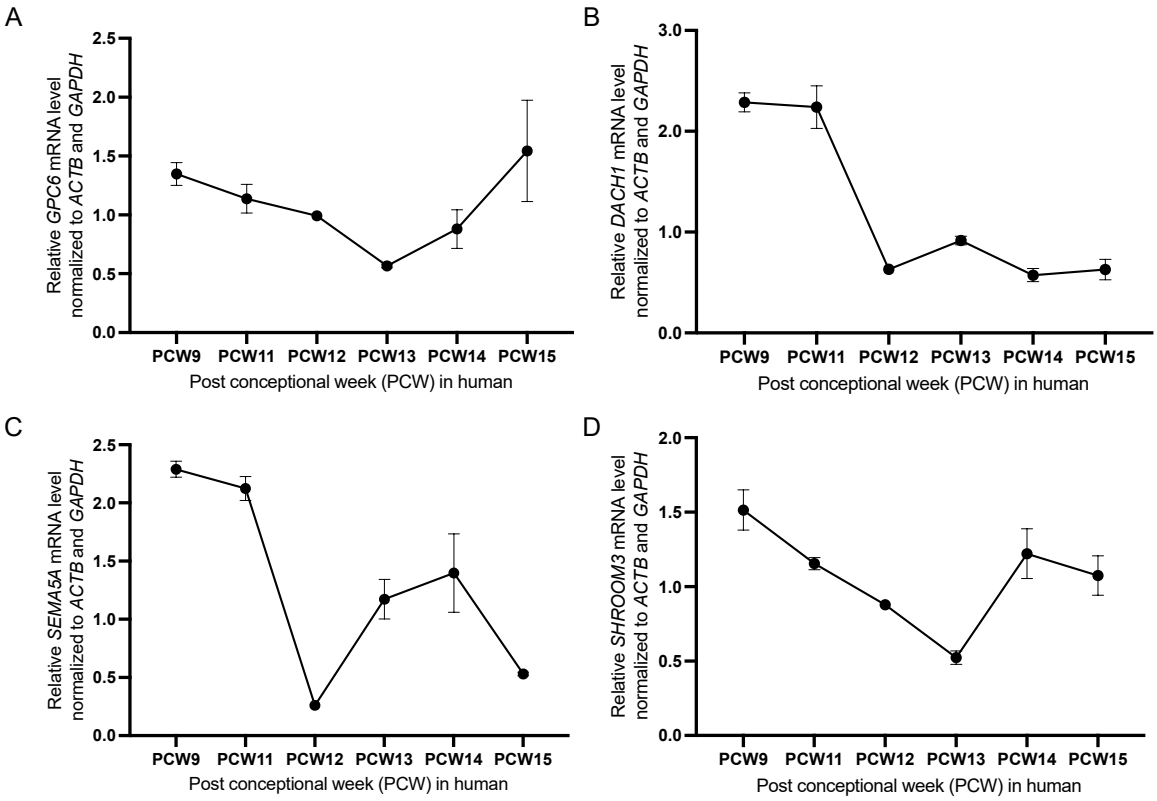

Figure 3. Temporal profile of the mRNA level of molecular targets in the fetal human neocortex (n = 1) revealed by single-nucleus RNA sequencing. Data were normalized to *GAPDH* and *ACTB.* Error bars, SEM of the three technical replicates.

A) RT-qPCR analysis of relative *GPC6* mRNA levels in the fetal human neocortex at the indicated developmental stages.

B) RT-qPCR analysis of relative *DACH1* mRNA levels in the fetal human neocortex at the indicated developmental stages.

C) RT-qPCR analysis of relative *SEMA5A* mRNA levels in the fetal human neocortex at the indicated developmental stages.

D) RT-qPCR analysis of relative *SHROOM3* mRNA levels in the fetal human neocortex at the indicated developmental stages.

Recombinant human GDF15 protein induces a dose-dependent increase in molecular target expression

The mRNA levels of the molecular targets in neocortical tissue incubated with various concentrations of recombinant human GDF15 protein (0, 1, 10, 100, 1000 ng/ml) were quantified (See 'Material & Methods' and 'Experimental Design' for details). I calculated the mean and SEM of three technical triplicates for three biological samples at the same concentration of recombinant human GDF15 protein, normalized to *GAPDH* and *ACTB*. Although the statistical tests did not reveal any significance, data from this range-finding experiment indicated a concentration-dependent increase in expression with the highest expression level of all molecular targets observed in neocortical tissue incubated with 100ng/ml recombinant human GDF15 protein. Specifically, in *GPC6*, 100ng/ml recombinant human GDF15 protein induced a ≈45% increase from control (Fig. 4A). Interestingly, *GPC6* also displayed a comparable ≈36% increase in expression at 1ng/ml compared to control (Fig. 4A). *DACH1* mRNA levels in 100ng/ml recombinant human GDF15 protein induced a ≈27% increase from control. In contrast, the other concentrations only showed up to ≈13% increase in *DACH1* mRNA levels from control (Fig. 4B). a ≈19% reduction in mRNA levels of *SEMA5A* was observed when incubated with 1ng/ml or 10ng/ml of recombinant human GDF15 protein compared to control (Fig. 4C). At 1000ng/ml, a more pronounced ≈32% reduction in *SEMA5A* mRNA levels compared to control was observed, while 100ng/ml resulted in a very modest reduction of ≈5% (Fig. 4C). Moreover, *SHROOM3* mRNA levels drastically increased ≈52% from control, when the fetal human neocortical tissue was incubated with 100ng/ml recombinant human GDF15 protein (Fig. 4D).

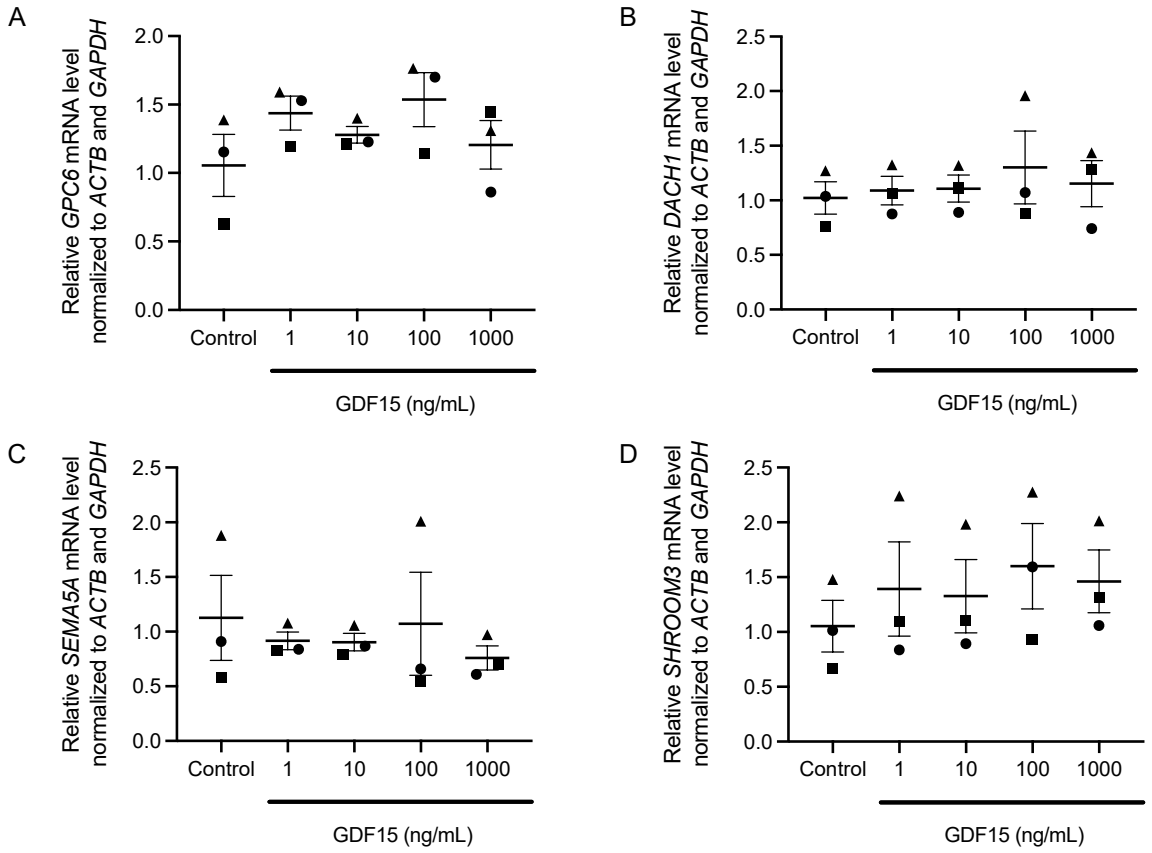

Figure 4. Relative mRNA levels of molecular targets in fetal human neocortical tissue at PCW12 and 13 (n = 3) incubated with 0, 10, 100, or 1000 ng/ml recombinant human GDF15 protein. The same symbol represents neocortical tissue from the same fetus. Data were normalized to *ACTB* and *GAPDH*. Error bars, SEM.

A) RT-qPCR analysis of the relative *GPC6* mRNA levels in fetal human neocortex incubated with 0, 10, 100, or 1000 ng/ml concentrations of recombinant human GDF15 protein.

B) RT-qPCR analysis of the relative *DACH1* mRNA levels in fetal human neocortex incubated with 0, 10, 100, or 1000 ng/ml concentrations of recombinant human GDF15 protein.

C) RT-qPCR analysis of the relative *SEMA5A* mRNA levels in fetal human neocortex incubated with 0, 10, 100, or 1000 ng/ml concentrations of recombinant human GDF15 protein.

D) RT-qPCR analysis of the relative *SHROOM3* mRNA levels in fetal human neocortex incubated with 0, 10, 100, or 1000 ng/ml concentrations of recombinant human GDF15 protein.

GDF15 knockout results in reduced expression of molecular targets

I examined the molecular targets' mRNA levels in E14.5 WT and GDF15 KO embryonic mice (See 'Material & Methods' and 'Experimental Design' for details). I calculated the mean and SEM of three technical triplicates for six WT and ten GDF15 KO biological samples at the same developmental stages, normalized to *Gapdh* and *Actb*. Analysis revealed that there is a change in the molecular target's mRNA levels in the GDF15 KO embryonic mouse neocortex compared to WT embryonic mouse neocortex (Fig. 5). Specifically, while *Gpc6* mRNA levels did not display significant results, there was still a ≈25% decrease in mRNA levels (Fig. 5A). GDF15 KO did result in a significant ≈40% decrease in *Dach1* mRNA levels compared to WT (Fig. 5B). *Sema5a* and *Shroom3* also displayed a significant ≈25% decrease in mRNA levels in the GDF15 KO embryonic mice compared to WT (Fig. 5C, D).

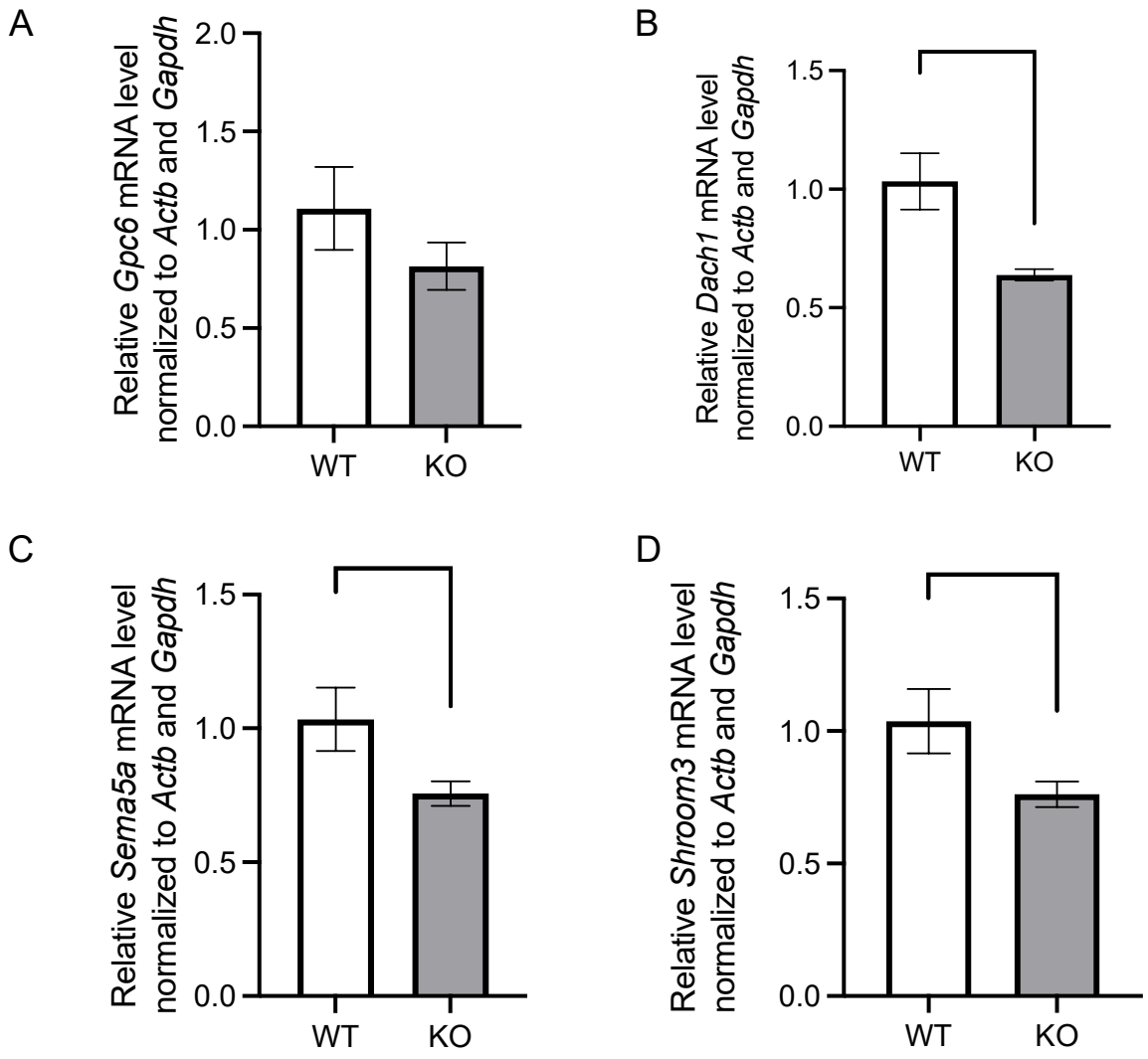

Figure 5. Relative mRNA levels of molecular targets in WT (n = 6) and GDF15 KO (n = 10) embryonic mouse neocortical tissue at E14.5. Data were normalized to *Gapdh* and *Actb*. Error bars, SEM.

A) RT-qPCR analysis of the relative *Gpc6* mRNA levels in WT and GDF15 KO embryonic mice at E14.5.

B) RT-qPCR analysis of the relative *Dach1* mRNA levels in WT and GDF15 KO embryonic mice at E14.5. ***, $p < 0.001$.

C) RT-qPCR analysis of the relative *Sema5a* mRNA levels in WT and GDF15 KO embryonic mice at E14.5. *, $p < 0.05$.

D) RT-qPCR analysis of the relative *Shroom3* mRNA levels in WT and GDF15 KO embryonic mice at E14.5. *, $p < 0.05$.

Adult GDF15 knockout mice exhibit decreased interneuron abundance

I examined the perimeter and thickness of the neocortex in seven WT and six GDF15 KO adult P56 mice (See 'Material & Methods' and 'Experimental Design' for details). Measurement of the cortical perimeter and thickness revealed no significant changes (Fig. 6A-C). Additionally, I analyzed the abundance of different types of neurons within the somatosensory cortex of three WT and three GDF15 KO adult P56 mice, as seen by the orange columns in Fig. 6A. Although the statistical tests did not reveal any significance, potentially due to the small sample size (n = 3), data from this neuron abundance analysis showed that as a consequence of GDF15 KO, the adult mouse neocortex exhibited a reduction in the numbers of Parvalbumin+ interneurons (Fig. 7A, C) as well as decreased numbers of Brn2+ neurons in layer IV of the somatosensory cortex (Fig. 7B, E). However, no noticeable change could be observed in Brn2+ neurons in layer II + III (Fig. 7B, D) or Ctip2+ neurons in layer V + VI (Fig. 7F). These data suggest that the downregulation of the expression of *Dach1*, *Sema5a* and *Shroom3* observed at E14.5 due to GDF15 KO (Fig. 5B-D) could lead to reduced abundance of Parvalbumin+ interneurons and Brn2+ neurons in layer IV in the somatosensory cortex.

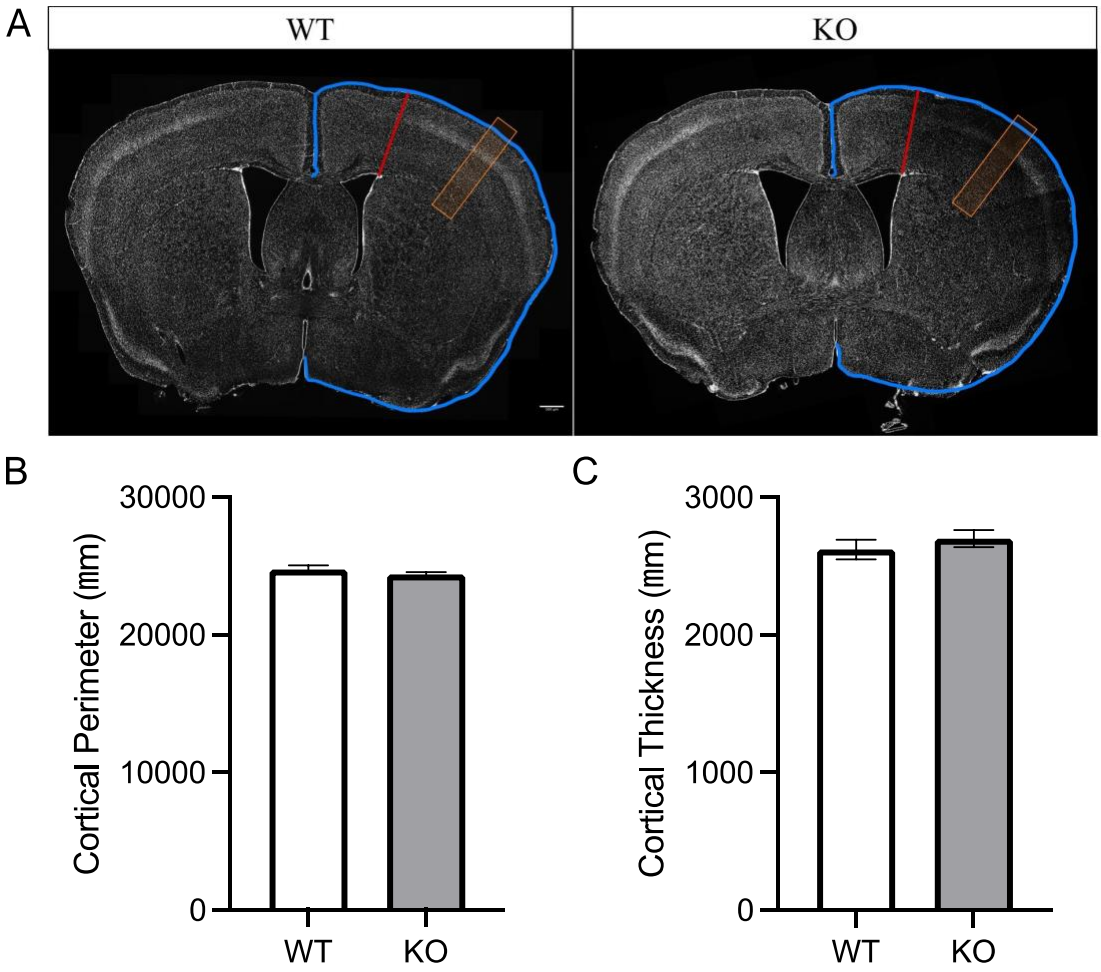

Figure 6. Quantifications of cortical perimeter and thickness in adult P56 WT (n = 3) and GDF15 KO (n = 3) mouse neocortex. Error bars, SEM.

A) Representative DAPI staining (white) of adult P56 WT and GDF15 KO mouse neocortex from confocal microscopy images illustrating the measurements of the perimeter (blue) and thickness (red) of the cortical hemisphere. The column (orange box) served for cell counting. Scale bar, 500 µm.

B) Cortical perimeter values measured from adult P56 WT and GDF15 KO mouse neocortex confocal microscopy images.

C) Cortical thickness values measured from adult P56 WT and GDF15 KO mouse neocortex confocal microscopy images.

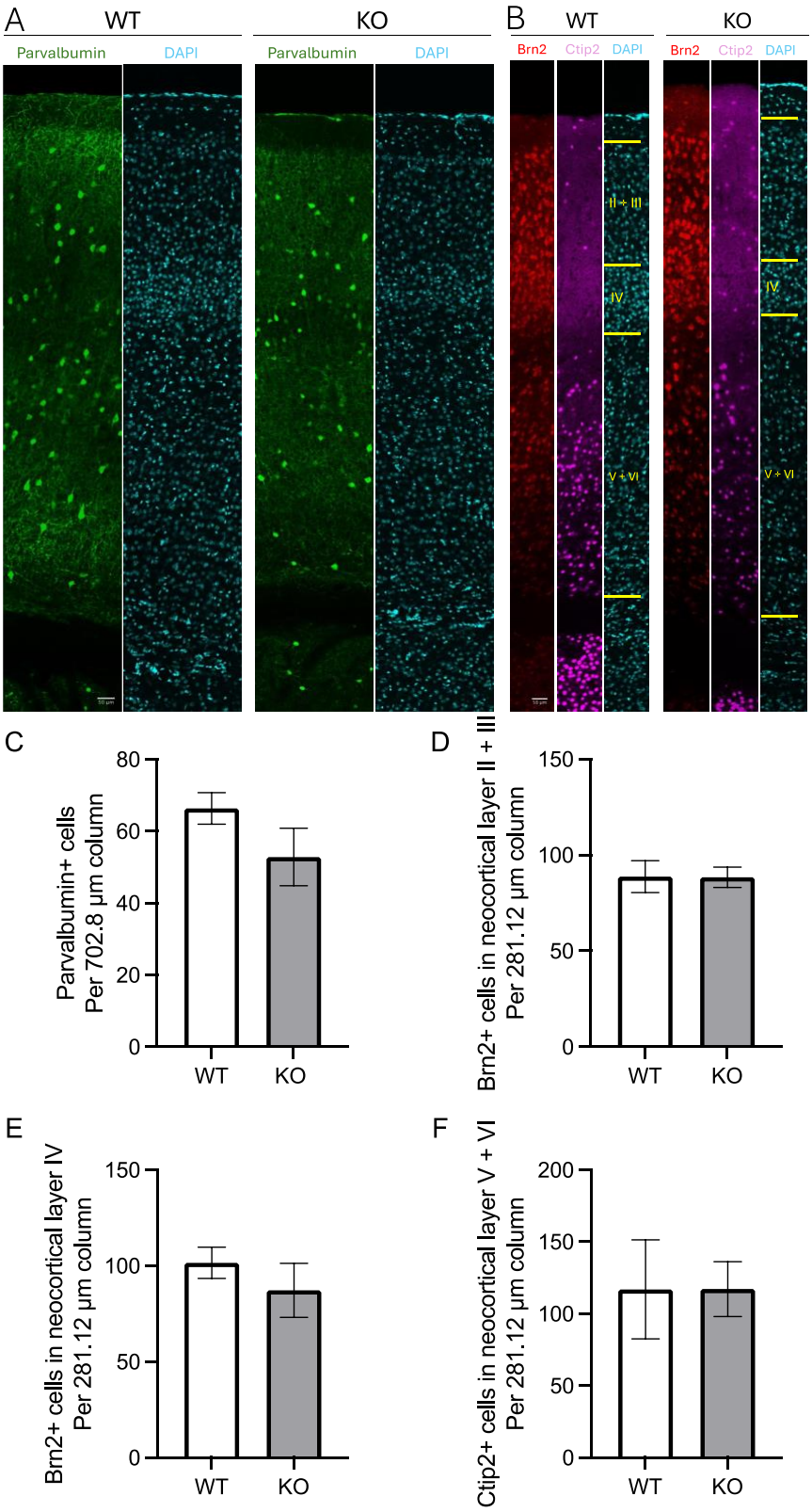

Figure 7. Quantification of Parvalbumin+, Brn2+, and Ctip2+ neurons in adult P56 WT (n = 3) and GDF15 KO (n = 3) mouse neocortex. Error bars, SEM.

A) Representative immunofluorescence for Parvalbumin (green) combined with DAPI staining (cyan) in a 702.8 μm-wide field of adult P56 WT and GDF15 KO mouse somatosensory cortex. Scale bar, 50 μm.

B) Representative immunofluorescence for Brn2 (red), Ctip2 (magenta), combined with DAPI staining (cyan) in a 281.12 μm-wide field of adult P56 WT and GDF15 KO mouse somatosensory cortex. Yellow lines indicate the boundaries between adjacent cortical layers of the somatosensory cortex. Scale bar, 50 μm.

C) Quantification of Parvalbumin+ neurons of adult P56 WT and GDF15 KO mouse neocortex.

D) Quantification of Brn2+ neurons in neocortical layer II + III of adult P56 WT and GDF15 KO mouse neocortex.

E) Quantifications of Brn2+ neurons in neocortical layer IV of adult P56 WT and GDF15 KO mouse neocortex.

F) Quantifications of Ctip2+ neurons in neocortical layer V + VI of adult P56 WT and GDF15 KO mouse neocortex.

Discussion

With increasing interest in the involvement of NPCs in neurodevelopmental diseases, this present study aimed to investigate the mechanisms by which cell-extrinsic factor GDF15 influences NPC proliferation in the developing brain. This study corroborates previous findings and provides new insights into the role of GDF15 in regulating NPC proliferation during embryonic development and its long-term effects on neuronal abundance in adulthood. I found that GDF15 modulates the expression of molecular targets *GPC6*, *DACH1*, *SEMA5A*, and *SHROOM3* during the peak cell proliferation period, causing downstream effects on neocortical development.

The basis of this study is the temporal profiling of GDF15 regulated genes during development. Among the molecular targets examined, *Dach1* and *Shroom3* showed the highest expression level during NPC proliferative stages in both mouse (E12.5-E14.5) and human (PCW9-11), relative to the other developmental stages (Fig. 2B, D and Fig. 3B, D). Previous studies have also reported high *DACH1* expression in proliferating NPC populations in the fetal human neocortex, with a significant decrease following the cell proliferative stages, further reinforcing this study's findings (Castiglioni *et al*., 2019). Another study on the human-specific gene *ARHGAP11B,* which also has a role in promoting BP proliferation, showed that *ARHGAP11B* levels peak highest at E12.5 in a similar manner as *Dach1* and *Shroom3* in this study (Xing *et al*., 2021). This similar temporal profile further validates that these downstream targets play an essential role in NPC proliferation. Likewise, *Sema5a* also exhibited high expression in cell proliferative stages E12.5 and similarly in humans at PCW9 (Fig. 2D and 3D), which builds on previous research showing that *SEMA5A* contributes to neurogenesis as well as other neurodevelopmental behaviour (Izzi & Charron, 2011; Limoni & Niquille, 2021; Zhu *et*

al., 2014). While *Gpc6* exhibited increased expression in embryonic mice (Fig. 2A), the human temporal profile revealed that it may not have as strong of an impact on cell proliferation as the other downstream targets (Fig 3A). While this study cannot confirm *Gpc6*'s role in neurogenesis, it may have roles in other developmental activities, such as synaptogenesis and organization, as previously described (Allen *et al*., 2012; Filmus, 2022). However, more work could be done to understand *Gpc6's* potential in neurogenesis. Overall, prior identification of *DACH1*, *SHROOM3*, and *SEMA5A* as potential downstream targets of GDF15 in the aRG, bRG, and bIPs, combined with my findings of their high expression during proliferative stages, provides a strong rationale for further investigation.

Furthermore, when the GDF15 protein level is manipulated pharmacologically using recombinant protein administration in culture media and genetically in the GDF15 KO mice, this study's results have confirmed the regulatory effects of GDF15 on the expression of target genes. Specifically, *Dach1* and *Shroom3* were upregulated in response to elevated GDF15 levels and suppressed by GDF15 deficiency (Fig. 4B, D and Fig. 5B, D), which further confirms GDF15's role in regulating these downstream targets. *Gpc6* also exhibited upregulation in response to elevated GDF15 levels and downregulation due to a lack of GDF15; however, the results were not statistically significant (Fig. 4A and Fig. 5A). These data align with previous studies on GDF15; Carrillo-García *et al*. (2014) reported that reduced levels or KO of GDF15 in mice resulted in decreased cell proliferation and migration in the hippocampal regions through decreased responsiveness of the epidermal growth factor signalling pathway, adversely affecting brain development. In a similar manner, this study demonstrates that GDF15 KO leads to a reduction in the expression levels of molecular targets *Dach1*, *Shroom3*, and

Sema5a, consequently decreasing their activity in NPC proliferation. These findings contribute to the existing literature on cell-extrinsic regulation of cell proliferation by reporting GDF15 as a key regulator of these molecular targets, influencing NPC proliferative behaviour in the developing neocortex.

In addition to investigating the molecular consequences of genetic ablation of GDF15 during development, I sought to evaluate how it leads to long-term effects on brain anatomy and function in adulthood. I observed that the lack of GDF15 protein in genetically modified mice does not cause pronounced changes in neocortical size. Measurements of cortical thickness and perimeter in adult P56 GDF15 KO mice revealed no significant differences from WT (Fig. 6A-C). This contradicts my hypothesis that GDF15 KO adult mice would exhibit smaller neocortical size. However, these morphological parameters may not fully capture the underlying cellular changes and, as such, should not be taken as evidence of unaltered cortical development. A more detailed analysis of neuron abundance in the adult GDF15 KO mouse neocortex revealed that specific neuron populations were impacted by GDF15 deficiency: reductions in Parvalbumin+ interneurons and Brn2+ neurons in layer IV of the somatosensory cortex were evident. It is worth noting that Parvalbumin plays an essential role in regulating excitatory transmission as a calcium-binding protein in inhibitory interneurons; as such, previous studies reported that a reduction of Parvalbumin allows for uninhibited excitability in the brain and, therefore, may be implicated in neurodevelopmental disorders like epilepsy (Godoy *et al*., 2022). Similarly, Brn2 is a transcription factor highly expressed in the neocortex and is thought to play a role in the proliferation of cortical precursors at E14.5 in embryonic mice (Hagino-Yamagishi *et al*., 1997; Sugitani *et al*., 2002). In line with its role in development, previous studies reported that depletion

of Brn2 may be implicated in neurodevelopmental pathology; organoids derived from schizophrenic individuals' tissue samples revealed a marked reduction in Brn2+ neurons (Notaras *et al*., 2021). Supplementing Brn2 restored neuron abundance in the organoids which rescued the phenotype, further supporting this link *(*Notaras *et al*., 2021).

Overall, my findings confirm that cell-extrinsic factor GDF15, through the regulation of downstream targets *GPC6*, *DACH1*, *SEMA5A*, and *SHROOM3*, is critical for influencing NPC proliferative activity and proper assembly of neocortical circuits during brain development. I have shown that manipulating GDF15 during critical periods of cell proliferation can alter neuron abundance, potentially leading to long-term phenotypical and pathological effects in adulthood. These results emphasize the importance of GDF15 as a modulator of embryonic neurogenesis. Future research could explore broader functions of GDF15 signalling within the brain, particularly by examining its activity in additional brain regions in order to determine whether GDF15 exerts region-specific modulation on regulating the expression of downstream molecular targets examined in this project. Additionally, investigating the role of GDF15 in shaping neurobehavioural outcomes in GDF15 KO mice is another important next step. Linking the molecular actions of GDF15 to higher-order brain functions could elucidate whether GDF15 influences long-term behavioural phenotypes. Furthermore, given its crucial role in cortical development, GDF15 shows potential as a target for early therapeutic interventions of neurodevelopmental disorders, particularly those caused by dysregulated developmental signalling.

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