Transcriptomic changes due to early, chronic ethanol exposure during cortical development implicate arealization, cell-type specification, axon guidance and synaptogenesis as primary determinants of Fetal Alcohol Spectrum Disorders

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Abstract

Ethanol (EtOH) exposure in early development can lead to profound consequences on neural progenitor cells (NPC) and the neurons that they generate. The clusters of neurological and anatomical hallmarks associated with prenatal EtOH exposure are collectively referred to as fetal alcohol spectrum (FAS). Because EtOH affects multiple developmental processes depending on dosage, frequency and timing of exposure, FAS symptoms exists on a spectrum from relatively benign to severely debilitating intellectual disability, including difficulties with learning, memory and executive function. While animal models successfully recapitulate multiple aspects of FAS including characteristically altered craniofacial morphogenesis and behavioral deficits, much less is understood regarding how EtOH exposure affects human NPCs and neuronal development. In this study, we utilize an in vitro human pluripotent stem cell-based (hPSC) model of early neurogenesis to probe the differences in the development of NPCs and post-mitotic neurons using bulk RNA sequencing. Our findings include specific alterations in the areas of WNT signaling, cortical regionalization and synapse formation.

Introduction

Fetal alcohol spectrum disorder (FASD) refers to a cluster of physical and mental symptoms affecting a person whose mother consumed alcohol during pregnancy. FASD is not a diagnostic term, but rather an umbrella term that can refer to fetal alcohol syndrome (FAS) with or without confirmed prenatal alcohol exposure, alcohol related neurodevelopmental disorder (ARND) and alcohol related birth defects (ARBD) (Kodituwakku 2007). According to estimates from the CDC, accidental and intentional drinking during pregnancy affects somewhere between 0.2 to 9 out of 1000 children in the U.S. and the added cost of care for individuals with FASD is approximately $2 million over the course of their lifetime (Popova 2017). FASD can present with a large variety of severity of symptoms, from relatively mild perturbations to adaptive learning, attention, executive function, social cognition and craniofacial morphogenesis, to severely debilitating disabilities (Lange 2017). Abstinence from alcohol during pregnancy can completely prevent the development of the disorder, but as many women do not know they are pregnant for several weeks after conception, it is critical to understand the types of neurological insults that can occur before that time (CDC 2009).

The cerebral cortex is thought to be a major target of prenatal alcohol exposure (PAE), as many of the social, affective and cognitive deficits exhibited by children with FASD involve cortical regions. Animal models of PAE have confirmed that ethanol (EtOH) produces significant alterations in cortical development at the gross morphological, cellular, and subcellular levels (Granato 2018). Furthermore, animal models of PAE display similar cognitive disabilities exhibited by FASD patients (reviewed in Marquardt and Brigman 2016). Impairments to executive function as well as spatial and reversal learning are indicative of cortically-mediated behaviors that are understood to be impaired in animal and human studies of PAE. However, the mechanisms

by which EtOH exerts these effects on the developing cortex remain unknown. Furthermore, it is critical to understand whether the effects of EtOH on animal models share cellular and molecular targets in a human context. To address this issue, multiple reports have used neurons differentiated from human pluripotent stem cells (hPSCs) as these are thought to recapitulate multiple aspects of *in vivo* neural patterning and specification (Erceg 2009, Zhang 2010, Studer 2017). Critically, default differentiation without the addition of patterning morphogens produces cerebral cortical neurons on a physiologically-relevant time scale (Vanderhaeghen 2015).

Studies of developing human cortical neurons have come to a wide variety of conclusions concerning the effects of EtOH on cell specification, patterning and regionalization as well as synaptogenesis (Yang 2012, Leigland 2013, Treit 2014)). Early studies in *in vitro* models of human cortical development have implicated Wnt and hedgehog signaling pathways (Vangipuram 2012) as well as synaptic functions such as NMDA receptor transcripts (Covault 2012) as being preferentially dysregulated following EtOH exposure. Impaired proliferation of radial glial cells in response to EtOH exposure has been shown to underlie a reduction of neurogenesis at higher (70-100 mM) doses, but not lower (13 mM) (Zecevic 2012). More recently Qin *et al.* found similar evidence for premature neural differentiation and impaired neurogenesis in a human cerebral organoid model treated with 50mM EtOH from day 10 forward following genetic analyses (2017).

Using qPCR-based RNA analysis, we previously demonstrated that chronic 50mM EtOH exposure to developing hPSNs led to a significant alteration in a number of mRNAs associated with ventral forebrain patterning and GABAergic neuron specification (Larsen et al. 2016). However, this was not accompanied by gross alterations in the number of GABAergic neurons generated nor as a functional excitatory-inhibitory imbalance. Thus, to probe for potential compensatory mechanisms and/or other mechanistic underpinnings of PAE, we used bulk RNA-

sequencing to determine how EtOH affects RNA expression at the transcriptome level. The current data support previous findings concerning targeted downregulation of transcripts related to GABAergic patterning and excitatory/inhibitory balance. Additionally, we report significant perturbation to transcripts associated with WNT signaling and the cortical arealization, which may work in concert to alter brain development in patients with FASD.

Results

Differentiation of Human Neurons and Experimental Pipeline

Figure 1A demonstrates the developmental timeline of neuronal differentiation from human pluripotent stem cells (hPSCs) to functional cortical neurons. We used a modified default differentiation paradigm using the serum-free embryoid body (SFEB) (Zhang 2001, Weick 2011) that causes cells to become a relatively pure (>95% $Pax6^{+}$) population of forebrain neuroectoderm by day 10 of differentiation (Lavaute 2009). Without the application of exogenous morphogens this protocol produces post-mitotic neurons between days 17-21 (Weick 2009) that display a cortical phenotype, comprised of a mixture of glutamatergic projection and GABAergic interneurons (Nadadhur 2018, Floruta 2017, Vanderhaeghen 2011). Furthermore, hPSC-derived neurons begin to display spiking and synaptic activity around 7-8 weeks of differentiation, comprised of both excitatory and inhibitory post-synaptic currents in ratios similar to those observed in cortex (Weick 2016). To determine the effect of EtOH on neuronal specification and patterning we applied 50mM EtOH daily throughout the differentiation protocol similar to previous reports and then examined changes to patterns of gene expression (Larson 2016). This is meant to mimic an early exposure during the periods of gastrulation and neurulation similar to those that may occur during early first trimester exposures (Okada 2009, Vaccarino 2012).

Global Findings for Transcripts Differentially Expressed Under EtOH Exposure

Comparisons of gene-level data for three biological replicates revealed 691 mRNA transcripts that were significantly altered (adjpval<0.05) at least 1.2-fold between untreated and EtOH-treated cells at day 50 (Fig. 1B). Interestingly EtOH had an overall positive impact on mRNA expression, with 477 transcripts upregulated compared to just 214 downregulated. This asymmetrical effect is

demonstrated also in Figure 1C which highlights the most differentially expressed (DE) gene transcripts, including a dramatic upregulation of WNT co-agonists R-spondin family members (RSPO1-3) as well as WNT8. In contrast, multiple GABAergic interneuron-related transcripts including DLX1, GAD1, and somatostatin (SST) as well as the WNT receptor FZD5 make up the most significantly downregulated transcripts (Fig. 1C). Lastly, unbiased hierarchical clustering of DE genes by treatment group (Fig. 1D) demonstrates that EtOH exposure leads to robust and reproducible patterns of change in the expression of these various transcripts.

Canonical Signaling Pathways Altered with EtOH Exposure

To gain insight into whether EtOH selectively altered the expression of genes within particular biological pathways, the list of DE transcripts was analyzed using the DAVID bioinformatics suite (v6.8); analytic tools designed to reference a list of genes and extract biological context from an integrated biological database using high throughput data-mining (Huang 2009). Functional annotation of DE transcripts utilizing the gene ontology-based "Biological Pathway" algorithm identified significant categories of enrichment (Supplemental Table 2). Some of the most selectively enriched pathways are listed in Figure 2A, such as "anterior/posterior axon guidance," "forebrain anterior/posterior pattern specification" as well as "cerebral cortex regionalization" (Fig. 2A). In addition to regional specifiers, abnormalities in GABAergic cell maturation and integration into the cortex were highlighted with categories like "cerebral cortex GABAergic interneuron differentiation" and "interneuron migration from the subpallium to the cortex." Together, these findings highlight previous changes observed that point to alterations in E/I balance, but also implicate more global alterations to cortical patterning. Also among the DAVID tools is the KEGG algorithm provided by the Kyoto Encyclopedia of Genes and Genomes, which relies on analysis of pathways rather than ontology terms (Kanehisa 2019).

Among the most enriched pathways according to KEGG were the categories corresponding to each the GABAergic and glutamatergic synapse as well as "synaptic vesicle cycle" and "focal adhesion," hinting perhaps at perturbation to the establishment and maintenance of synapses. Additionally the pathway associated with "alcoholism" and "Wnt signaling" were found to be highly enriched (Fig. 2B, Supplemental Table 3).

Lastly we took advantage of Qiagen's proprietary software, Ingenuity Pathway Analysis (IPA) to further contextualize the changes reported in the differential gene lists with another orthogonal algorithm. These analyses augment the GO and KEGG findings by providing additional biological context, as the IPA database is manually curated and based on peer-reviewed literature rather than high-throughput, *in silico* data-mining (Kramer 2014). According to IPA's analysis of the significantly enriched pathways, some of the most altered again included categories relating to WNT signaling, GABA receptor signaling, and synaptogenesis (Fig. 2C, Supplemental Table 4). In addition to pathway analyses, IPA enables users to identify potential upstream transcriptional regulators of DE genes as well as whether those transcription factors show up or downregulation in the given dataset. Given our list of DE mRNAs, the transcription factors *ASCL1* and GSX2 were not only predicted to be regulating our cultures with high statistical confidence but were also shown to be 1.76-fold and 2.97-fold downregulated with EtOH, respectively (Fig. 2D, Supplemental Table 5). Importantly, these transcription factors are both known to be involved in GABAergic interneuron patterning, as well as axonogenesis and synaptic patterning (Mizuguchi 2006, Kessaris 2014, Sun 2016). Conversely, EOMES (TBR2) the mostly highly predicted regulator was found to be upregulated 1.76-fold with EtOH. SOX2 and SHH, critical regulators of neural progenitor cells and regional patterning of the cortex, were additionally found to be potential upstream targets of EtOH in our dataset. Interestingly however, these were not significantly altered

in the abundance of their transcripts by EtOH; it is worth noting than neither SHH, nor any of the hedgehog ligands, were found to be expressed to detectable levels (Xu 2010, Nadadhur 2018). This suggests a unique type of patterning of cortical progenitors in default-generated hPSNs that is independent of SHH (Floruta 2017).

EtOH Exposure Alters Processing of GABAergic and Glutamatergic Differentiation-Associated Genes

It has been previously demonstrated that EtOH transcriptional induces alterations to multiple genes known to affect the patterning, differentiation and migration of GABAergic interneurons in early development (Yeh 2008, Vangipuram 2011, Larsen 2016, Qin 2017). In the current study, our data suggest multiple levels of regulation at which this GABAergic developmental program is altered (Fig. 3A). Markers of mature IN subtypes, such as NPY and SST, were found to be downregulated. SST is interesting given its important role in GABA interneuron specification. However, SST is post-transcriptionally suppressed in many cell-types and the protein is not found in default-generated hPSNs (Floruta et al., 2017). Perhaps most importantly, numerous transcription factors (TFs) associated with IN development were also significantly *down*regulated, including ASCL1, GSX2, SIX3 as well as homeobox TFs DLX1/2/5/6. In addition to alterations in the expression of markers of mature post-mitotic neurons, notably both mRNAs coding for the GABA-synthetic enzymes glutamic acid decarboxylase 65/67 (GAD1/2) were more than 2-fold downregulated with EtOH exposure. In contrast, several IN markers were found upregulated, including NDNF and RELN. Recent single-cell RNA-sequencing of rat cortex was used to discover that both markers are found co-expressed in a newly identified population of INs (2017 study), which may suggest compensatory changes that maintain E/I balance in default-generated hPSNs (see discussion).

In addition to mRNAs associated with GABAergic patterning, a large number of transcripts involved in the specification of glutamatergic cortical neurons were found to be significantly *up*regulated in the EtOH-treated group (Fig. 3B). These included the homeobox domain containing transcription factors *EMX2*, *LHX1/2/5/9* and *OTX2*, the zinc finger protein *FEZF2*, basic helix-loop-helix transcription factors *NEUROD1/2/6*, *NEUROG2* (*NGN2*) and importantly the t-box binding proteins *TBR1* and *TBR2* (*EOMES*). Interestingly, OTX2 as well as the DLX family of TFs, have been recognized for their role not only in brain development, but also craniofacial development, specifically of the rostral aspects of the head. This is noteworthy as dysregulation of these processes is a hallmark of individuals with FAS (Matsuo 2018). The proteins coded for by a number of these genes have also been identified by other groups as showing altered expression with prenatal EtOH exposure, such as TBR2 (Rakic 2011), FEZF2, NEUROD2, and NEUROG2 (Mandal 2018). Of note, NEUROG2 was shown to be sufficient to convert pluripotent stem cells into cortical glutamatergic projection neurons (Zhang 2013).

Transcripts involved with synaptic function further support E/I imbalance hypothesis

In addition to the perturbations of genes related to neuronal specification, KEGG and IPA analyses revealed that EtOH had a highly significant effect on mRNAs involved both GABAergic and glutamatergic synaptic function (Figure 2B). Figure 4B shows a diagrammatic summary of data list in Figure 4A, which illustrates an overall diminution of GABA signaling and increased glutamatergic transmission based on number of transcripts altered, similar to the effect of EtOH on patterning genes. Both synthesizing enzymes for GABA (*GAD1* and *GAD2*), along with the vesicular GABA transporter *VGAT*/*SLC32A1*, were all significantly downregulated by more than 2-fold. In contrast, both vesicular glutamate transporters, *VGLUT1*/*SLC17A7* and *VGLUT2*/*SLC17A6*, showed concurrent upregulation. In addition, a number of transcripts related

to glutamatergic synapse formation and function were upregulated. Of particular interest are the multiple members of the Glypican family (GPC1, 3, 4); these are significant as they are astrocyte-secreted factors that have been shown to be sufficient for inducing functional glutamatergic synapse formation (Eroglu). Collectively, these data add to the hypothesis that altered E/I imbalance may be a crucial mechanism underlying FASD pathologies.

EtOH primarily alters the WNT family of Secreted Morphogens

Appropriate spatial and temporal regulation of multiple secreted morphogens are required for proper cell type specification and regionalization of the brain and spinal cord during development (Lumsden 2005). As noted, the WNT signaling pathway was identified via multiple unbiased analyses (Fig. 2B, C) as significantly enriched in EtOH-treated hPSNs (Fig. 5E). Other morphogen pathways known to be critical for proper regionalization of the cerebral cortex include bone morphogen proteins (BMPs), fibroblast growth factors (FGF), Notch/Delta signaling and hedgehog signaling (Wilson 2004, Mallamaci 2006). Figure 5A-E highlights selected members of each of these respective morphogen categories that have been shown to facilitate cortical patterning and are expressed in default-differentiated hPSN cultures. While some individual members were altered, the preponderance of the genes within each pathway except for WNT signaling remained relatively unchanged (Fig. 5A-D).

The Hedgehog pathway is an established inducer of ventral fates in developing forebrain (Varga 2001, Mullor 2002). Similar to our previous report (Floruta et al., 2017), no individual hedgehog ligand was found to be expressed above background levels (see methods). Interestingly however, the dorsal marker GLI3 (Quinn 2009, Wang 2011) was found upregulated in EtOH-treated cells. Among the FGF family of molecules, FGF8 is known to be highly expressed in the anterior neural ridge (ANR), a secreted morphogen-rich organizing center at the most anterior

extent of neural ectoderm (Pownall 2010). However, it is also highly expressed in the isthmic organizer to help maintain the midbrain-hindbrain boundary. Interestingly, FGF8 was expressed well below background levels suggesting neither a highly anterior nor posterior default phenotype of hPSN forebrain progenitors. In contrast, its family member FGF receptor 3 (FGFR3), which has been shown to exist on a low to high gradient between the most anterior to the most caudal poles of the developing forebrain, was not only found to be robustly expressed, but also to be significantly upregulated (1.46-fold) with EtOH (Fig. 5B) (Alzu'bi 2017).

In contrast, 14 transcripts associated with WNT signaling were altered by EtOH treatment, with 85.7% (12/14) being upregulated. Interestingly, all three members of the secreted WNT co-agonist R-spondins (RSPO1-3) were among the most significantly upregulated transcripts. In addition, the WNT ligand proteins WNT5A, 7A and 8B (Fig. 5E) were all significantly upregulated. Importantly, not only were secreted ligands altered, downstream transcription factor targets of WNT signaling were also upregulated with EtOH treatment, including TCF3/4 and LEF1. One of the main downregulated WNT factors was the Frizzled receptor (FZD5), which was lowered to nearly undetectable levels in EtOH-treated cells. This receptor is interesting based on its anatomical enrichment in the ventro-lateral temporal lobe, near the anti-hem, and may indicate a potential compensatory mechanism to global WNT upregulation. However, the more highly expressed FZD2 receptor showed a minor but significant increase in expression.

EtOH exposure specifically increases transcripts with posterodorsal enrichment and decreases anteroventral markers

As WNT signaling is a critical modulator of regional patterning in the developing forebrain, we sought to examine how EtOH exposure affected expression of gene transcripts with regional enrichment along the anterior-posterior (A-P) and dorsal-ventral (D-V) neuraxes

(O'Leary 2007, Harrison-Uy 2012, Bocchi 2017). As mentioned, the canonical anterior marker FGF8 as well as ventral markers SHH and Nkx2.1, were not found to be expressed above background levels (Figure 5). Thus, it is possible that this simplified model system is not ideal for detecting changes across the A-P/D-V axes, where regional 'identity' is relatively biased toward posterodorsal cortex. However, many other markers expressed in default-generated hPSNs do display regional enrichment and are altered by EtOH. For instance, FGFR3, NR2F1/2 and EMX2 are enriched in caudal brain regions compared to SIX3, ROBO1, CHD19, which are enriched in anterior forebrain. Similarly, the transcription factor ventral-anterior homeobox 1 (VAX1) functions to specify ventral domains of the developing forebrain shows a nearly 4-fold downregulation (Hallonet 1999). These transcripts were both highly expressed and altered in our dataset in a complementary fashion, with posterior markers significantly increased and anterior markers decreased (Fig. 6A). Interestingly, many transcripts implicated in GABAergic IN development including members of the DLX family (DLX1/2/5/6) and ASCL1, are also enriched in the anteroventral forebrain and significantly decreased with EtOH (Figure 3B).

In addition, WNT signaling proteins themselves display highly restricted expression patterns in developing CNS, including the most altered WNTs in our dataset (WNT8b; Lako 1998). As mentioned, the roof-plate specific Spondin family (RSPO1-3) were among the most altered transcripts, are WNT co-agonists, and display largely posterodorsal expression in nervous tissue (Kamata 2004). In addition, many WNT ligands and receptors themselves are relatively restricted to the cortical hem, a dorsomedial structure that is critical for hippocampal formation in rodent brains. For instance, WNT2b, WNT5A, and FZD9 are all restricted to the hem and are significantly upregulated by EtOH (Fig. 5). Interestingly, alteration in expression or function of DLX1/2/5/6,

R-Spondins and VAX1 have all been shown to alter craniofacial morphology, a canonical feature full-blown FAS (Depew 2002, Jeong 2008, Jin 2011, Geohegan 2018).

Model of regional patterning changes underlying PAE/FASD.

Figure 7 illustrates summarized data for previously established transcripts that display regional enrichment or restriction. Significantly upregulated (red) and downregulated (blue) transcripts are superimposed on a sagittal view of a rodent brain for simplicity, including the ganglionic eminences. Included are canonical markers for regional identity including SHH, NKX2.1, FGF8, GLI3, PAX6, and BMP4, many of which are not expressed above background levels (indicated by *), or those expressed but not changed in our dataset (black). However, while there are examples of opposite patterns of expression (e.g. BRN2), the preponderance of evidence supports the fact that EtOH promotes the expression of posterodorsal transcripts at the expense of anteroventral genes in the telencephalon, at least at early timepoints during development.

Discussion

The transcriptomic data reported here provide unique insights into the potential mechanisms of cortical dysfunction in patients with FASD. We found that EtOH-induced changes could be clustered into a relatively narrow group of biologically relevant categories such as regionalization, cell fate decisions and synapse specificity. These findings provide testable hypotheses regarding potential compensatory mechanisms that help maintain E/I balance despite robust indications of overall decreases to GABAergic and increases to glutamatergic specification. Consistent with previous reports, WNT signaling, known to be critical in multiple aspects of development such as self-renewal and cell fate commitment, proved to be disproportionately affected by the presence of EtOH compared to other secreted morphogens and mitogens. Based on

the role of WNT signaling in determination of areal identity, we examined the regulation of a number of genes with regionally restricted patterns of expression and found evidence for a spectrum of up- and down-regulation along the nascent neuraxis. The most rostro-ventral transcripts in normal development showed notable downregulation, while more dorso-caudal mRNAs showed concurrent upregulation. Taken together, these data point to a potential primary effect of EtOH on WNT signaling, with the downstream consequence being overall caudalization of developing cerebral cortex. This is consistent with many anatomical and behavioral findings of animal models and patients with FAS/D, and may provide a mechanistic explanation for some of the functional deficits observed in these populations.

E/I Imbalance and PAX6-Dependence

A major hypothesis for several developmental disorders including FASD stems from the notion of excitatory-inhibitory imbalance, with multiple lines of evidence supporting enhanced excitation and reduced inhibition (reviewed thoroughly in Valenzuela 1997, Sadrian 2013). There is phenotypic evidence for this from clinical studies where patients with FAS/D display significantly increased rates of seizure disorders compared to health individuals (Nicita 2014). Furthermore, in rodent models Kim *et al*. first reported increased rates of glutamatergic differentiation and specification with comparable EtOH exposures and hypothesized that this proceeded in a PAX6-dependent manner (Kim 2010). Interestingly, despite the fact that the vast majority of neural precursors in this differentiation scheme express PAX6, we found no EtOH-induced difference in PAX6 expression in the current study, nor in our previous report, suggesting an alternative mechanism of E/I compensation apparent in these studies (Larsen 2016).

E/I Compensation by IN Subtype Shift

Recently developed single-cell sequencing techniques have begun to uncover the multitude of neuronal cell types making up the circuitry of the mammalian cortex (Wang 2018, Tang 2018). These early results present the hypothesis that E/I compensation may rely on differential patterning of neuronal subtypes rather than simply altering the number or proportion of principle neurons. Specifically, one possible explanation for this disparity is an upregulation of GABAergic neurons that are less affected by the loss of many upstream regulators thought to be required for GABA IN specification. While multiple TFs thought to be required for nearly all GABA IN fates were downregulated (e.g. ASCL1, GSX2, DLX1/2/5/6), studies with DLX1/2 knockout mice, while leading to an embryonic lethal phenotype, surprisingly still demonstrate the generation of GABAergic neurons (Le 2007). Interestingly, one study additionally found an accumulation of INs in the ganglionic eminences, though this may be due to impaired migration to the cortex rather than a proliferative effect per se (Le 2007, 2017). Strengthening this hypothesis, a recent report from Tasic *et al.* utilized single-cell RNA sequencing and clustering algorithms to assess cellular diversity in the mouse visual cortex and identified 49 distinct cell types, including two novel $NDNF^+$ IN subtypes (2.61-fold upregulated with EtOH) (Tasic 2016). Skorput *et al.* also noted an increase in the $parvalbumin^+$ (PV) interneurons in layers V and VI or adult PAE mice, indicating long-lasting effects of these patterning deficits (1.24-fold upregulated with EtOH) (2017). Perhaps the paradoxical upregulation of NDNF+, PV+ and RELN+ cells can point to unique classes of INs upregulated despite the TF repression, that help restore GABAergic tone and E/I balance in developing EtOH-exposed brains. Elucidating these cell types and the functional consequences of such shifts in IN diversity will prove critical for understanding the molecular mechanisms underlying brain functions altered in FAS/D patients.

WNTs in Synapse Formation

Key among the findings in this report was the preferential dysregulation of WNT signaling with EtOH, an alteration with many potential implications. The secreted family of WNT signaling molecules and their downstream effectors are known to regulate diverse developmental processes from the delineation of the initial germ layers to maintenance of stem cell proliferation and overall patterning of the forebrain (Lindsley 2006, Harrison-Uy 2012, Merrill 2012). Additionally, WNTs and their receptors have been found to influence synaptogenesis and synapse strengthening/maintenance (Ahmad-Annuar 2006). WNT ligands necessary for cell cycle regulation (WNT1/3A) were not affected by ethanol exposure, but additional family members with influence on synaptogenesis did show surprising degrees of dysregulation in these data. For instance, the highly expressed WNT7A ligand promotes synaptic assembly from the presynaptic bouton as well as synaptic vesicle recycling (Hall 2000). Although WNT7A ligand expression was unaltered with EtOH, shRNA knockdown of the receptor protein frizzled5 (FZD5) suggests that FZD5 is necessary for WNT7A's activity at nascent synapses (Sahores 2010). Interestingly, FZD5 is one of the only WNT-related transcripts to show significant reduction with EtOH exposure. This suggests that despite WNT7A not being regulated directly by EtOH, its effect through the FZD5 receptor is likely compromised, potentially reducing WNT7A's positive effect on excitatory glutamatergic tone overall as one potential mechanism of E/I compensation through WNT signaling. Another WNT protein that has been extensively studied for its role in synapse formation and maintenance is WNT5A – among the most highly upregulated in our study (Salinas 2012). In a FZD5-independent manner, WNT5A promotes synaptic assembly through binding Ror tyrosine receptors, which are also highly upregulated (Paganoni 2010). Interestingly, WNT5A increases the clustering of PSD95 and GABAA receptors when applied to hippocampal neurons in vitro,

increasing mini inhibitory postsynaptic current (mIPSC) amplitude but not frequency (Farias 2009, Cuitino 2010). While this does not directly explain the E/I compensation observed, it does clearly indicate the convergence of multiple WNT proteins with EtOH-regulated expression on GABA- and glutamatergic tone rather than cell cycle and proliferation.

WNTs in Arealization

In addition to WNT proteins' roles in synapse formation and maintenance, the preponderance of literature on WNT signaling focuses on their involvement with nervous system patterning (reviewed in Mulligan 2012). Specifically, one WNT family member, WNT5A, has been demonstrated in knockout models to be necessary for the development of caudal brain structures (Kumawat 2016). Broadly speaking however, WNTs tend to antagonize the ventralizing actions of sonic hedgehog in the neural tube, as well as FGF8 signaling in the anterior pole of the telencephalon (Danesin 2009). Expression of FGF8 at the anterior pole of the developing telencephalon is sufficient to coordinate the assembly of an entire regional signaling center referred to as the anterior neural ridge (ANR), (Shanmugalingam 2000). Interestingly, neither SHH nor FGF8 were expressed to detectable levels in neuroepithelial cells derived from the H9 cell line when differentiated via default methods (Supplemental Table 1, Floruta et al., 2017). The lack of FGF8 and SHH expression in these data is interesting as it potentially indicates an inherently caudal cell type due to either the H9 cell line or the default differentiation protocol that is enhanced by EtOH exposure. It is possible that other e/iPSC cell lines that express these factors to a greater basal extent could buffer the phenotype more effectively against caudalizing WNT signaling pathways.

TFs in Arealization (human)

In addition to WNT signaling, TFs such as NR2F2 (COUP-TFII) and EMX2 are known to coordinate regional cortical identity, as knockout of these factors demonstrably expands the extent of the caudal forebrain anteriorly (Mallamaci 2000). These current data were obtained in a human developmental model system however, so it is important to review the emerging work concerning regional distribution of TFs and morphogens in *in utero* human cortex. The Clowry lab in Newcastle, UK has demonstrated that in human developing cortex, the genes for NR2F2 and FGFR3 are among the most significantly enriched in caudal forebrain and both show significant upregulation with EtOH (Alzu'bi 2017). The same study showed that the DLX family, along with ASCl1 and ROBO1 to be among the most anteriorly enriched. All of these were downregulated by EtOH exposure in this study. ROBO1, downregulated here with EtOH, has been shown to suppress WNT signaling in human NPCs, potentially further exacerbating the EtOH-mediated caudalization (Andrews 2006). Furthermore, SP8 transcriptional repression by EMX2 has been reported to underlie some of the earliest A-P patterning in developing mouse brain (Sahara 2007) and knockout of SP8 expands caudal CoupTFI/II expression into more anterior cortical regions (Zembryzcki 2007). Taken together, these data suggest that TFs, in addition to morphogens like WNTs, are coordinating a caudalized cortical phenotype with EtOH exposure.

Behavioral Consequences

While this publication represents the most comprehensive transcriptomic analysis of EtOH-exposed developing human neurons to date, these findings are only particularly meaningful in context of functional, behavioral outputs later in life. We have described multiple putative mechanisms for the dysregulation and subsequent compensation of E/I balance as well as the WNT-mediated alterations to synapses and regionalization, but importantly, behavioral studies

have shown that PAE-associated behavioral changes closely mimic the deficits predicted by these genetic alterations (Marquardt 2016). Hamilton *et al.* recently concluded that a variety of moderate PAE-associated behavioral changes in rats, such as social behavior and cognitive flexibility in spatial tasks, seem to converge upon abnormal connectivity in ventro-lateral frontal cortex, a regional signature markedly reduced with EtOH treatment in these data (Hamilton 2014). Indeed these effects can be quite subtle – another report investigating behavioral correlates of PAE described no gross morphological, motor or sensory alterations, but rather more subtle changes to learning and behavior like reverse learning (Marquardt 2015). Neuroimaging and computation studies alike have linked these phenotypes to differences in cortical patterning and connectivity, exactly of the variety expected based on these findings (Astley 2009, Rodriguez 2016). The effects on transcripts associated with synaptogenesis, axon guidance and synaptic maintenance beg further investigation into the potential effect of EtOH on the synaptic architecture of new neurons, in terms of number and subtypes of synapses as well as their synaptic protein machinery. While the molecular changes highlighted in our study offer a possible mechanism for brain dysfunction, it is critical to link these to behavioral changes observed in PAE animals and individuals with FAS/FASD.

References:

1. Ahmad-Annuar A, Ciani L, Simeonidis I, Herreros J, Fredj NB, Rosso SB, et al. Signaling across the synapse: a role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. J Cell Biol. 2006 Jul 3;174(1):127–39.

2. Alzu'bi A, Lindsay SJ, Harkin LF, McIntyre J, Lisgo SN, Clowry GJ. The Transcription Factors COUP-TFI and COUP-TFII have Distinct Roles in Arealisation and GABAergic Interneuron Specification in the Early Human Fetal Telencephalon. Cerebral Cortex. 2017 Oct 1;27(10):4971–87.

3. Astley SJ, Richards T, Aylward EH, Olson HC, Kerns K, Brooks A, et al. Magnetic resonance spectroscopy outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. Magnetic Resonance Imaging. 2009 Jul;27(6):760–78.

4. Bocchi R, Egervari K, Carol-Perdiguer L, Viale B, Quairiaux C, De Roo M, et al. Perturbed Wnt signaling leads to neuronal migration delay, altered interhemispheric connections and impaired social behavior. Nat Commun. 2017 Dec;8(1):1158.

5. Cuitino L, Godoy JA, Farias GG, Couve A, Bonansco C, Fuenzalida M, et al. Wnt-5a Modulates Recycling of Functional GABAA Receptors on Hippocampal Neurons. Journal of Neuroscience. 2010 Jun 23;30(25):8411–20.

6. Cuzon VC, Yeh PWL, Yanagawa Y, Obata K, Yeh HH. Ethanol Consumption during Early Pregnancy Alters the Disposition of Tangentially Migrating GABAergic Interneurons in the Fetal Cortex. Journal of Neuroscience. 2008 Feb 20;28(8):1854–64.

7. Danesin C, Peres JN, Johansson M, Snowden V, Cording A, Papalopulu N, et al. Integration of Telencephalic Wnt and Hedgehog Signaling Center Activities by Foxg1. Developmental Cell. 2009 Apr;16(4):576–87.

8. Depew MJ. Specification of Jaw Subdivisions by Dlx Genes. Science. 2002 Oct 11;298(5592):381–5.

9. Erceg S, Ronaghi M, Stojković M. Human Embryonic Stem Cell Differentiation Toward Regional Specific Neural Precursors. Stem Cells. 2009 Jan;27(1):78–87.

10. Farías GG, Alfaro IE, Cerpa W, Grabowski CP, Godoy JA, Bonansco C, et al. *Wnt-5a* /JNK Signaling Promotes the Clustering of PSD-95 in Hippocampal Neurons. J Biol Chem. 2009 Jun 5;284(23):15857–66.

11. Floruta CM, Du R, Kang H, Stein JL, Weick JP. Default Patterning Produces Pan-cortical Glutamatergic and CGE/LGE-like GABAergic Neurons from Human Pluripotent Stem Cells. Stem Cell Reports. 2017 Nov;9(5):1463–76.

12. Gaspard N, Vanderhaeghen P. From stem cells to neural networks: recent advances and perspectives for neurodevelopmental disorders: Review. Developmental Medicine & Child Neurology. 2011 Jan;53(1):13–7.

13. Granato A, Dering B. Alcohol and the Developing Brain: Why Neurons Die and How Survivors Change. IJMS. 2018 Sep 30;19(10):2992.

14. Hall AC, Lucas FR, Salinas PC. Axonal Remodeling and Synaptic Differentiation in the Cerebellum Is Regulated by WNT-7a Signaling. Cell. 2000 Mar;100(5):525–35.

15. Hallonet M, Hollemann T, Pieler T, Gruss P. Vax1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system. Genes & Development. 1999 Dec 1;13(23):3106–14.

16. Hamilton DA, Magcalas CM, Barto D, Bird CW, Rodriguez CI, Fink BC, et al. Moderate Prenatal Alcohol Exposure and Quantification of Social Behavior in Adult Rats. JoVE. 2014 Dec 14;(94):52407.

17. Harrison-Uy SJ, Pleasure SJ. Wnt Signaling and Forebrain Development. Cold Spring Harbor Perspectives in Biology. 2012 Jul 1;4(7):a008094–a008094.

18. Hashimoto-Torii K, Kawasawa YI, Kuhn A, Rakic P. Combined transcriptome analysis of fetal human and mouse cerebral cortex exposed to alcohol. Proceedings of the National Academy of Sciences. 2011 Mar 8;108(10):4212–7.

19. Jeong J, Li X, McEvilly RJ, Rosenfeld MG, Lufkin T, Rubenstein JLR. Dlx genes pattern mammalian jaw primordium by regulating both lower jaw-specific and upper jaw-specific genetic programs. Development. 2008 Sep 1;135(17):2905–16.

20. Jin Y-R, Turcotte TJ, Crocker AL, Han XH, Yoon JK. The canonical Wnt signaling activator, R-spondin2, regulates craniofacial patterning and morphogenesis within the branchial arch through ectodermal–mesenchymal interaction. Developmental Biology. 2011 Apr;352(1):1–13.

21. Kamata T, Katsube K, Michikawa M, Yamada M, Takada S, Mizusawa H. R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression. 2004 Jan;1676(1):51–62.

22. Kanehisa M, Sato Y, Furumichi M, Morishima K, Tanabe M. New approach for understanding genome variations in KEGG. Nucleic Acids Research. 2019 Jan 8;47(D1):D590–5.

23. Kiecker C, Lumsden A. Compartments and their boundaries in vertebrate brain development. Nat Rev Neurosci. 2005 Jul;6(7):553–64.

24. Kim KC, Go HS, Bak HR, Choi CS, Choi I, Kim P, et al. Prenatal exposure of ethanol induces increased glutamatergic neuronal differentiation of neural progenitor cells. J Biomed Sci. 2010;17(1):85.

25. Kodituwakku PW. Defining the behavioral phenotype in children with fetal alcohol spectrum disorders: A review. Neuroscience & Biobehavioral Reviews. 2007 Jan;31(2):192–201.

26. Kramer M, Dutkowski J, Yu M, Bafna V, Ideker T. Inferring gene ontologies from pairwise similarity data. Bioinformatics. 2014 Jun 15;30(12):i34–42.

27. Kumawat K, Gosens R. WNT-5A: signaling and functions in health and disease. Cell Mol Life Sci. 2016 Feb;73(3):567–87.

28. Lange S, Rovet J, Rehm J, Popova S. Neurodevelopmental profile of Fetal Alcohol Spectrum Disorder: A systematic review. BMC Psychol. 2017 Dec;5(1):22.

29. Larsen ZH, Chander P, Joyner JA, Floruta CM, Demeter TL, Weick JP. Effects of Ethanol on Cellular Composition and Network Excitability of Human Pluripotent Stem Cell-Derived Neurons. Alcohol Clin Exp Res. 2016 Nov;40(11):2339–50.

30. LaVaute TM, Yoo YD, Pankratz MT, Weick JP, Gerstner JR, Zhang S-C. Regulation of Neural Specification from Human Embryonic Stem Cells by BMP and FGF. Stem Cells. 2009 Aug;27(8):1741–9.

31. Le TN, Du G, Fonseca M, Zhou Q-P, Wigle JT, Eisenstat DD. *Dlx* Homeobox Genes Promote Cortical Interneuron Migration from the Basal Forebrain by Direct Repression of the Semaphorin Receptor Neuropilin-2. J Biol Chem. 2007 Jun 29;282(26):19071–81.

32. Le TN, Zhou Q-P, Cobos I, Zhang S, Zagozewski J, Japoni S, et al. GABAergic Interneuron Differentiation in the Basal Forebrain Is Mediated through Direct Regulation of Glutamic Acid Decarboxylase Isoforms by *Dlx* Homeobox Transcription Factors. J Neurosci. 2017 Sep 6;37(36):8816–29.

33. Leigland LA, Ford MM, Lerch JP, Kroenke CD. The Influence of Fetal Ethanol Exposure on Subsequent Development of the Cerebral Cortex as Revealed by Magnetic Resonance Imaging. Alcohol Clin Exp Res. 2013 Jun;37(6):924–32.

34. Lieberman R, Levine ES, Kranzler HR, Abreu C, Covault J. Pilot Study of iPS-Derived Neural Cells to Examine Biologic Effects of Alcohol on Human Neurons In Vitro. Alcohol Clin Exp Res. 2012 Oct;36(10):1678–87.

35. Lindsley RC. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development. 2006 Oct 1;133(19):3787–96.

36. Mallamaci A, Stoykova A. Gene networks controlling early cerebral cortex arealization. European Journal of Neuroscience. 2006 Feb;23(4):847–56.

37. Mandal C, Park KS, Jung KH, Chai YG. Ethanol-related alterations in gene expression patterns in the developing murine hippocampus. Acta Biochim Biophys Sin. 2015 Aug;47(8):581–7.

38. Mariani J, Simonini MV, Palejev D, Tomasini L, Coppola G, Szekely AM, et al. Modeling human cortical development in vitro using induced pluripotent stem cells. Proceedings of the National Academy of Sciences. 2012 Jul 31;109(31):12770–5.

39. Marquardt K, Brigman JL. The impact of prenatal alcohol exposure on social, cognitive and affective behavioral domains: Insights from rodent models. Alcohol. 2016 Mar;51:1–15.

40. Marquardt K, Sigdel R, Caldwell K, Brigman JL. Prenatal Ethanol Exposure Impairs Executive Function in Mice into Adulthood. Alcohol Clin Exp Res. 2014 Dec;38(12):2962–8.

41. Matsuo I, Kuratani S, Kimura C, Takeda N, Aizawa S. Mouse Otx2 functions in the formation and patterning of rostral head. Genes & Development. 1995 Nov 1;9(21):2646–58.

42. Merrill BJ. Wnt Pathway Regulation of Embryonic Stem Cell Self-Renewal. Cold Spring Harbor Perspectives in Biology. 2012 Sep 1;4(9):a007971–a007971.

43. Mizuguchi R, Kriks S, Cordes R, Gossler A, Ma Q, Goulding M. Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. Nat Neurosci. 2006 Jun;9(6):770–8.

44. Mo Z, Milivojevic V, Zecevic N. Enforced Pax6 Expression Rescues Alcohol-Induced Defects of Neuronal Differentiation in Cultures of Human Cortical Progenitor Cells. Alcohol Clin Exp Res. 2012 Aug;36(8):1374–84.

45. Mulligan KA, Cheyette BNR. Wnt Signaling in Vertebrate Neural Development and Function. J Neuroimmune Pharmacol. 2012 Dec;7(4):774–87.

46. Mullor JL, Ruiz i. Altaba A. Growth, hedgehog and the price of GAS. Bioessays. 2002 Jan;24(1):22–6.

47. Nadadhur AG, Alsaqati M, Gasparotto L, Cornelissen-Steijger P, van Hugte E, Dooves S, et al. Neuron-Glia Interactions Increase Neuronal Phenotypes in Tuberous Sclerosis Complex Patient iPSC-Derived Models. Stem Cell Reports. 2019 Jan;12(1):42–56.

48. Nicita F, Verrotti A, Pruna D, Striano P, Capovilla G, Savasta S, et al. Seizures in fetal alcohol spectrum disorders: Evaluation of clinical, electroencephalographic, and neuroradiologic features in a pediatric case series. Epilepsia. 2014 Jun;55(6):e60–6.

49. O'Leary DDM, Chou S-J, Sahara S. Area Patterning of the Mammalian Cortex. Neuron. 2007 Oct;56(2):252–69.

50. Paganoni S, Bernstein J, Ferreira A. Ror1-Ror2 complexes modulate synapse formation in hippocampal neurons. Neuroscience. 2010 Feb;165(4):1261–74.

51. Popova S, Lange S, Probst C, Gmel G, Rehm J. Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis. The Lancet Global Health. 2017 Mar;5(3):e290–9.

52. Quinn JC, Molinek M, Mason JO, Price DJ. Gli3 is required autonomously for dorsal telencephalic cells to adopt appropriate fates during embryonic forebrain development. Developmental Biology. 2009 Mar;327(1):204–15.

53. Sadrian B, Wilson D, Saito M. Long-Lasting Neural Circuit Dysfunction Following Developmental Ethanol Exposure. Brain Sciences. 2013 Apr 29;3(4):704–27.

54. Sahores M, Gibb A, Salinas PC. Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis. Development. 2010 Jul 1;137(13):2215–25.

55. Shanmugalingam S. Fgf8/Ace in forebrain patterning. :13.

56. Skorput AGJ, Gupta VP, Yeh PWL, Yeh HH. Persistent Interneuronopathy in the Prefrontal Cortex of Young Adult Offspring Exposed to Ethanol In Utero. Journal of Neuroscience. 2015 Aug 5;35(31):10977–88.

57. Smith DK, Yang J, Liu M-L, Zhang C-L. Small Molecules Modulate Chromatin Accessibility to Promote NEUROG2-Mediated Fibroblast-to-Neuron Reprogramming. Stem Cell Reports. 2016 Nov;7(5):955–69.

58. Sun AX, Yuan Q, Tan S, Xiao Y, Wang D, Khoo ATT, et al. Direct Induction and Functional Maturation of Forebrain GABAergic Neurons from Human Pluripotent Stem Cells. Cell Reports. 2016 Aug;16(7):1942–53.

59. Suzuki IK, Vanderhaeghen P. Is this a brain which I see before me? Modeling human neural development with pluripotent stem cells. Development. 2015 Sep 15;142(18):3138–50.

60. Tang H, Zeng T, Chen L. High-Order Correlation Integration for Single-Cell or Bulk RNA-seq Data Analysis. Front Genet. 2019 Apr 26;10:371.

61. Tasic B, Menon V, Nguyen TN, Kim TK, Jarsky T, Yao Z, et al. Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. Nat Neurosci. 2016 Feb;19(2):335–46.

62. Tchieu J, Zimmer B, Fattahi F, Amin S, Zeltner N, Chen S, et al. A Modular Platform for Differentiation of Human PSCs into All Major Ectodermal Lineages. Cell Stem Cell. 2017 Sep;21(3):399-410.e7.

63. Treit S, Lebel C, Baugh L, Rasmussen C, Andrew G, Beaulieu C. Longitudinal MRI Reveals Altered Trajectory of Brain Development during Childhood and Adolescence in Fetal Alcohol Spectrum Disorders. Journal of Neuroscience. 2013 Jun 12;33(24):10098–109.

64. Valenzuela CF. Alcohol and Neurotransmitter Interactions. RESEARCH WORLD. 1997;21(2):5.

65. Vangipuram SD, Lyman WD. Ethanol Affects Differentiation-Related Pathways and Suppresses Wnt Signaling Protein Expression in Human Neural Stem Cells: ETHANOL SUPPRESSES WNT SIGNALING PROTEINS. Alcoholism: Clinical and Experimental Research. 2012 May;36(5):788–97.

66. Varga ZM. Zebrafish smoothened. :13.

67. Wang H, Ge G, Uchida Y, Luu B, Ahn S. Gli3 Is Required for Maintenance and Fate Specification of Cortical Progenitors. Journal of Neuroscience. 2011 Apr 27;31(17):6440–8.

68. Wang M, Liu X, Chang G, Chen Y, An G, Yan L, et al. Single-Cell RNA Sequencing Analysis Reveals Sequential Cell Fate Transition during Human Spermatogenesis. Cell Stem Cell. 2018 Oct;23(4):599-614.e4.

69. Weick JP, Liu Y, Zhang S-C. Human embryonic stem cell-derived neurons adopt and regulate the activity of an established neural network. Proceedings of the National Academy of Sciences. 2011 Dec 13;108(50):20189–94.

70. Weick JP. Functional Properties of Human Stem Cell-Derived Neurons in Health and Disease. Stem Cells International. 2016;2016:1–10.

71. Wilson SW, Houart C. Early Steps in the Development of the Forebrain. Developmental Cell. 2004 Feb;6(2):167–81.

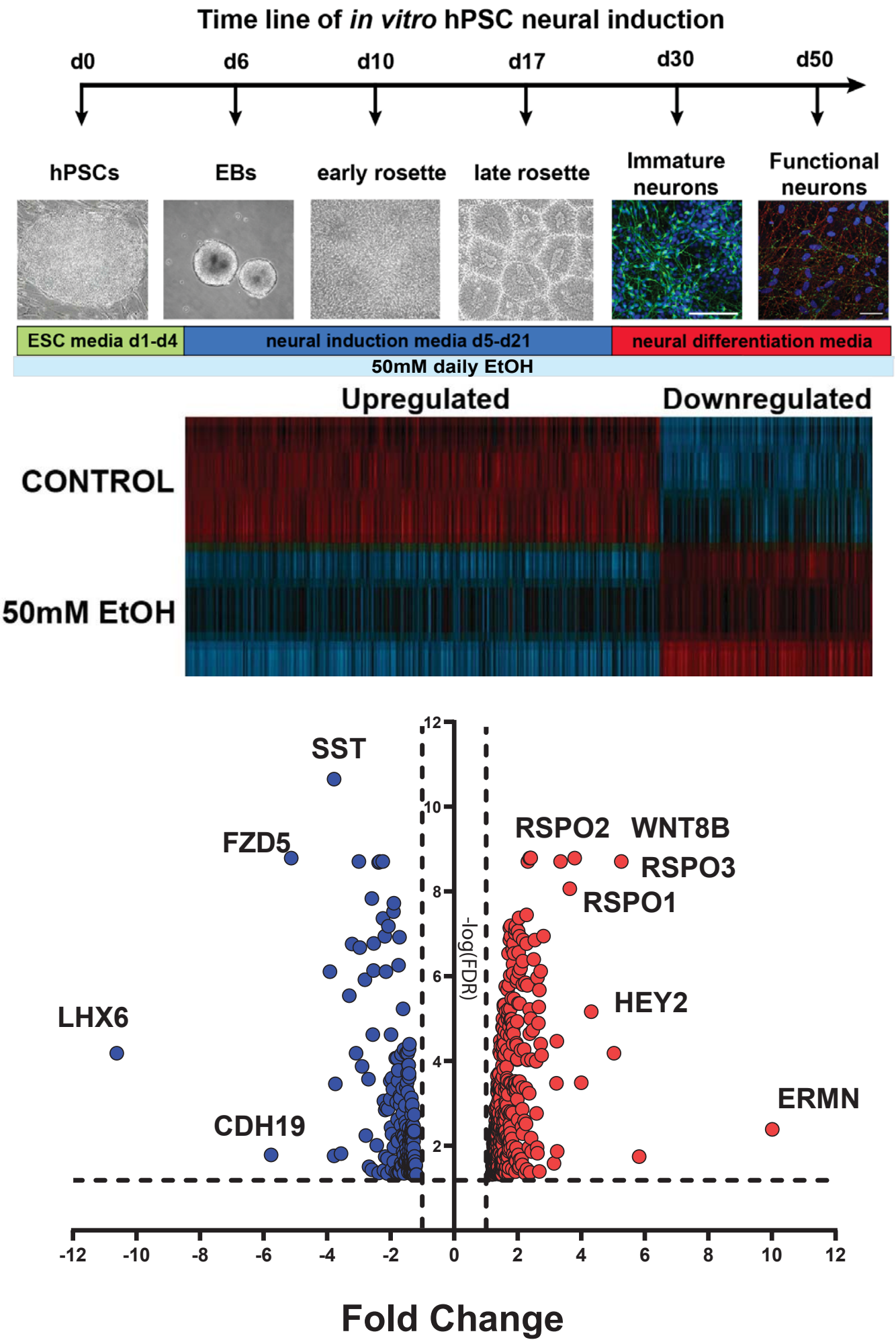
72. Xu Q, Guo L, Moore H, Waclaw RR, Campbell K, Anderson SA. Sonic Hedgehog Signaling Confers Ventral Telencephalic Progenitors with Distinct Cortical Interneuron Fates. Neuron. 2010 Feb;65(3):328–40.

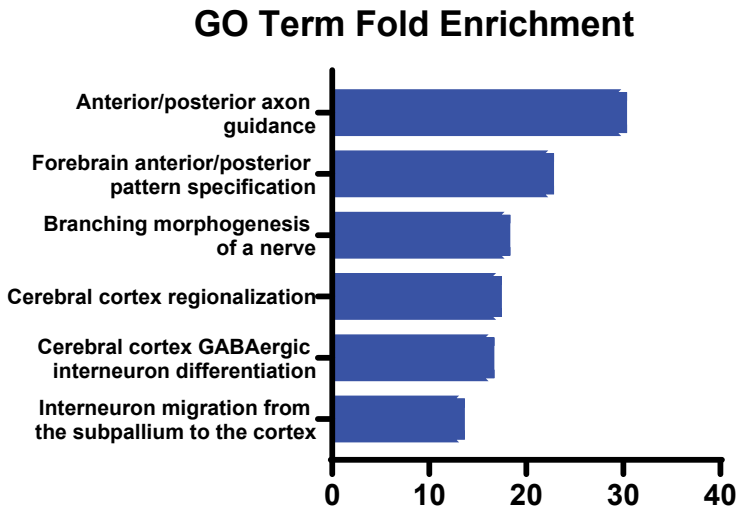
73. Zembrzycki A, Griesel G, Stoykova A, Mansouri A. Genetic interplay between the transcription factors Sp8 and Emx2 in the patterning of the forebrain. Neural Dev. 2007;2(1):8.

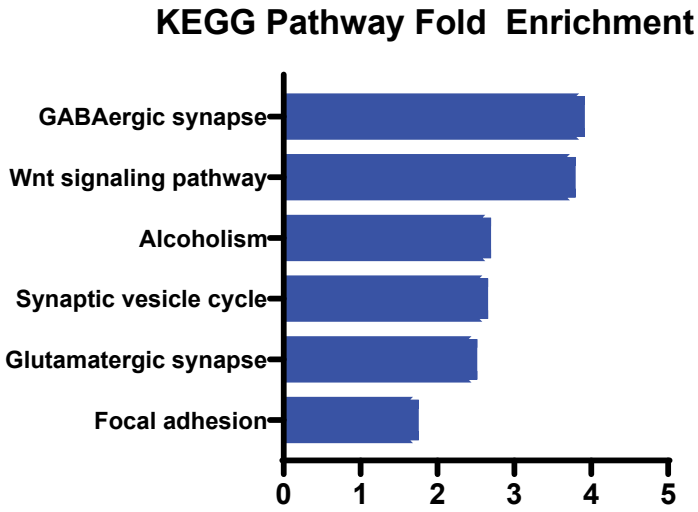
74. Zhang S-C, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001 Dec;19(12):1129–33.

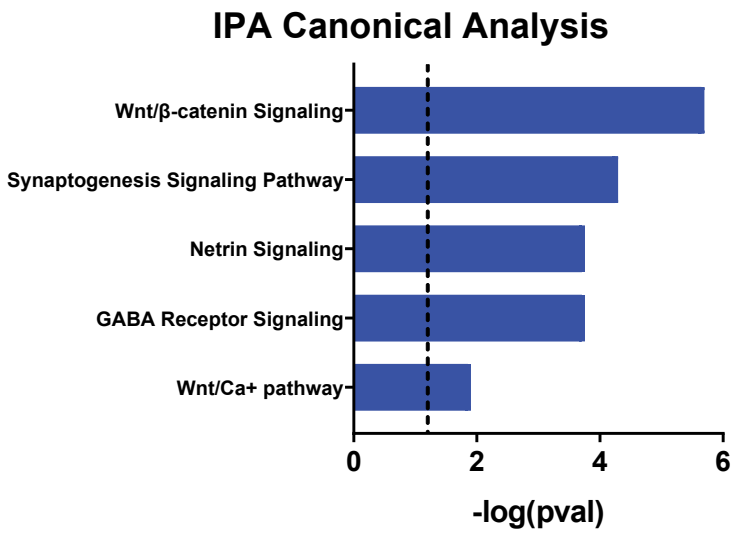
75. Zhang X-Q, Zhang S-C. Differentiation of Neural Precursors and Dopaminergic Neurons from Human Embryonic Stem Cells. In: Turksen K, editor. Human Embryonic Stem Cell Protocols [Internet]. Totowa, NJ: Humana Press; 2009 [cited 2019 Sep 30]. p. 355–66. Available from: http://link.springer.com/10.1007/978-1-60761-369-5_19

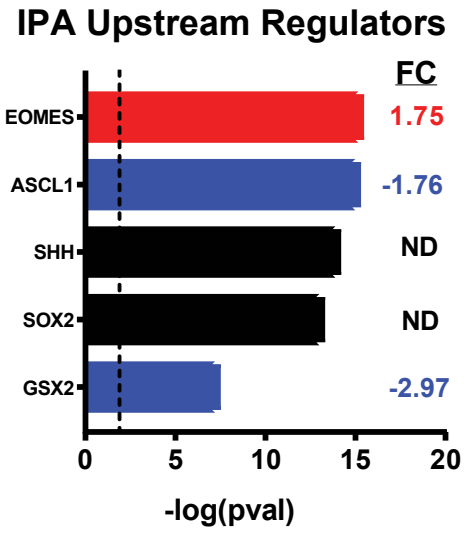
76. Zhu Y, Wang L, Yin F, Yu Y, Wang Y, Shepard MJ, et al. Probing impaired neurogenesis in human brain organoids exposed to alcohol. Integr Biol. 2017;9(12):968–78.

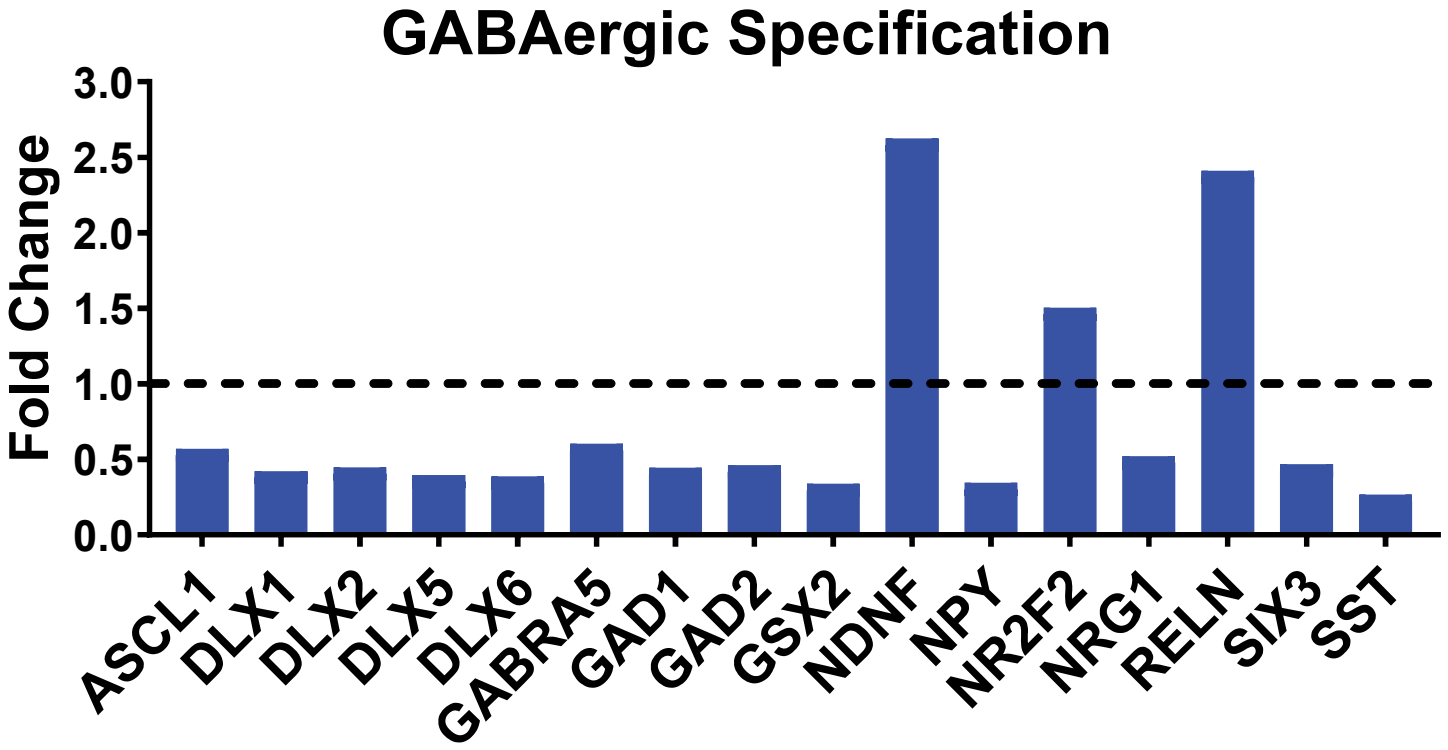

Time line of *in vitro* hPSC neural induction
d0
d6
d10
d17
d30
d50
hPSCs
EBs
early rosette
late rosette
Immature neurons
Functional neurons
ESC media d1-d4
neural induction media d5-d21
neural differentiation media
50mM daily EtOH
Upregulated
Downregulated
CONTROL
50mM EtOH
SST
FZD5
LHX6
CDH19
RSPO2
WNT8B
RSPO3
RSPO1
HEY2
ERMN
-log(FDR)
Fold Change

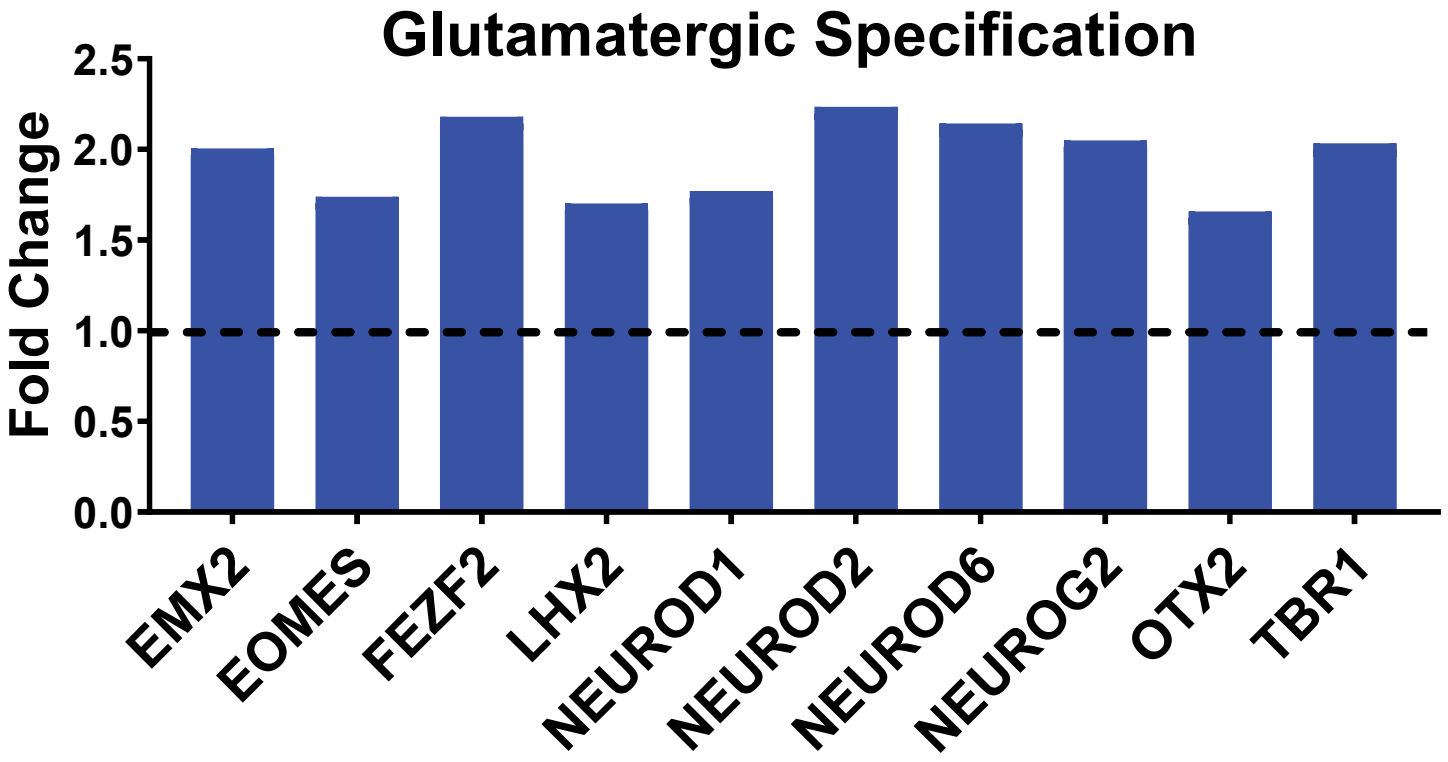

GO Term Fold Enrichment
Anterior/posterior axon guidance
Forebrain anterior/posterior pattern specification
Branching morphogenesis of a nerve
Cerebral cortex regionalization
Cerebral cortex GABAergic interneuron differentiation
Interneuron migration from the subpallium to the cortex
0
10
20
30
40


KEGG Pathway Fold Enrichment
GABAergic synapse
Wnt signaling pathway
Alcoholism
Synaptic vesicle cycle
Glutamatergic synapse
Focal adhesion
0
1
2
3
4
5

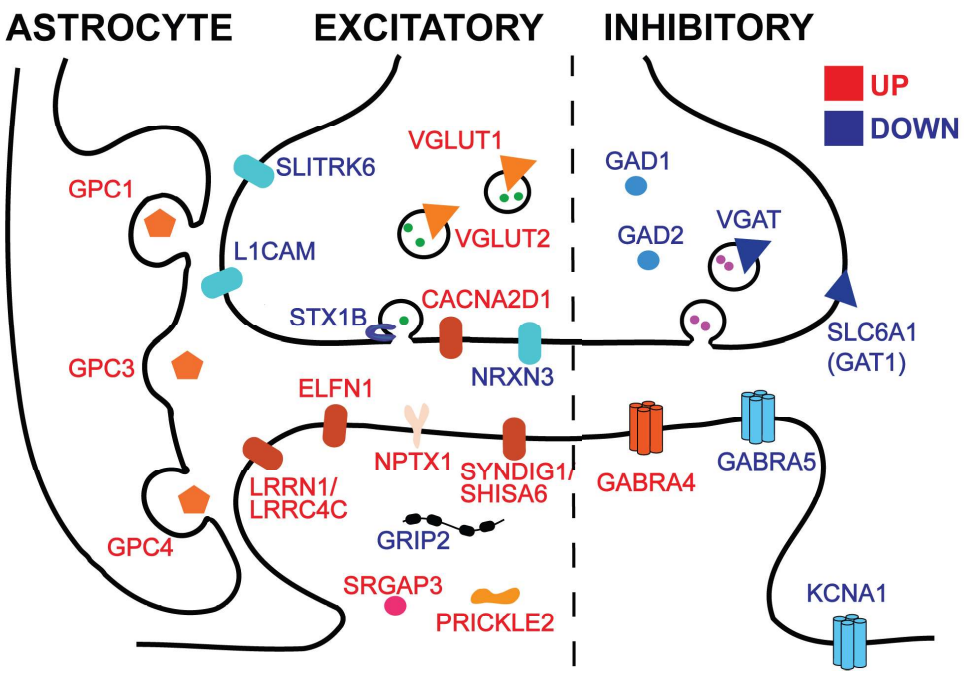

IPA Canonical Analysis
Wnt/β-catenin Signaling
Synaptogenesis Signaling Pathway
Netrin Signaling
GABA Receptor Signaling
Wnt/Ca+ pathway
0
2
4
6
-log(pval)

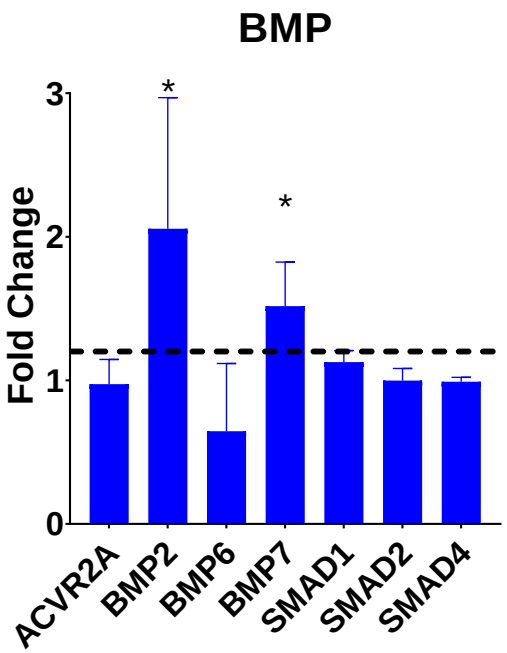

IPA Upstream Regulators
FC
EOMES
1.75
ASCL1
-1.76
SHH
ND
SOX2
ND
GSX2
-2.97
0
5
10
15
20
-log(pval)

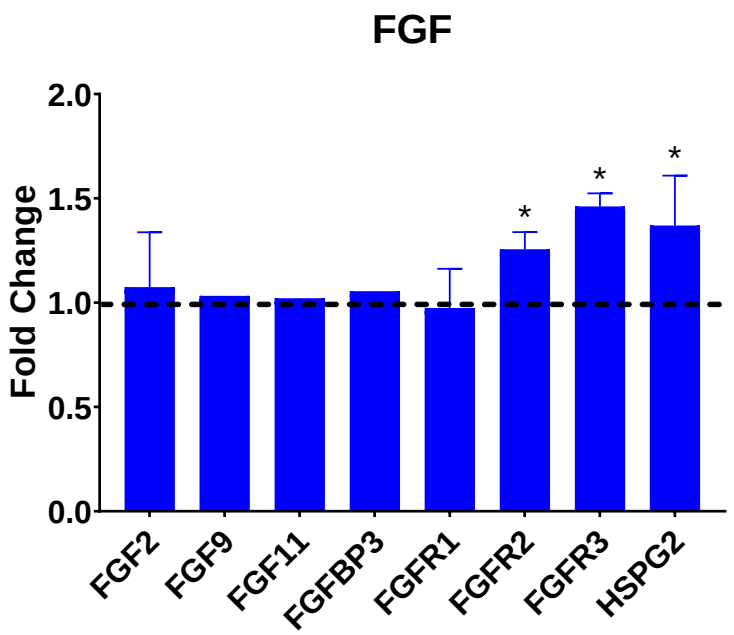

GABAergic Specification
Fold Change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ASCL1
DLX1
DLX2
DLX5
DLX6
GABRA5
GAD1
GAD2
GSX2
NDNF
NPY
NR2F2
NRG1
RELN
SIX3
SST

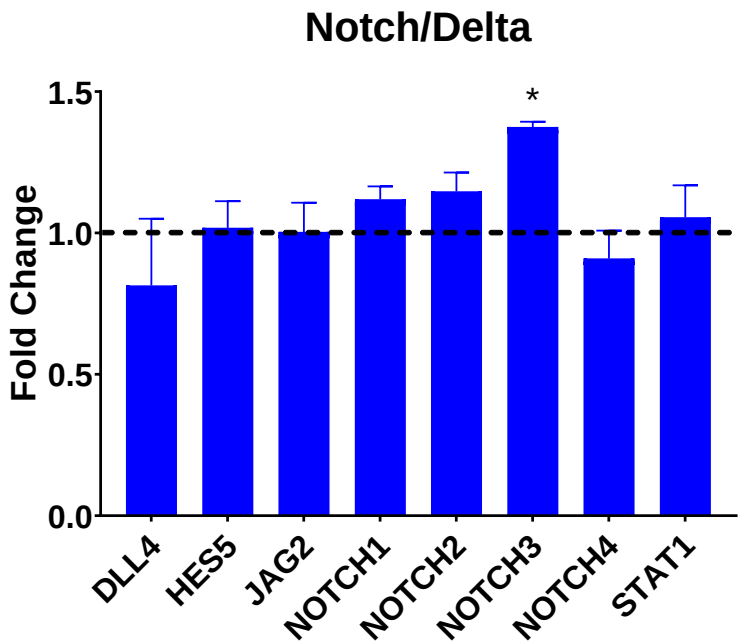

Glutamatergic Specification
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
EMX2
EOMES
FEZF2
LHX2
NEUROD1
NEUROD2
NEUROD6
NEUROG2
OTX2
TBR1

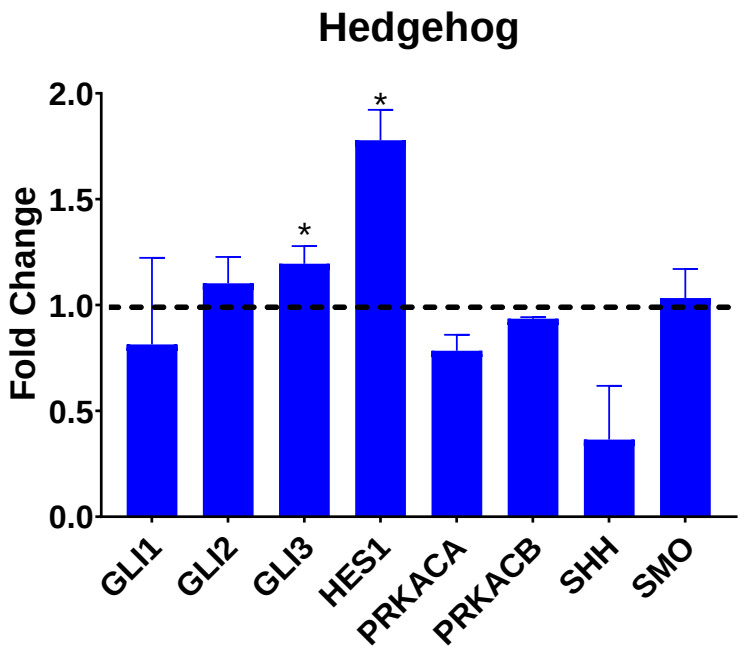
Column1	Gene Symbol	Fold Change	FDR
Excitatory	CACNA2D1	1.771	5.92E-06
	ELFN1	1.290	1.79E-02
	GRIP2	-1.258	4.69E-02
	L1CAM	-1.268	4.84E-03
	LRRC4C	1.649	1.33E-03
	NPTX1	1.816	2.04E-05
	NRXN3	-1.914	3.00E-08
	PRICKLE2	-1.551	7.29E-03
	SHISA6	1.667	4.08E-04
	SLITRK6	-1.632	5.59E-03
	SRGAP3	1.365	2.25E-04
	STX1B	-1.241	3.00E-02
	SYNDIG1	2.147	4.27E-04
	VGLUT1	1.650	1.84E-03
	VGLUT2	1.434	7.91E-03
Inhibitory	GABRA4	1.681	1.68E-02
	GABRA5	-1.662	2.56E-02
	GAD1	0.460	4.05E-05
	GAD2	0.459	2.13E-06
	GAT1	-1.502	4.27E-04
	KCNA1	-1.994	3.74E-03
Glial	GPC1	1.305	1.32E-03
	GPC3	1.520	3.58E-02
	GPC4	1.425	3.99E-04

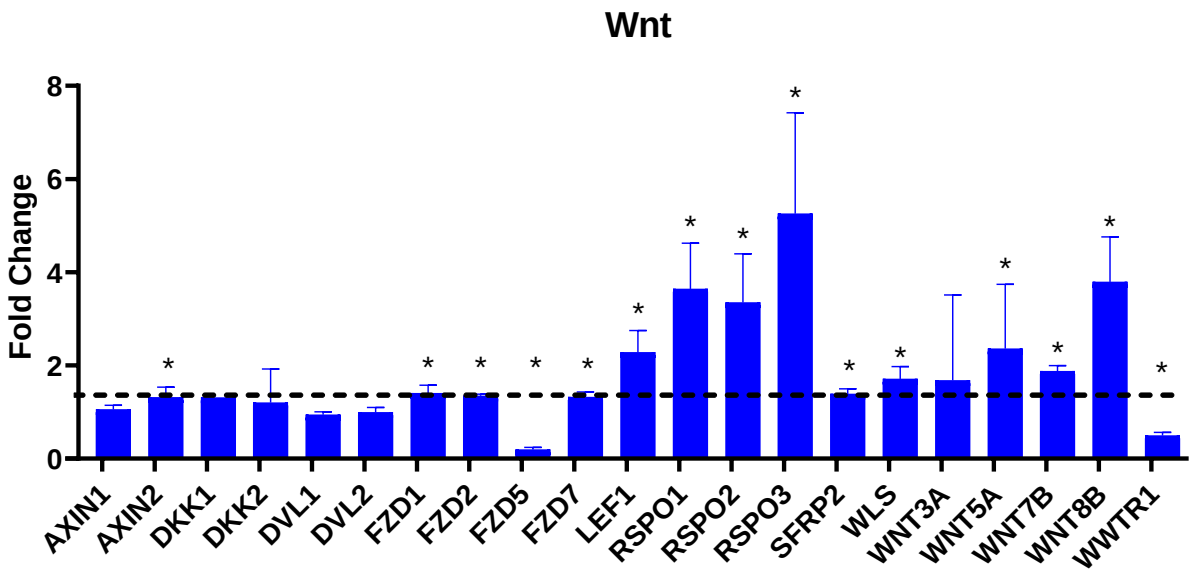



BMP
Fold Change
0
1
2
3
*
*
ACVR2A
BMP2
BMP6
BMP7
SMAD1
SMAD2
SMAD4

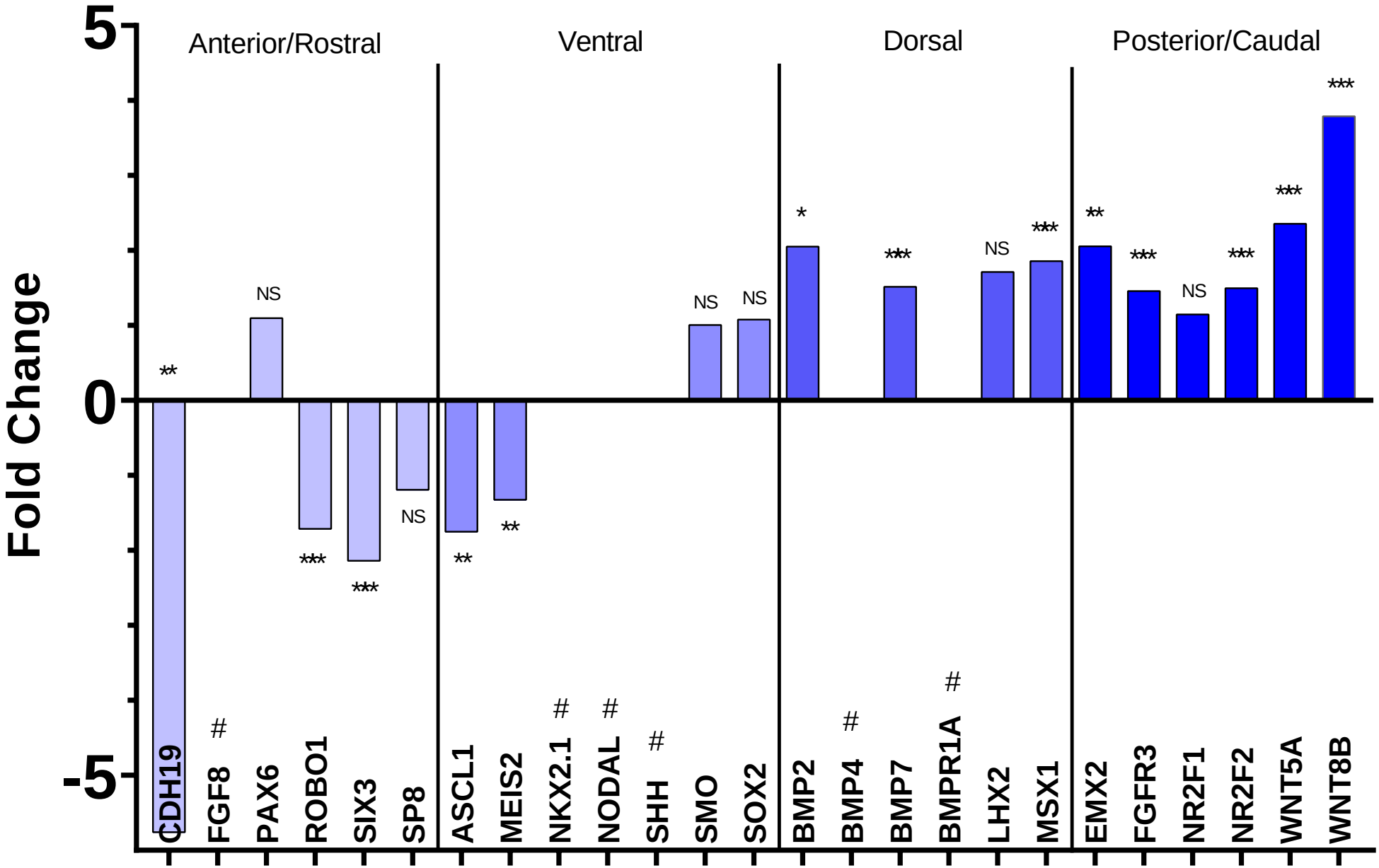

FGF
Fold Change
0.0
0.5
1.0
1.5
2.0
*
*
*
FGF2
FGF9
FGF11
FGFBP3
FGFR1
FGFR2
FGFR3
HSPG2

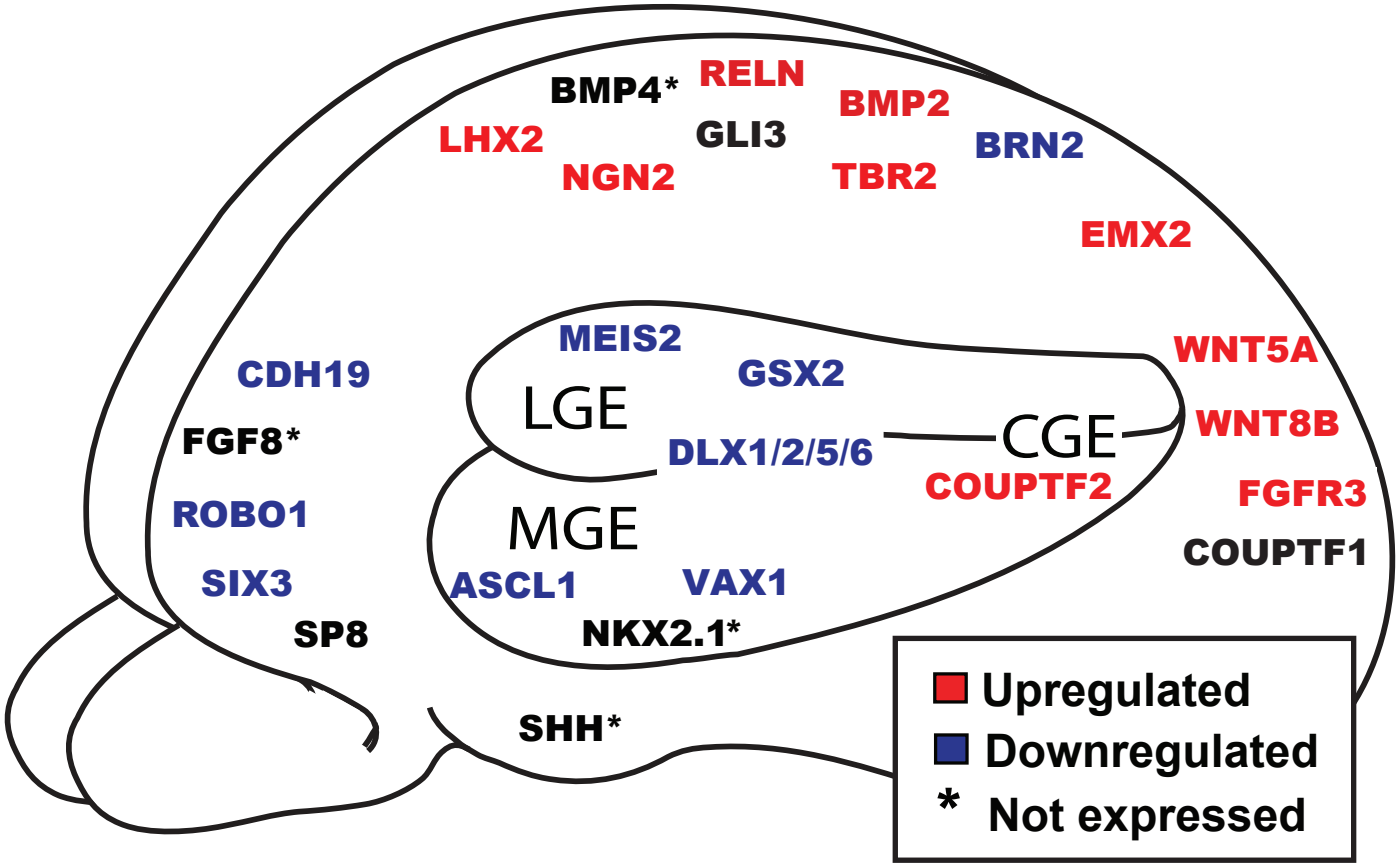

Notch/Delta
Fold Change
0.0
0.5
1.0
1.5
*
DLL4
HES5
JAG2
NOTCH1
NOTCH2
NOTCH3
NOTCH4
STAT1


Hedgehog
Fold Change
0.0
0.5
1.0
1.5
2.0
*
*
GLI1
GLI2
GLI3
HES1
PRKACA
PRKACB
SHH
SMO


Wnt
Fold Change
0
2
4
6
8
*
*
*
*
*
*
*
*
*
*
*
*
*
*
*
AXIN1
AXIN2
DKK1
DKK2
DVL1
DVL2
FZD1
FZD2
FZD5
FZD7
LEF1
RSPO1
RSPO2
RSPO3
SFRP2
WLS
WNT3A
WNT5A
WNT7B
WNT8B
WWTR1

Regionally Expressed Transcripts




BMP4*
RELN
BMP2
LHX2
GLI3
BRN2
NGN2
TBR2
EMX2
MEIS2
WNT5A
CDH19
GSX2
LGE
CGE
WNT8B
FGF8*
DLX1/2/5/6
COUPTF2
FGFR3
ROBO1
MGE
COUPTF1
SIX3
ASCL1
VAX1
SP8
NKX2.1*
SHH*
Upregulated
Downregulated
* Not expressed