SECRETED FACTORS FGF *AND WNT IN CORTICAL INTERNEURON SPECIFICATION*

By

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Dedication

I dedicate this thesis to my daughter, Olivia.

Everyday you inspire me anew.

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I'd like to thank:

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Abstract

Cortical Interneurons are an incredibly diverse population of locally connecting GABAergic inhibitory neurons. In rodents, cortical interneurons originate from the ventral telencephalon during embryogenesis, and migrate tangentially into the neocortex following their specification. Despite our understanding of the early patterning of the telencephalon, established through sonic hedgehog (SHH), fibroblast growth factor (FGF) signaling, and wingless-int (WNT) we still know very little about the downstream effectors responsible for establishing interneuron diversity. This work has aimed to elucidate the role of secreted morphogens in interneuron specification, specifically FGF and WNT.

I began by investigating the role of FGF signaling in the specification of cortical interneurons by targeting downstream effectors, a critical adaptor protein, and receptors for FGF signaling. In particular, I examined the role of two candidate transcription factors classically found downstream of FGF: *Ets1* and *Ets2*. Previously identified by microarray as enriched in cortical interneurons at developmental timepoints, *Ets1* and *Ets2* single and double mutants had no obvious defects in interneuron specification as assessed by immunohistochemistry. Using both forebrain and interneuron specific *Cre* recombinase drivers, I also generated conditional knockouts of the adaptor protein *FRS2*α, which is critical for FGF signaling through the MAP kinase

and PI3 kinase signaling pathways (Hadari *et al*, 2001). Interestingly, pan-forebrain loss of *FRS2α* failed to replicate the phenotype of forebrain removal of *FGF receptors 1,2,*and *3*. Similarly, interneuron specific removal of *FRS2α* did not affect interneuron migration or fate. Additionally, through a complex set of genetic crosses, I generated an interneuron specific triple knockout of *FGFR*s *1,2,* and *3*; this animal also did not exhibit any gross interneuron specification defects. These results together suggest that the development of cortical interneurons is likely not regulated by FGF signaling, at least not after their initial specification.

Previous work in the developing spinal cord has shown that cell identity can be conferred by exposure to diffusible morphogen gradients. Despite previous attempts, delineation of cell types by morphogen gradient in a “spinal cord” fashion has not yet been discovered in the forebrain. We have discovered a novel rostral-caudal regionality within the medial ganglionic eminence (MGE) that delineates the specification of the two main classes of cortical interneuron subtypes based on their exposure to a non-canonical WNT signaling gradient. Caudally located MGE progenitors receiving high levels of WNT signaling give rise to cortical interneurons labeled by somatostatin (SST). Parvalbumin (PV) expressing basket cells, in contrast, originate primarily from the most rostral region of the MGE, and do not signal highly through WNT pathways. Interestingly, canonical WNT

signaling through β-catenin is not required for this process. WNT signals transmitted via cleavage of the intracellular domain of the non-canonical WNT receptor RYK, however, are sufficient to drive interneuron progenitors to a SST fate.

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Introduction

Developmental Biology and the Brain: History and Philosophy

Embryology has fascinated humankind for centuries. The fourth century B.C. philosopher Aristotle is credited with being the first embryologist for his anatomic comparisons of embryos (Gilbert, 2010). Little advancement was made until the advent of microscopy in the mid 17th century. This era was characterized by the idea of preformation, or that eggs or sperm (and which of the two was hotly debated) provided microscopic versions of fully formed beings. This theory was incredibly attractive because of its simplicity. There was no need for an external organizing force to organize the developing embryo and the idea of nested preformed beings like Russian dolls provided conveniently for the stable continuity of the race. However, this relatively simple idea was inconsistent with various observations of heredity, namely the mixing of parental characteristics in offspring. Later 17th century philosophers and scientists Immanuel Kant and Johann Friedrich Blumenbach began to argue for an intrinsic organizing force in the embryo that could guide its development. It was the development of cell theory and microscope improvements that put the preformation theory to rest. We could finally appreciate that sexual reproduction was even more complicated and fraught with opportunities to

disrupt the continuity of the human race than originally thought. The capacity for complex organisms to arise from the humble beginning of a single cell is awe inspiring and arguably the most important evolutionary advance in life on earth.

Though it has happened again and again throughout evolutionary time, the transition of unicellular organisms to cooperative multicellular 'colonial' structures was critical to set the stage for the origin of complex, multi-organ animals like us. An important facet to this transition, particularly for organ systems is the ability of similar cells to differentiate from their neighbors and specialize their function. While every complex organ has a unique and interesting developmental path, from this perspective, few biological systems have captured the imagination like the brain.

Again this fascination began with early philosophers debating some version of the developmental questions of the mind that still interest us today. How much of our understanding of the world is determined and shaped by our neural circuits, and how much is determined by experience? The idea of the *tabula rasa* (John Locke), that we are born 'a blank slate' and learn all aspects of our thinking from experience was a popular view among early philosophers. More recently, an understanding of genetics and neuroscience has suggested a more complicated answer to this question, namely that many aspects of our 'nature' and experience are at least

partially determined by our genes and circuits. It was Kant who proposed a unifying theory that more closely reflects what we currently understand about this question. Kant proposed that we have innate constructs that allow us to view and experience the world in a predictable way (Burnham, 2008). For example, our eyes and ears allow us to experience those aspects of the world that fall into detectable wavelengths. As a result, our perception of reality is limited by our biology, and then refined and informed by our experience of the world, as interpreted by our brain. Despite our increasing knowledge about the biological underpinnings of this question and its many nuances, the enthusiasm and debate over this question has not diminished. The degree to which we are a product of nature and nurture remains an important point of discussion in philosophical, biological and neuroscientific circles, as well as in the mainstream media. Though we are far from fully understanding the brain, a more thorough comprehension of the brain and its process of self-assembly are a prerequisite for a deeper understanding of ourselves that society has been searching for since the beginning of organized thought. That is a grand statement to introduce a study as specialized as this; though I do not purport to make any headway on understanding brain development in a philosophical sense, I hope I have made a step in the right direction.

Gradients as organizers

Philosophic questions aside, from a purely biological perspective, development is a fascinating and extremely complex task. The embryo is a self-assembling machine. A single cell must divide and differentiate with almost no outside instruction. One of the major requirements for a process of this scale is the generation of polarity. This occurs at both the single cell level as well as the multi-cellular level. One classic paradigm for how this might occur in a field of cells is through the coordinated actions of secreted morphogens. Alan Turing first described this possible mechanism mathematically in 1953 (TURING and Wardlaw, 1953). Lewis Wolpert later refined Turing's description and proposed the "French flag model": a point source of a secreted factor diffuses across a field, establishing regions where the concentration of the factor is highest, less concentrated, and least concentrated (Wolpert, 1969) (Figure 1). A field of identical cells expressing the receptor for this secreted factor could be delineated along step-wise responses according to their exposure. The difference in response is translated within the cells to differences in gene expression and ultimately, cell fate. In this way, though this is a great oversimplification of the way gradients work *in vivo*, a point source of a single factor could establish multiple different cell types. In reality, in order to generate the cellular diversity found in nature, gradients involve multiple layers of

regulation and necessarily yield patterns much more complex than a French flag (reviewed in (Kondo and Miura, 2010; Lander, 2007)). Following Turing's morphogen models, eponymous Meinhardt-Gierer mechanisms describe more complex, interacting morphogen gradients capable of yielding spots and stripes (Gierer and Meinhardt, 1972) (Figure 1).

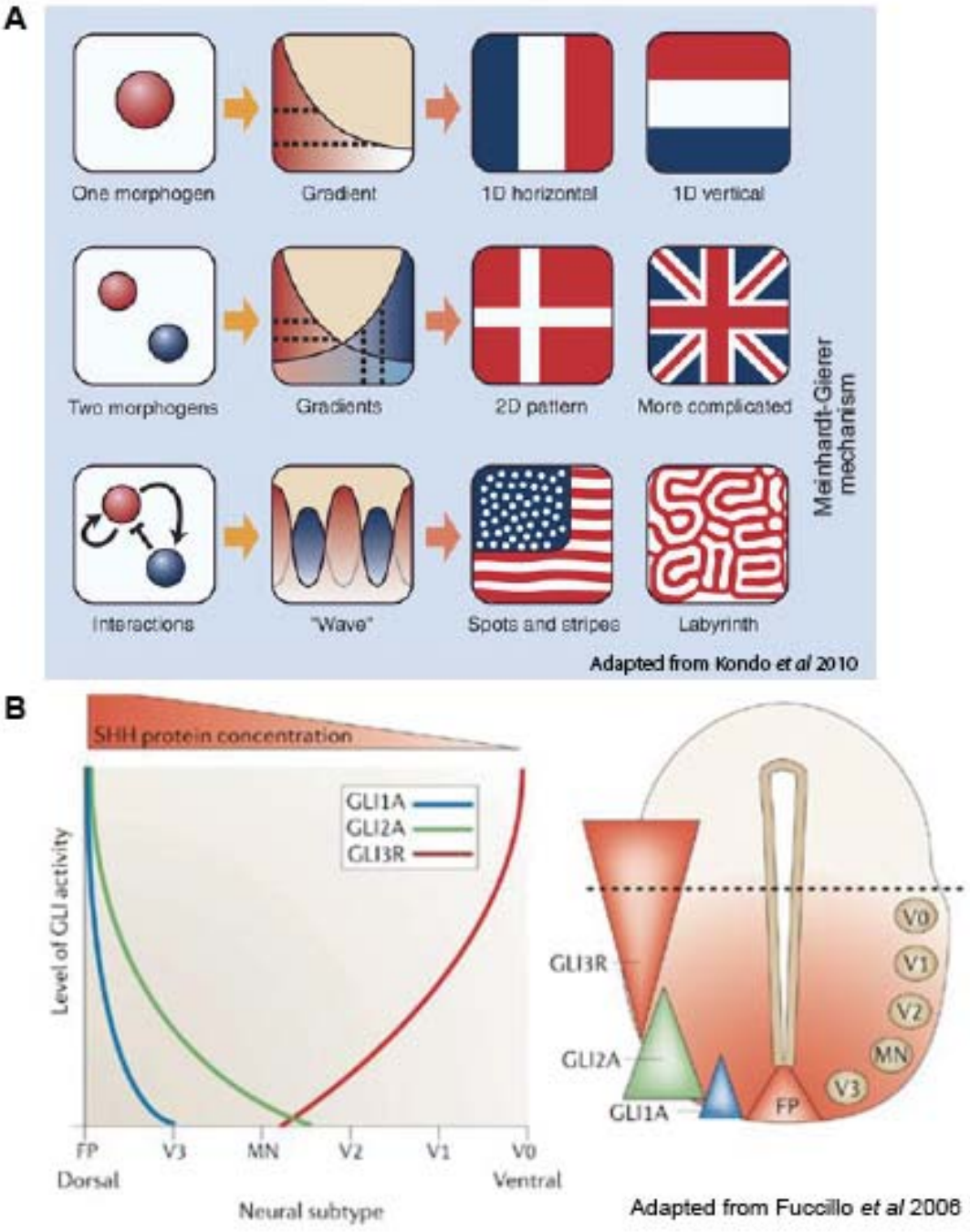

Figure 1 The French flag model and its more complex variants

(**A**) A single morphogen molecule can produce a gradient system with multiple vertical or

horizontal positional differences. Two morphogens overlaid on one another can produce more complex patterns still, and if these molecules interact, patterns such as stripes can arise spontaneously. (**B**) SHH gradient in the neural tube produces different activity levels of downstream GLI activators and repressors and delineate multiple spinal cord cell types.

The first demonstration of a patterning factor that works this way is the anterior-posterior axis specified by *bicoid* (*bcd)* in *Drosophila melanogaster* embryo. Bicoid is truly an idealized form of a morphogen. *Bcd* mRNA is already localized in an anterior to posterior gradient within the syncytial drosophila embryo before zygotic transcription begins. As a transcription factor that diffuses through a syncytium of cells, Bicoid isn't required to diffuse through the extracellular matrix and activate receptors in a graded fashion (Driever and Nüsslein-Volhard, 1988). Most mammalian morphogens, facing these obstacles, are hard-pressed to meet the Bicoid ideal of a morphogen gradient. Even complex, spontaneous strip-forming Meinhardt-Gierer models fail to capture all of the complexity present *in vivo*. The mammalian morphogen WNT, for example, can be post-translationally modified in a way that affects its diffusion through the extracellular space, interactions with secreted inhibitors, and binding to multiple receptors and co-receptors, and those are just levels of regulation present on the extracellular side of membrane (for general review (Lander, 2007)). Nevertheless, gradients of secreted growth factors have been shown again

and again to be critical for embryonic development. The universality of these systems across phyla highlights their importance and adaptability to multiple types of organisms, and is the focus of the entire burgeoning field of evo-devo (Evolution and Development).

In the development of the nervous system, there are many instances of secreted factors acting in a graded fashion. In particular, spinal cord development is a prototypical example of this mode of patterning and cell specification. The generation of multiple neuronal types within the spinal cord is dependent on their level of exposure to sonic hedgehog (SHH) protein secreted from the ventral notochord and floor plate (Briscoe et al., 1999; Roelink et al., 1995). Pitted against this SHH gradient is the opposing dorsal gradient of bone morphogenic protein (BMP) secreted from the roof plate (Liem et al., 2000; 1995). As expected, the French flag model isn't sufficient for the understanding of SHH and spinal cord development. Modeling of SHH gradient signaling in the spinal cord suggests that cell fates are specified even before the SHH gradient could theoretically reach stability (Saha and Schaffer, 2006). Furthermore, nerve cord cells closest to the initial SHH source begin expressing *Shh* themselves, the neural tube continues to divide and newly born cells move within the emerging gradient. In addition, cells are sensitive to the amount of time they are exposed to

SHH (Dessaud et al., 2007). This is a fascinating example of the spatial and temporal dynamics that characterize embryonic development.

Of the patterning signals important for establishing cellular identity in the developing nervous system, SHH may be the most famous, but it certainly isn't unique. This is also true in the formation of the forebrain, the focus of this study. Much is known about the earliest steps of neural patterning: the general induction of the neurectoderm, followed by specification of the forebrain primordium, and then patterning events that establish the pallium and subpallium (Figure 4). These early processes are accomplished through the coordinated actions of multiple signaling factors, including FGF, SHH, WNT, and BMP, among others. Interestingly, after the initial regions are established, many of these signaling pathways remain active in the brain. In some cases their expression patterns become more complex, as the structures themselves become more complex (Guillemot and Zimmer, 2011). This begs the question: do these same signaling factors have a later patterning role in cell type specification? In my thesis work, I have sought to answer this question specifically with respect to the iterative roles of FGF and WNT signaling in forebrain development, particularly with regard to the genesis of GABAergic cortical interneurons.

Fibroblast Growth factor (FGF)

FGF involvement in the induction and patterning of neural tissue is evolutionarily ancient (Itoh and Ornitz, 2004). Recently, FGF homologues in the sea anemone have been shown to be required for the formation of the sensory organ in that organism (a cnidarian) suggesting that the association of FGF signaling with nervous system development predates the separation of protostomes and deuterostomes (Rentzsch et al., 2008). Additionally, the hemi-chordate acorn worm *Saccloglossus kowalevskii*, a still-ancient but more recent evolutionary ancestor of vertebrates was recently found to pattern its ectoderm using the same pattern and sequence of secreted factors used to pattern the vertebrate brain (Pani et al., 2012).

FGFs are a vast family comprising 22 family members and 7 subfamilies (Mason, 2007) in mouse with a total of 4 active receptors (FGFR1-4). To complicate matters, FGF receptors undergo alternative splicing as well as post-translational modifications, including glycosylation, creating dramatic effects on their ligand binding properties. Systematic analysis characterizing the binding specificities of each ligand/receptor pair has been performed *in vitro* (Ornitz et al., 1996; Zhang et al., 2006). *In vivo*, ligand-binding specificity is likely modified by the presence or absence of various co-factors, accounting for differences between *in vivo* and *in vitro* binding specificities (Dailey et al., 2005; Knox et al., 2002). While there is

always some degree of promiscuity of FGF ligands for various receptors, interestingly, a recent study revealed that specific FGFR/ligand pairs could specifically promote either excitatory or inhibitory synapse development in the hippocampus (Terauchi et al., 2010).

In many ways, FGF signaling is a stereotypical signaling cascade. Most FGF ligands have classic N-terminal signal peptides and are secreted constitutively, and the receptors act via tyrosine kinase domains to activate various positive signaling pathways including RAS, PLC-γ, and PI3K (Mason, 2007; Tsang and Dawid, 2004) (Figure 2). Connection between signaling at the receptor and downstream pathways is accomplished through specific adaptor proteins, including FRS2α and β (Ong et al., 2000). FRS2α and β are structurally very similar, and both contain functional domains that bind to the FGF and Nerve Growth Factor (NGF) receptors (Hadari et al., 2001; Ong et al., 2000). FRS2α binds to FGFR1 constitutively, irrespective of the activity of the receptor. In contrast, FRS2 association with the NGF receptor is dependent on ligand binding. Lacking the appropriate domain to recruit the essential Grb-Sos complex, FGFRs absolutely require the docking protein FRS2 to activate the MAPK pathway (Schlessinger, 1993). Loss of *FRS2α* results in a lethal defect in trophoblast development very early in embryogenesis (Gotoh et al., 2005). Creating a

tetraploid chimera can rescue the trophoblast defect, resulting in a viable mouse with FGF signaling related phenotypes (Gotoh et al., 2005).

Further downstream, FGF activity influences the transcription of a number of target genes. Many of the gene products of FGF signaling also serve as negative feedback loops. *Sprouty*, *Sef*, and *MAP kinase phosphatase 3* (*MKP3)*, for example, all act to dampen FGF signaling. In addition, several families of transcriptional activators can be induced in response to FGF signaling, including members of the Ets family. FGF signaling has been co-opted for numerous cellular processes including cell survival, where it functions as a growth factor; division and differentiation, as a pro-proliferative factor; patterning during development as a pro-neural factor, and also neuronal migration and synapse formation (reviewed in (Guillemot and Zimmer, 2011; Hebert, 2011)).

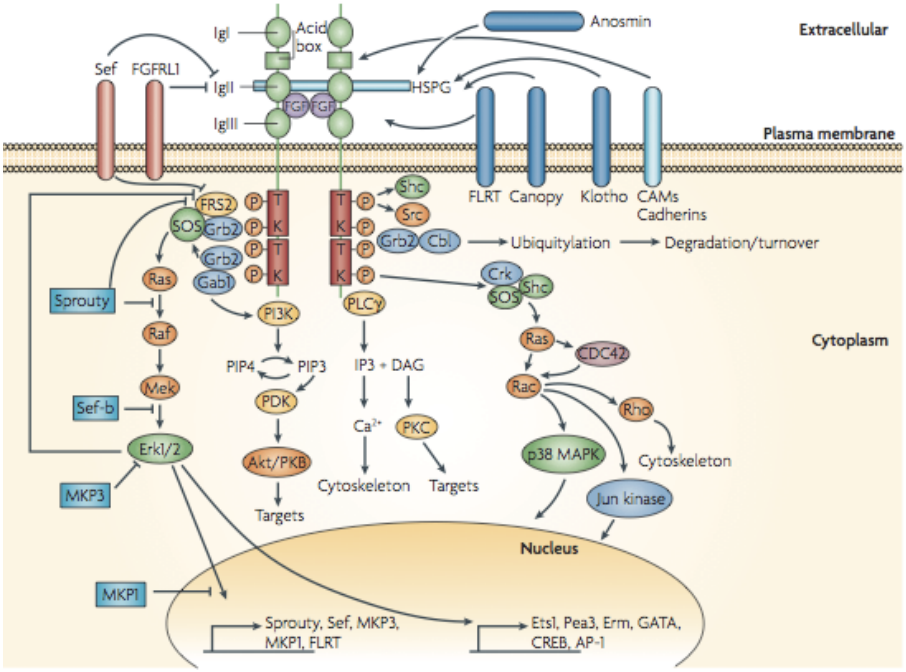

Figure 2 FGF Signaling

Scheme of WNT signaling pathways its components, reproduced from (Mason, 2003)

Wingless-INT (WNT)

The Wingless-INT (WNT) signaling pathway is a similarly ancient developmental mechanism. All multicellular eukaryotic lineages (metazoans) express WNT proteins, including sponges (Adell et al., 2007). This predates even FGF's ancient arrival, arising at or prior to the development of true multi-cellularity, far before the emergence of bilateral symmetry, three embryonic germ layers, and the divergence of protostomes and deuterostomes.

As with the FGF proteins, there are many (19) WNT ligands encoded in the mouse and human genome, and multiple receptors. All WNT ligands are defined by their sequence similarity, specifically by the presence of 22 conserved cysteine residues, which are important for the formation of the protein's secondary structure. The recent elucidation of the structure of a WNT ligand bound to a Frizzled (FZD) receptor has shed light on the many mysteries of WNT structure including that these cysteine residues, once hypothesized to be involved in intermolecular binding - perhaps by forming WNT dimers - are mostly involved in internal disulfide bonds (Cha et al., 2008; Janda et al., 2012). WNT proteins undergo multiple post-translational modifications prior to their secretion, mostly glycosylation and acylation (Willert and Nusse, 2012). Glycosylation modifications vary between different WNT ligands, with variable effects on their activity and ability to be secreted (reviewed in (Willert and Nusse, 2012)). Acylation, on the other hand, is essential for the secretion of all WNT ligands (Proffitt and Virshup, 2012; Willert et al., 2003). The acylation of WNT proteins is accomplished by the membrane bound O-acyl transferase (MBOAT) protein Porcupine (PORCN), originally identified as a segment polarity gene during *Drosophila* development (Barrott et al., 2011; Kadowaki et al., 1996; van den Heuvel et al., 1993). These fatty acid modifications contribute to the relative hydrophobicity of native WNT proteins. In fact, based solely on their amino

acid sequence, WNT proteins should be very hydrophilic, as they have a number of charged residues and a predicted isoelectric point of 9 (Willert and Nusse, 2012). As a result, there are many theories about how WNT ligands travel through the extracellular space (they do:(Solis et al., 2013)). In *Drosophila*, the *Wnt* homologue *Wingless* (*Wg*) has been shown to associate with lipid rafts, much like the apoB proteins in mammals (Panáková et al., 2005). There are many other extracellular proteins that could also serve to transport WNT over long distances, including HSPGs and even potentially the WNT inhibitory protein sFRP (secreted frizzled receptor protein) (reviewed in (Logan and Nusse, 2004)).

The usual receptor for the WNT ligands is one of the FZD family of receptors, of which there are 10 in humans and mice. FZD receptors are G-coupled receptors with a conserved extracellular cysteine rich domain (CRD), that binds to WNT ligands. The recently solved structure of a WNT ligand bound to a FZD receptor confirmed that there are two domains on the WNT protein that interact with the frizzled CRD. One of these WNT domains is thought to be palmitoylated in many different WNTs (Janda et al., 2012). Based on the structure of the FZD bound WNT, there is reason to think there are little to no differential binding properties between various WNT ligands and receptors, though not much is known on this topic. As a result, although WNT signaling downstream can take canonical or non-

canonical forms, this is likely a function of the cell that receives the signal, rather than the signal itself. One contributing factor may be the cellular complement of FZD co-receptors; including LRP proteins (5 and 6 in vertebrates), ROR1/2, or RYK. LRP proteins function in a multimeric structure with various FZD receptors, and are phosphorylated in response to WNT binding. ROR1/2 and RYK, in contrast, while they also have confirmed or hypothesized co-receptor activity, also each contain an extracellular domain that binds directly to WNT and can act as receptors for WNT in the absence of FZD (reviewed in (Green et al., 2014)). The ROR1/2 WNT binding domain is similar to the CRD of the FZD receptors; RYK, however, contains a domain similar to that of the WNT antagonist WNT inhibitor factor (WIF). Despite the differences between these receptors, interestingly, they are thought to interact with similar portions of the WNT ligand (Janda et al., 2012; Malinauskas et al., 2011).

Canonical WNT signaling downstream of FZD receptors regulates the level of the intracellular signaling molecule β-catenin (Figure 3). Under basal conditions, β-catenin is targeted for degradation by GSK3β. When WNT is bound to the FZD-LRP multimeric complex, a conformational change in the FZD intracellular domain results in the subsequent phosphorylation of LRP. As a result, adaptor proteins such as axin are recruited to the complex. These changes lead to a decrease in the activity

of GSK3β and the degradation of β-catenin. Ultimately, the accumulation of β-catenin in the cell leads to the activation of TCF/LEF transcription factors and the transcription of their target, WNT-responsive, genes. As with most signaling cascades, many of these target genes are part of the signaling machinery itself, so the activation of TCF/LEF transcription factors further leads to the transcription and production of more TCF/LEF transcription factors.

As a β-catenin independent (thus non-canonical) WNT receptor, RYK is an interesting case. While similar to receptor tyrosine kinases, RYK's intracellular tyrosine kinase domain is functionally dead. In the absence of a functional kinase domain, it was hypothesized that RYK would function primarily as a co-receptor. Recent studies have shown, however, that RYK's intracellular domain can be cleaved and participate in transcriptional regulation following its translocation to the nucleus (Lyu et al., 2008) (Figure 3). While the cleavage event, mediated through γ-secretase activity, seems not to be dependent on WNT signaling, the nuclear translocation of the cleaved intracellular domain appears to require a WNT signal (Lyu et al., 2008). The details of RYK signaling, including the cytoplasmic and nuclear partners for the RYK intracellular domain, the mechanism for WNT dependent nuclear translocation, and the transcriptional targets of RYK activation are still largely unknown. It is clear

that RYK signaling mechanisms are quite heterogeneous, as with other WNT receptors. RYK receptor expression has been shown to activate canonical (β-catenin based) signaling in some *in vitro* contexts and not in others, depending on the cell line (Andre et al., 2012; Lu et al., 2004; Macheda et al., 2012). In addition to the RYK specific non-canonical signaling via its intracellular domain, RYK is also known to signal through other WNT-related signaling pathways. The two most well characterized non-canonical pathways are the planar cell polarity pathway (PCP) and the convergent extension pathway (CE). The PCP pathway was first characterized in the fly wing, and involves the lateral transfer of positional information across a field of cells through extracellular/transmembrane signaling components. In addition to FZD receptors, which can signal via the PCP pathway, RYK also seems to participate in this mode of signal transduction. RYK protein pull-downs have uncovered PCP components and *Ryk* mutants have classic ear-hair cell abnormalities characteristic of defects in PCP (Andre et al., 2012; Macheda et al., 2012). The CE pathway is interesting, as it signals via Ca^{++} released from intracellular calcium stores in response to Phospholipase C and Inositol triphosphate (IP3). The release of Ca^{++} activates any number of common calcium responsive pathways, giving WNT signaling the opportunity to interact with and modulate/be modulated by a number of independent signaling modalities.

Interestingly, a number of common core components of the various WNT signaling pathways allows the cross-repression of one signaling cascade by another in some cellular contexts. One excellent example of this cross-repression is the repulsive action of RYK and the attractive effect of FZD receptors on the growth cone during axon guidance (reviewed in (Bovolenta et al., 2006)). The Drosophila homologue of *Ryk*, *Derailed* also acts in this way during commissural crossing of axons in the ventral nerve cord (Bovolenta et al., 2006; Callahan et al., 1995). While these effects are likely due to changes in the local cytoskeletal architecture rather than through changes in gene expression, they serve as examples for how divergent WNT ligand effects can be achieved through cellular context/receptor expression.

As with SHH, FGF and other signaling factors important in neural development, WNT's role in patterning the brain changes dynamically over time.

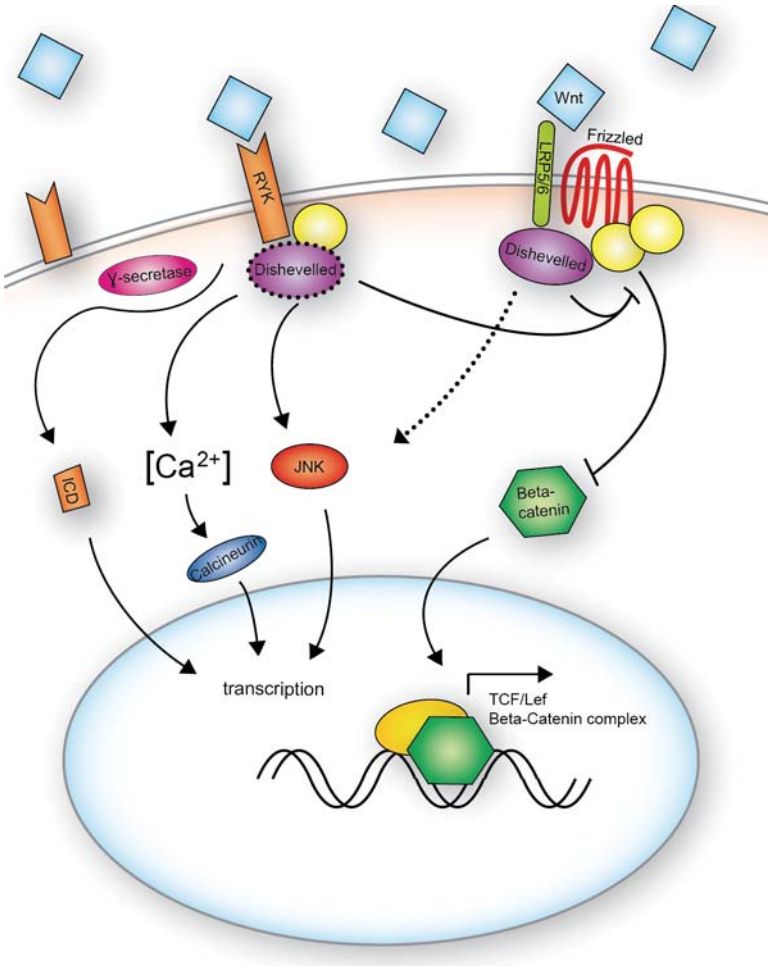

Figure 3 WNT Signaling

Scheme of WNT signaling pathways downstream of FZD receptors and RYK.

Telencephalic Development

Early patterning

So far, we have considered two different patterning signals independently. However, much as the brain is more complex than the French flag, it is patterned by multiple, interacting gradients. That being said, each morphogen has it's own characteristic effect, which can allow for some holistic simplifying, especially at early embryonic stages. For example: at e8, FGF can broadly be thought of as the telencephalic inducer (reviewed in (Hebert, 2011; Hebert and Fishell, 2008)), WNT as specifying dorsal fates (Grove et al., 1998; Lee et al., 2000), and SHH as the ventralizer (Aoto et al., 2002; Ericson et al., 1997) (reviewed (Sousa and Fishell, 2010)) (Figure 4).

FGFs emerge as an organizing factor in telencephalic patterning starting when neurectoderm first separates from ectoderm (Hebert, 2011; Hebert and Fishell, 2008; Paek et al., 2009; Shimamura and Rubenstein, 1997). At this early stage, around e8, cells expressing FGF8 collect at the anterior neural ridge (ANR), the border zone between the anterior ectoderm and the neurectoderm, and act as an organizer to pattern the emerging telencephalon (Maruoka et al., 1998; Storm, 2006). Adjacent to the ANR, the anterior neural plate expresses the early telencephalic transcription factor *forkhead box G1 transcription factor* (*Foxg1*) (Shimamura and

Rubenstein, 1997). The relationship between *Foxg1* and the ANR's FGF expression is reciprocally activating, in that *Foxg1* induces the expression of *Fgf8* in the ANR and FGFs reciprocally activate the expression of *Foxg1* in a positive feedback loop (Martynoga et al., 2005; Shimamura and Rubenstein, 1997). This is critical for forebrain development. In fact, the removal of *FGFR1,2,* and *3* specifically in the FoxG1 domain, causes failure of the entire forebrain to form (*FGFR4* is not expressed in the developing telencephalon) (Paek et al., 2009). Similarly, mice expressing hypomorphic *Fgf8* alleles have smaller telencephalons as a result of decreased FOXG1 expression (Storm, 2006). Early on, (also around e8) canonical WNT signaling through β-catenin is also necessary for the cell-survival signal of FGF8 from the ANR; removal of β- catenin via a *FoxG1* cre driver completely eliminates the telencephalon by e10 (Paek et al., 2011).

Later, as the initial pattern of the telencephalon is laid out and the hemispheres grow, WNT and FGF signaling begin to specialize (Figure 4). SHH expressed in the ventral midline continuously during telencephalic development acts to bolster FGF signaling and *Foxg1* expression. It does this indirectly by inhibiting the proteolytic coversion of Gli3 from a transcriptional activator to a repressor, which acts antagonistically to FGF signaling (reviewed in (Hebert and Fishell, 2008)). FGF and SHH signaling pathways together induce ventral fates, including expression of the

transcription factor *Nkx2.1,* which defines the medial ganglionic eminence (Corbin et al., 2000; Gutin et al., 2006; Shimamura and Rubenstein, 1997). The restricted domain of these secreted factors is important to establish normal boundaries; ectopic expression of *Shh* in the cortex can induce ventral specification markers such as *Nkx2.1* (Kohtz et al., 2001). Similarly, ectopic expression of WNT mediators at the ANR induces midbrain type fate markers (Paek et al., 2012). Meanwhile, *Wnt* expression from the cortical hem promotes dorsal fates, including the expression of cortical markers *Emx2*, *Sp8*, and *CouptfI* (O'Leary et al., 2007). WNT signaling at the hem is dependent on the expression of *Gli3*. In *Gli3* mutant mice, *Wnt* expression at the hem is abolished and the cortex is ventralized (Kuschel et al., 2003; Tole et al., 2000). *Gli3*$^{-/-}$ mutants also display ectopic *Fgf8* expression, another indication of the complex interconnected web formed by these signaling centers. The dorsalizing effect of WNT doesn't appear to be simply downstream of a fading SHH signal, as a ventral induction of ectopic WNT induces the expression of dorsal markers (Backman et al., 2005).

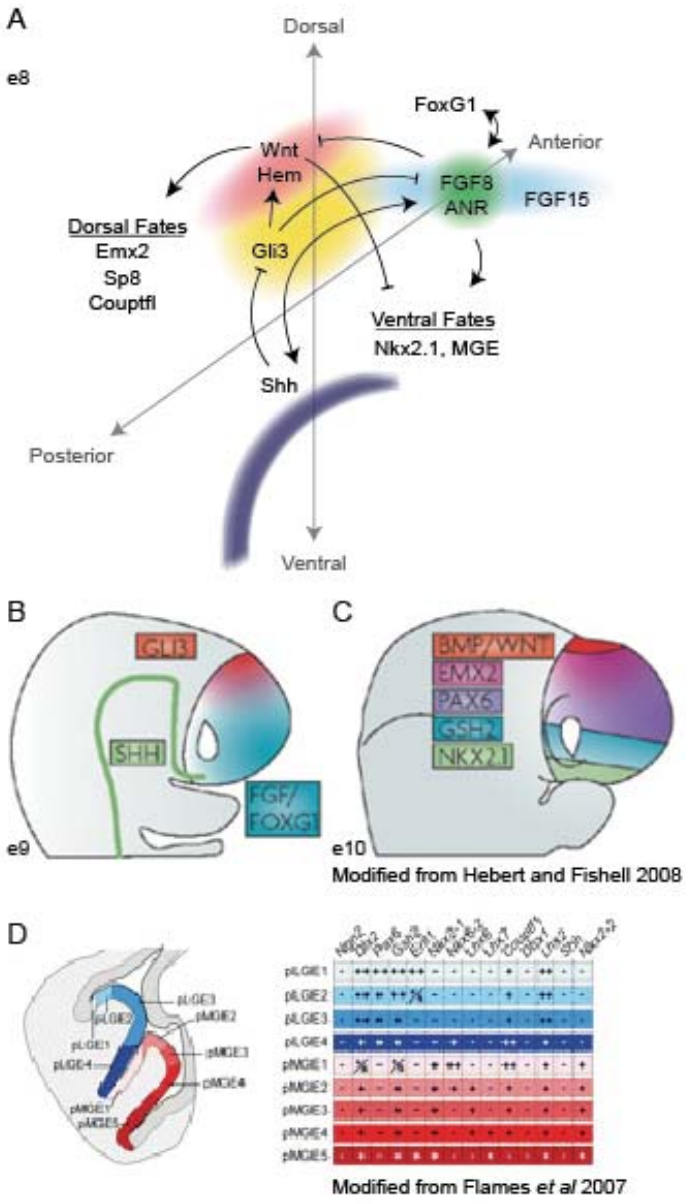

Figure 4 Telencephalic patterning and the subpallial subdomain hypothesis

(A) Schema indicating the relationship of extrinsic patterning signals to transcription factor expression and cell fates in the telencephalon. (B) Simplified scheme of key patterning factors in the telencephalon at e9,

which are further refined into (C) transcription factor domains. (D) Scheme from Flames *et al.* 2007 showing further subdivision of the eminences into subdomains by expression of different transcription factors in a coronal section of an e12.5 embryonic mouse brain. Chart indicates expression of different factors in proposed subdivisions.

After patterning of these regions is accomplished, the distribution of early signaling molecules, including FGF ligands, changes to fulfill later roles in neural development (in cortical arealization, for example (O'leary and Sahara, 2008; Okada et al., 2008)). Despite the crucial role of these early signals in the establishment of the ventral forebrain, little is known about whether these same signals play a role in specifying the cell diversity that arise later from these regions. WNT signaling, for example, while an important dorsalizing factor that has been well characterized, is also important for cell-survival within the MGE. A *β-catenin* knock-out specifically in the NKX2.1 domain results in a massive reduction of the size of the eminence (Gulacsi and Anderson, 2008). Considering the limited repertoire of patterning molecules utilized throughout development, it seems logical that their roles are constantly shifting to accommodate the newly created needs as time passes and the embryo changes. An observation that

supports this hypothesis in the telencephalon is the dynamic expansion and shifting of ligand expression. *Wnt7a* for example, is expressed in the ventral telencephalon at e9.5, however, by e12.5 *7a* is expressed dorsally throughout the cortical ventricular zone, yet is excluded from the hem, which expresses high levels of other WNT ligands (reviewed in (Harrison-Uy and Pleasure, 2012)). FGF ligands also expand their reach and influence. *Fgf15*, for example, early on is expressed anteriorly from the midline, just outside the expression domain of *Fgf8.* Later, its expression includes a caudo-lateral domain at the pallial-subpallial boundary (reviewed in (Iwata and Hevner, 2009)). During cortical arealization, FGF8 and FGF15 have opposing effects on neuronal proliferation and differentiation (Borello et al., 2008).

Together, these pathways coordinately act to promote ventral identities and establish the basic organization of the telencephalon (Figure 4) (reviewed in (Hebert and Fishell, 2008; Sousa and Fishell, 2010)). This includes the induction of multiple transcription factor domains that will specify dorsal and ventral regions of the telencephalon, including the ventral ganglionic eminences. Cortical interneurons are born in the ventral ganglionic eminences, and then migrate tangentially into the cortex, located dorsally.

Interneuron specification

Most cells in the cortex are like members of a tight-knit family in a small town; they don't move far from their brothers and sisters and stay connected to each other in the area they were born (Yu et al., 2012). Pyramidal excitatory cells of the cortex comprise about 70% of cortical neurons. Born at the ventricular surface, they migrate in an inside-out fashion building, layer by layer, the multilaminate cortex (Rakic, 1988). Part of what gives the cortex its beautiful layered organization is the steady and predictable radial migration of postmitotic cells from the pallial progenitor zones. GABAergic inhibitory interneurons, the minority cell type, are migrants into this highly structured community. Born in the subpallial ventricular zone, they migrate tangentially an exceptionally long distance to integrate into the cortex in a dispersed salt-and-pepper like pattern (Anderson et al., 1997; 2001; Corbin et al., 2001).

Cortical Interneurons are an incredibly diverse population, comprising multiple cell types characterized by numerous immunohistochemical, morphological, and electrophysiologic signatures (for review, see (Fishell and Rudy, 2011)). Taking each of these basic categories into account, and factoring the additional element of layering and cortical area, one can appreciate the 'snowflake' quality of interneuron identity. From a practical standpoint, generalizations must be made to talk

coherently about cell types at the expense of thoroughness. Immunohistochemical marker expression is one commonly used method to delineate interneuron subtypes. Though each of these ‘cardinal cell types’ is truly a somewhat heterogeneous group, using this fairly crude measure to distinguish cell types has advantages (Kepecs and Fishell, 2014). The availability of transgenic mice to target subpopulations of interneurons works at this basic level of specificity (Taniguchi et al., 2011). Additionally, antibody staining is a simple and easily quantifiable way of identifying cells, reflects the regional origin of cells, and allows us to infer something about each labeled cell’s other properties. It is important, however, to appreciate that each cell type plays its own unique role in the cortical circuit it inhabits, such that even cells of the same immunohistochemical type occupying different layers can have vastly different output effects. For example, Somatostatin (SST) positive interneurons in the major thalamorecipient layer of the cortex, layer 4 (L4), restrict their axonal arbors to L4 and target mostly L4 fast-spiking interneurons. Through this activity, these L4 SST interneurons function in a disinhibitory capacity. In contrast, layer 2/3 SST interneurons have more widespread axonal arbors and target mostly pyramidal cells (Xu et al., 2013).

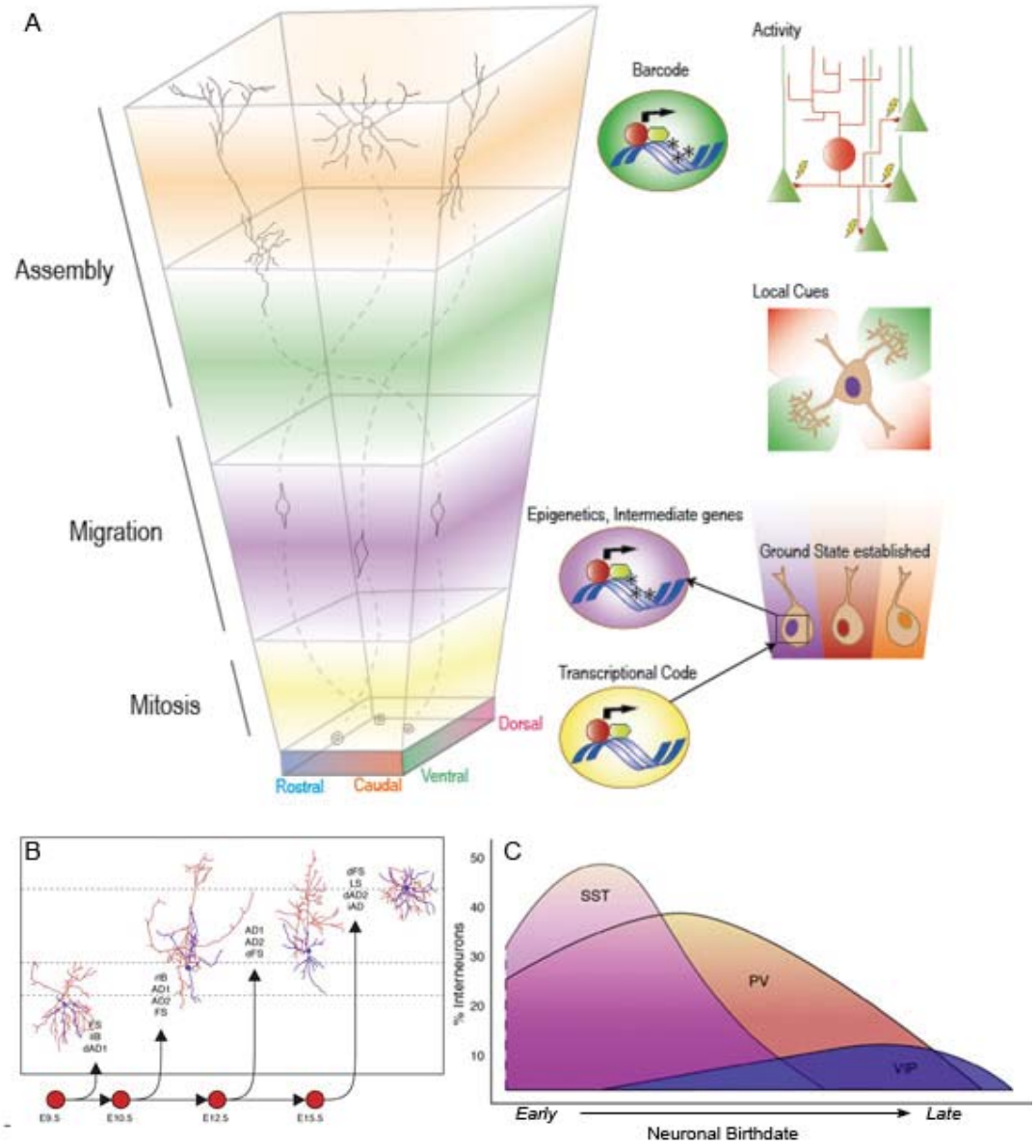

Figure 5 Interneuron development- progression from mitosis to circuit assembly

Scheme indicating the progression of interneurons from their cardinal or ground state subtypes, specified by gradients of patterning signals as interpreted by the transcriptional code through migration into their final

circuit role. The expanding cone represents the expansion of the diversity space occupied by the cell types.

Cortical interneurons and human disease

The tremendous functional diversity exhibited by interneurons likely accounts for the many disparate neuropsychiatric disorders that can be linked to their malfunction (Marín, 2012). The most famous, and most established connection of these is schizophrenia (Inan et al., 2013; Lewis, 2013). Schizophrenic patients have gamma band activity abnormalities in the pre-frontal cortex that are largely attributed to failures in fast-spiking, parvalbumin-positive (PV) interneuron function (Georgiev et al., 2014; Lewis, 2013; Wang et al., 2010). In addition, molecular analysis has revealed a number of interneuron-related changes in affected individuals including decrease in the levels of GABA mRNA (González-Burgos et al., 2010). Additionally, the phenotypes of a number of mouse models of autism spectrum disorders have been attributed to interneuron specific deficits. *Cntnap2* and more recently, *Cntnap4* mice display *GABA*ergic or PV cell synaptic defects, respectively (Peñagarikano et al., 2011). The phenotype of the *Mecp2* null mouse model of human Rett syndrome (a syndrome with autistic features) can be replicated in an interneuron specific knockout

(Chao et al., 2010). Both *Mecp2* and *Cntnap2* mice also display epilepsy, mirroring the high degree of co-morbidity between epilepsy and autism in human patients (Gabis et al., 2005). Recent human data has also supported the hypothesis of a connection between interneuron dysfunction and autism spectrum disorders (Stoner et al., 2014). In rodent models, deletion or other manipulation of cortical interneurons often leads to epilepsy or seizure susceptibility (Batista-Brito et al., 2009; Close et al., 2012). Defects in the GABAergic system are becoming a more common finding in human patients with epilepsy as well (Powell, 2013). The incredible diversity of interneuron function reflected in the varied interneuron related disorders signifies the importance of treating interneurons as a mixed population. Though they share common origins, it is the signals and pathways responsible for diverting subgroups to their particular fate that are crucial for understanding the nuance of interneuron function in cognition.

Interneuron birthplace and identity

Even though they travel far from their birthplace to settle in the cortex, the region where a cortical interneuron is born specifies its 'cardinal' cell type (Nery et al., 2002; Wichterle et al., 2001; Xu et al., 2004)(review (Kepecs and Fishell, 2014))(Figure 5). As mentioned above, interneurons

are born in the medial and caudal ganglionic eminences: anatomically defined structures that are present transiently during development. These regions are also defined molecularly by their expression of specific transcription factors, induced by the coordinated actions of secreted growth factors. The medial ganglionic eminence (MGE) is well defined by its expression of the homeobox transcription factor *Nkx2.1*, which is induced by ventral SHH (Shimamura and Rubenstein, 1997; Sousa and Fishell, 2010; Xu et al., 2010; 2005). *Nkx2.1* is a master regulator of cell fates arising from the MGE. Removal of *Nkx2.1* even right before progenitors exit the cell cycle can induce a fate-shift to dorsal cell types (Butt et al., 2008; Sussel et al., 1999). Further downstream, the genetic cascade of *Lhx6* activating *Sox6* and *Satb1* downstream of *Nkx2.1* has been followed to unique cell type specification effects on mature PV and SST cells (Batista-Brito et al., 2009; Close et al., 2012).

The caudal ganglionic eminence (CGE) is molecularly characterized by the expression of *couptfII*, *Sp8*, *Serotonin receptor 3a (5HT3aR),* and *Prox1* though none of these factors acts as a master regulator *à la Nkx2.1* in the MGE ((Cai et al., 2013; Flames et al., 2007; Lee et al., 2010; Ma et al., 2011; Nery et al., 2002; Rudy et al., 2011), Miyoshi *et al.,* in preparation). To date, *Prox1* is unique as the only CGE transcription factor with a specific cortical interneuron phenotype (Miyoshi *et al.*, in

preparation). The lateral ganglionic eminence, which gives rise to interneurons of the olfactory bulb (Wichterle et al., 2001), shares many molecular markers with the CGE as well as the MGE, leading some in the field to conceptualize the CGE as a caudal extension of the LGE (Flames et al., 2007; Nery et al., 2002). However, thus far, disrupting transcription factors like *Nkx2.1* and *Gsh2,* which are absolutely required for the proper development of the MGE and LGE, respectively, has had little to no effect on the CGE (Nery et al., 2002). While there is a clear anatomic sulcus that physically separates the MGE and LGE, the CGE is in fact a physical extension of both the MGE and LGE, beginning where the sulcus ends. The sulcus, while we will not address its specific contribution in detail here, is an interesting region in and of itself. The sulcus expresses the homeodomain factor *Nkx6.2* and is enriched for the production of somatostatin (SST) calretinin (CR) double positive neurons (Sousa et al., 2009). That these separate regions give rise to non-overlapping interneuron subtypes was first shown via classic developmental biology transplantation techniques. Each of these regions was dissected from ubiquitously labeled mice and transplanted into unlabeled recipient embryos (Nery et al., 2002; Wichterle et al., 2001; Xu et al., 2004). Heterotopically or heterochronically transplanted cells migrate to the cortex and the cell types produced remain faithful to their place and time of origin (Nery et al., 2002).

Interneuron specification: temporal effects and clonality

As with all developmental processes, the production of cells from the eminences varies over time. Cortical interneurons, despite their long-range migration also observe the temporal inside-out pattern of cortical development. MGE cells, born early in development, tend to populate early-born, deep cortical layers (Miyoshi et al., 2007)(Figure 5). Interestingly, heterochronic transplantation of MGE cells can influence the layers those cells migrate to- suggesting that layering aspects of cellular identity are not intrinsically specified, but influenced by the environment (Valcanis and Tan, 2003). CGE cells, born later in development, tend to occupy more superficial layers, but follow a different migratory pattern. Unlike MGE cells that incorporate into their cognate layer as they migrate, CGE cells adopt a temporary state of even distribution within all cortical layers before sorting into the more superficial layers (Miyoshi and Fishell, 2011). Within the MGE, the cell types produced vary over time. SST positive cells tend to predominate the earliest born MGE neurons, slowly shifting toward a PV positive bias that characterizes the cells produced at e12.5, the peak of cell production from the MGE (Miyoshi et al., 2007; 2010)(Figure 5). There is some indication that the very earliest MGE cells born are SST positive interneurons that act as highly connected “Hub” cells important for circuit

formation in the hippocampus (Picardo et al., 2011). Interestingly, the latest born cells from the MGE have recently shown to also be a unique population of neurons, cortical chandelier cells (Inan et al., 2012; Taniguchi et al., 2013). Whether these unique temporal ratios of cells produced reflects a changing population of progenitor cells or the changing output of a stable progenitor set (or some combination thereof) was long a matter of debate. Recent studies using sparse viral infection in the MGE to label single progenitors and their progeny suggested that clones are temporally closely related and the majority are a mixture of cell types (Brown et al., 2011; Ciceri et al., 2013). Another interesting finding of this study was that clones appeared to settle in clusters in the cortex. In fact, one difficulty of studying interneurons in this way, compared with studying the clonal relationship of radially migrating pyramidal cells, is that the progeny migrate, making it difficult to be certain of a cell's likely origin by its location. Live imaging of cells as they migrate from the progenitor zone is similarly impossible with current technology. Regardless, the Shi study identified a number of cells clustered in such a striking way that would predict a clonal relationship just based on the statistical rarity that 2 random cells would end up so close to one another. Recent work in the Fishell lab using a similar technique but with a viral catalogue that allows for the conclusive identification of clonal relationships by unique genetic tags has found that

while cells born at similar time points *can* cluster, individual clones are often, but not always, widely dispersed even across forebrain structures. Using this technique, it also seems that individual clones are often small, comprised of single cells or a couple of cells (Christian Mayer, personal communication).

Activity refines interneuron phenotypes

As previously mentioned, CGE interneurons are born later in development and occupy the superficial layers of the cortex. Recent work has shown that these later born interneurons are influenced in a cell type specific way by the activity they encounter in the cortex. Dampening intrinsic excitability of newly born CGE interneurons by overexpressing an inward rectifying potassium channel causes cell type specific defects in migration and morphological development (De Marco García et al., 2011). This is some of the best evidence supporting the long held hypothesis that interneuron specification is not completely internally programmed by progenitor zone genetics. Given the vast diversity of cortical interneuron subtypes and their varied functional roles in the circuit it seems impossible that every nuanced difference could be predetermined from the progenitor stage. The influence of activity and local cortical cues is likely important in shaping the final phenotype of an individual interneuron (Figure 5). So far,

the previously mentioned experiments have borne this out in CGE derived interneurons, which integrate into a cortical circuit that is much more advanced than that encountered by MGE born interneurons. It will be interesting to see how similar or different the refining path of MGE derived interneurons will be.

The subdomain hypothesis of interneuron specification

Before activity dependent refinement takes place, we know much of a cell's identity is already determined. Given the regional difference between cells produced within the different eminences, it is reasonable to hypothesize that there may be subdivisions of the eminences that could be more specific for individual, genetically defined subtypes, similar to the scheme in the spinal cord. Work from multiple labs has previously characterized putative subdomains within the MGE, both in terms of cell types produced as well as combinatorial gene expression (Bulfone et al., 1993; Flames et al., 2007; Inan et al., 2012; Puelles and Rubenstein, 1993; Rubenstein et al., 1994)(Figure 4). Perhaps not surprisingly, there have been a few studies showing biases in cellular production from subdomains of the MGE, yet none have elucidated a particular factor or genetic signature responsible for the differences observed between subregions.

Evidence has borne out that interneuron fates are partially determined by the regions and time they are generated, and later refined by extrinsic signals from the developing circuits they migrate into. The initial determination of cardinal cell types seems to be intrinsically determined by a genetic code downstream of secreted morphogens, at least from a MGE/LGE/CGE level of distinction. Additional evidence suggests that this level could be refined further. Clonal tracing by viruses has suggested that progenitors in these regions give rise to very small clones of potentially mixed cell types. Many questions remain about how this diversity is achieved from a progenitor perspective. One common mechanism in the CNS is based on a temporal progression of progenitor competence. Through a series of intrinsic and extrinsic cues, pluripotent neuronal progenitors ratchet through different competencies becoming more restricted over developmental time. This has been demonstrated in many vertebrate systems including the spinal cord, retina, and forebrain (Edlund and Jessell, 1999; Livesey and Cepko, 2001; McConnell, 1995). In both retina and forebrain, heterochronic transplants of neuronal progenitors have found that progenitors can produce later cell types, but cannot revert to produce earlier cell types (Austin et al., 1995; McConnell and Kaznowski, 1991). This type of mechanism has also been shown in the fly embryonic neuroblast (Isshiki et al., 2001), and more recently, fly medulla suggesting

that this could be a general strategy for generating neuronal diversity (Li et al., 2013). However, temporal progression of different transcription factors has not been observed in the subpallial proliferative zone. Viral lineage tracing experiments in the MGE result in mixed clones, indicating that single progenitor cells give rise to multiple cell types. In the future, it will be interesting and important to understand how regional and temporal differences in cell type production biases interact to produce the full diversity of subpallial lineages. Though thus far, and in the following, we have restricted our discussion to cortical interneuron diversity, it should be noted that the ventral eminences also give rise to a number of ventral neuronal lineages. The MGE, for example, also gives rise to a number of striatal and globus pallidus neurons (Flandin et al., 2010; Wichterle et al., 2001). In this study, for simplicity's sake, we will focus on regional differences in cortical interneuron production that may arise due to secreted growth factor gradients.

Every part of an organism has the same developmental goals. Anyone with even a passing understanding of physiology can appreciate the difficulty of building a mammal from scratch. One must take a single, incredibly potent cell, and turn it into millions of different highly specified cell types, reliably, predictably, again and again. The brain is among one of the

most highly specified structures that needs to accomplish this task. In this study we will look at this complex problem from a very narrow perspective. The nervous system, and the telencephalon specifically, make use of a gradient strategy from the onset of their developmental organization. The process of this initial parsing of large portions of the telencephalic anlage has already been partially elucidated. Interneurons, a small but incredibly diverse set of cells produced from a restricted area of that anlage present an interesting test case to take initial patterning to its final output. In the following, we will aim to see if and how patterning strategies employed in the early patterning of the telencephalon are employed in the development of interneuron diversity.

FGF in cortical interneuron specification

Melissa McKenzie Chang and Gord Fishell

Introduction

FGF is a developmental patterning molecule that has been central to neural patterning in even the simplest of organisms (Pani et al., 2012; Rentzsch et al., 2008). In rodents, FGF signaling is critical for the early development and the patterning of the forebrain, including the induction of transcription factor domains that specify the ventral ganglionic eminences (Hebert, 2011; Hebert and Fishell, 2008; Paek et al., 2009). The diverse

populations of cortical interneurons that are born in the ventral eminences show temporal and regional variation in their mature subtype. These cell fate decisions appear, in part, to be determined close to the time of their final mitosis (Nery et al., 2002; Wichterle et al., 2001; Xu et al., 2008). Despite our understanding of the earlier role of FGF signaling in the patterning of the telencephalon, we still know little about many of the effectors important for the generation of interneuron diversity. What role, if any, FGF ligands present in the forebrain play in interneuron specification remains a mystery. At this stage the role of FGF signaling becomes difficult to assess. In addition to the challenge of redundancy in the receptors and ligands, FGF signaling is absolutely required for ventral patterning. Conditional removal of *Fgf receptors 1* and *2* using CRE recombinase driven by *FoxG1* eliminates the ventral telencephalon (Gutin et al., 2006). The work of the Fishell lab and others has elucidated many of the pathways downstream of another early patterning signal, SHH (Rallu et al., 2002; Sousa and Fishell, 2010; Xu et al., 2010). This study explores whether FGF, like SHH, plays a role in the specification of cortical interneuron subtypes. We took three major approaches to address this question: targeting downstream effector transcription factors, intracellular signaling adaptor proteins, or the receptors themselves.

Downstream transcription factors

First, we sought to look for transcription factors downstream of FGF signaling that might be important for cell specification. In particular, we looked at *Ets1*, *Ets2*, and *Sp8*.

The Ets family of transcriptional regulators has 30 members in mammals, all of which include the highly conserved *ets* DNA binding domain (Watson et al., 1988). Many *Ets* family members, including *Ets1* and *Ets2* also have a conserved *pointed* domain that forms a helix-loop-helix structure at the protein's N-terminus to facilitate protein-protein interactions (Oikawa and Yamada, 2003; Watson et al., 1988). The action of a particular *Ets* protein is dependent on the cell signaling pathways activated in the cell as well as the other transcriptional proteins present, making them particularly intriguing factors in developmental biology as an integration point of extrinsic and intrinsic cellular information. *Ets* proteins interact with multiple other transcription factor families and act downstream of numerous signaling cascades including Ras signaling, JNK, p38 and PI3K pathways (Oikawa and Yamada, 2003). *Ets1* and *2* are currently being extensively studied for their role in tumor progression and metastasis, due to modulation of their activity by Ras signaling as well as their known ability to regulate matrix metalloproteinases (MMPs), cadherins, and other cellular migration factors (Chakraborti et al., 2003; Foos et al., 1998). In

addition, *Ets* family genes are critical for multiple developmental processes, such as motor neuron network establishment in the spinal cord, several stages of hematopoesis, and neural crest migration in chick and *Xenopus* (Lin et al., 1998; Oikawa and Yamada, 2003; Remy and Baltzinger, 2000).

Sp8 is a member of the family of *Specificity Protein, Kruppel-like factor* (*SP/KLF)* zinc finger transcription factors (Suske et al., 2005). *Sp* genes are important for many developmental processes; including, interestingly, the arealization of the cortex. *Sp5, Sp9,* and *Sp8* are each associated with various signaling centers during the establishment of different regions within the cortex. *Sp8* is particularly interesting in this context because its cortical expression is reciprocally activating with *Fgf8* located at the rostral pole, where it contributes to the specification of rostro-medial cortical fates (Sahara et al., 2007). *Sp8* is also important for the specification of olfactory bulb interneurons, derived from the LGE(Waclaw et al., 2010).

We removed each of these factors specifically in postmitotic interneuron progenitors and looked for changes in interneuron subtypes or numbering by immunohistochemistry. While immunostaining for all of these factors revealed their enrichment in cIN populations; mutants for any of these factors, including the compound *ets1* and *ets2* mutant did not have any gross interneuron specification phenotypes. *Sp8* was recently found to

likewise show little phenotype when removed in interneurons despite being enriched in CGE derived populations (Ma et al., 2011).

Adaptor Proteins and FGF receptors

FGF ligands are extremely numerous and have promiscuous and overlapping binding specificities (Dailey et al., 2005; Knox et al., 2002; Ornitz and Itoh, 2001; Ornitz et al., 1996; Zhang et al., 2006), resulting in interpretation and practical challenges after their genetic manipulation. FGF receptors, with three of four receptors expressed in the nervous system, present a surmountable, but not insignificant, technical hurdle for genetic targeting. The docking protein FRS2α by contrast, as a single protein essential for linking FGF receptors to canonical MAPK signaling, presents a simple way to broadly disrupt FGF signaling. In combination with downstream effector knockouts, either broad manipulation of the receptors or of *FRS2α* theoretically permits the determination of both FGF signaling generally and the 'sub-functions' of different arms of the pathway.

To our surprise, manipulation of either *FGFR* or *FRS2α* in interneurons postmitotically failed to reveal any significant cell-specification phenotype. We also made attempts to remove FGF signaling before interneuron progenitors became postmitotic through an electroporation scheme, though it never yielded viable offspring. Considered together, these results suggest that cardinal interneuron cell types, as defined by

immunohistochemical labeling, are not mutable by changes in FGF signaling after neurons become postmitotic. This leaves open the question of whether loss of FGF signaling at the progenitor stage could influence cell fate.

Materials and Methods

In Situ Hybridization

Embryos ages e15.5 or later and adult mice were transcardially perfused with 4% paraformaldehyde in PBS (4%PFA). Brains were dissected and post-fixed in 4%PFA overnight at 4°C, then cryoprotected in 30% sucrose W/V in PBS overnight at 4°C. Prepared brains were mounted in Tissue-Tek (Takara) frozen at -80°C for storage until cryo-sectioned at -20°C in 12μm sections for embryonic tissue and 20μm sections for adult tissue. In embryos younger than e15.5, brains were left *in situ* and heads were removed and fixed overnight in 4%PFA, then treated as above. *In situ hybridization* using DIG labeled probes was performed as previously described (Hanashima et al., 2004). *Ets1* was generated by digesting full length cDNA (open Biosystems 3676286) with RsrII and transcribing with *T7* polymerase. *Ets2* probes designed to a unique portion of the mRNA sequence and was amplified by PCR from the full length cDNA (open Biosystems 3486021)(PCR primers: CTGGATTCTGTCTCCCATG, AGCTGATGAAAGATTGACA). A standard PCR protocol was followed:

95°C for 3 minutes, [95°C 20sec, 56°C 1.5min, 72°C 1.5min] repeated 30 times, 72°C 10minutes. PCR product of 400bps was cloned into TOPO vector (Invitrogen) following manufacturers instructions. Correctly oriented clones were identified by sequencing and used to generate DIG labeled probes (Roche).

Immunohistochemistry

Tissue for immunohistochemistry was prepared as described above, except adult tissue at p21 was post-fixed for 1hour before sucrose cryoprotection. Cryosections were allowed to dry for 1-2 hours then frozen at -20°C until they were used. Frozen sections were defrosted and dried at room temperature for 1hour then rehydrated in blocking solution (4%serum, .1% TritonX-100 in PBS) for 1-4 hours. Primary antibody incubation followed in blocking solution overnight at 4°C. Primary antibodies were used at the following concentrations: Rabbit anti Calretinin (Millipore 1:1500), Rat anti SST (Millipore 1:500), mouse anti CR50 (reelin) (Marine Biological Laboratories 1:500), Guinea Pig anti Parvalbumin (gift from David Lewis, University of Pittsburgh 1:1500), Rabbit anti VIP (1:1000), Rabbit anti FRS2α (.01% Triton-X was used for this staining 1:100), Mouse anti-Ets1 (sections were pre-incubated in generic anti-mouse alkaline-phosphatase conjugated antibodies to reduce background reactivity with the secondary antibody). Sections washed repeatedly in PBS then incubated in secondary

antibodies conjugated to Alexafluor 488 or 594, their host matching the serum used for blocking, for 45minutes at room temperature. When nuclear labeling was desired, DAPI was applied after secondary antibody at 1:10,000 in PBS for 5 minutes. Slides were promptly mounted with Fluoromount-G (Southern Biotech) and coverslipped.

Animals and breeding

All animal maintenance and use followed regulations set forth by the Institutional Animal Care and Use Committee at NYU School of Medicine. All lines were maintained on a mixed background of Swiss Webster and C57/B6 and genotyped as previously described- *Dlx5/6 CRE* (Stenman et al., 2003), *RCE* (Sousa et al., 2009), *FoxG1 CRE* (Hébert and McConnell, 2000), *Gad67GFP*(Tamamaki et al., 2003)*, Ets2flox* (Yamamoto et al., 1998)*, Ets1 null* (Barton et al., 1998)*, FRS2α* (Lin et al., 2007)*,* and *FGFR1flox* (Trokovic et al., 2003)*, FGFR2flox*(Yu et al., 2003) and *FGFR3*$^{-/-}$ (Deng et al., 1996)*.*

Ets2 Antibody production

Antibody design was done in collaboration with Susan Morton of the Jessell lab, Columbia University. *Ets2* protein sequence was analyzed by Abie Pro 3.0 software for antigenic regions, of sequences returned, a 17 peptide fragment from the N-terminal region was chosen for it's uniqueness and likely exposure to intracellular space in the intact *Ets2* protein. Global

Peptide/Pi Proteomics generated the peptide fragment sequence H^+-LLILRRRIRKQAHAHSK-OH, verified by analytical HPLC. Rabbits were immunized with B-pertussis before injection with *Ets2* peptide, and serum samples were acquired every 7 days and tested for specificity.

Results

Ets1 *and* Ets2 *are enriched in cortical interneuron precursors*

Prior to the Fishell lab's publication (Batista-Brito et al., 2008) of an interneuron specific microarray, no systematic analysis of the genes expressed in cortical interneurons during development had been undertaken. That study took advantage of the knowledge that interneurons are the only cells expressing *Dlx* family genes in the cerebral cortex. Large numbers of developing cortical interneurons were purified through their expression of EGFP directed by a *Dlx5/6* enhancer driving a minimal promoter (Zerucha et al., 2000). RNA expression profiling of the purified population through microarray analysis then enabled the sampling of genes enriched in multiple subtypes of interneurons (Batista-Brito et al., 2008). At the timepoints cells were isolated for the microarray analysis, CGE derived interneurons are largely absent from the cortex, thus this scheme preferentially identified genes expressed in MGE derived progenitors (Miyoshi and Fishell, 2011).

I participated at the validation stage of this screen. In order to identify key regulators of cell fate, my analysis focused on transcription factors that were likely to be downstream of the major signaling pathways involved in the patterning of the telencephalon. One gene identified in the screen, *Sox6,* was later shown by Batista-Brito *et al.* to be important for the maturation of specific MGE derived subtypes of interneurons (Batista-Brito et al., 2009). *Sox6* is an *HMG-box* transcription factor, a family that has been shown in some contexts to act downstream of the SHH signaling pathway, a critical regulator of early telencephalic development. While the importance of the SHH pathway in neural patterning has been well characterized, its role in patterning of the telencephalon appears to be passive. It works largely in the telencephalon by negatively regulating *Gli3* repressor activity and thus preventing dorsalization (Rallu et al., 2002). On the other hand, FGF signaling, which is also important for establishing early patterning of the telencephalon, acts mainly through the activation of the various positive signaling pathways, including *Ras, PLC-γ,* and *PI3K* (Tsang and Dawid, 2004). I focused on candidate downstream effectors of "positive" FGF signaling. With this in mind, I identified two particularly interesting transcriptional activators, *Ets1* and *Ets2* of the genes upregulated in interneurons in the microarray screen mentioned above. These genes are members of the highly conserved *Ets* superfamily and are

known downstream effectors of FGF signaling (Watson et al., 1988; Yordy and Muise-Helmericks, 2000)

Data from the microarray experiments revealed that *Ets1* and *2* are differentially regulated between e13.5 and e15.5 (Figure 6). *Ets1* and *Ets2* are both increasingly upregulated during this time period; however, the degree of increase and their specificity for interneurons is disparate. *Ets1* shows a two-fold upregulation over this time period, with increasing specificity for interneurons (compared to expression in sorted cortical cells not expressing the *Dlx* transgene). *Ets2* signal increases to a much smaller degree, however, both *Ets1* and *Ets2* are not highly expressed when absolute signal intensity is compared to that of known MGE specific transcription factor *Lhx6* (Figure 6). Signal intensity is not a precise predictor of expression levels, as probe quality will affect this measure, however it does give a rough estimate.

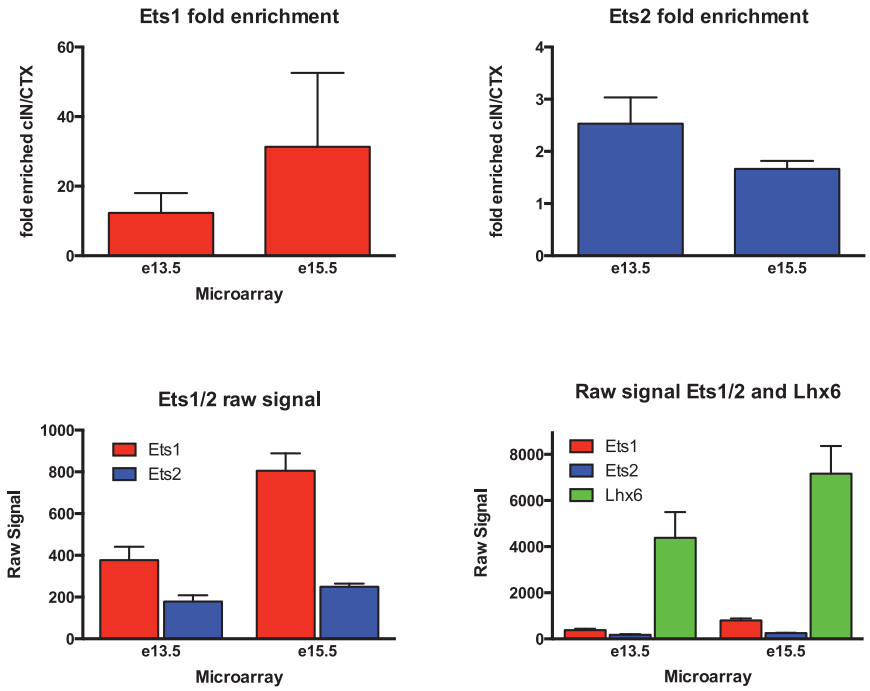

Figure 6 *Ets1/2* expression by microarray

A) *Ets1* mRNA signal fold enrichment in sorted interneurons compared to non-labeled cortex at e13.5 and e15.5 B) *Ets2* mRNA fold enrichment. C) Raw signal fluorescence of *Ets1* and *Ets2* probes at e13.5 and e15.5. D) Same raw signal compared to the highly-expressed and MGE derived interneuron specific transcription factor Lhx6

Ets1 protein is expressed embryonically in what appear to be migrating cells at the cortical hem. This is consistent with the presumed expression of *Ets1* in postmitotic interneurons. Interestingly, protein expression was not evident in embryonic cortex despite the microarray data

(Figure 7). Similarly, perhaps reflecting the low absolute levels of mRNA present, *in situ* hybridization for *Ets1* showed little embryonic expression levels (Figure 7). In adult cortex, *Ets1* is expressed throughout the cortex at low levels in a non-specific pattern that suggests it is expressed in multiple cell types.

In the absence of any commercially available antibodies to Ets2, our initial observations of *Ets2* expression were limited to *in situ* hybridization. At embryonic ages, much like *Ets1*, the expression levels were low and not easily detected with this method. This was again consistent with the signal levels we observed with the microarray analysis. To further our *Ets2* expression analysis, we immunized rabbits with an 11 amino acid long peptide of Ets2. The peptide, selected for it's uniqueness and specificity to Ets2, is located in the N-terminal portion of the *Ets2* protein and predicted to have a high level of antigenicity (data not shown). Embryonic staining was consistent with the *in situ* hybridization results, showing little expression in the cortex or eminences, save a small number of cells in the mantle (Data not shown). In adult animals, *Ets2* expression is widespread by *in situ* hybridization. Protein expression appears to be similarly widespread in the cortex using our antisera, present in both pyramidal cells and interneurons (Figure 7).

Sp8 has a remarkably specific expression pattern that had been described previously in the lateral ganglionic eminence (LGE), where it is important for the specification of LGE derived olfactory bulb interneurons (Waclaw et al., 2006). The LGE expression extends caudally into the CGE during embryonic timepoints. Not surprisingly, it is co-expressed with the CGE marker *CoupTFII* (Figure 7).

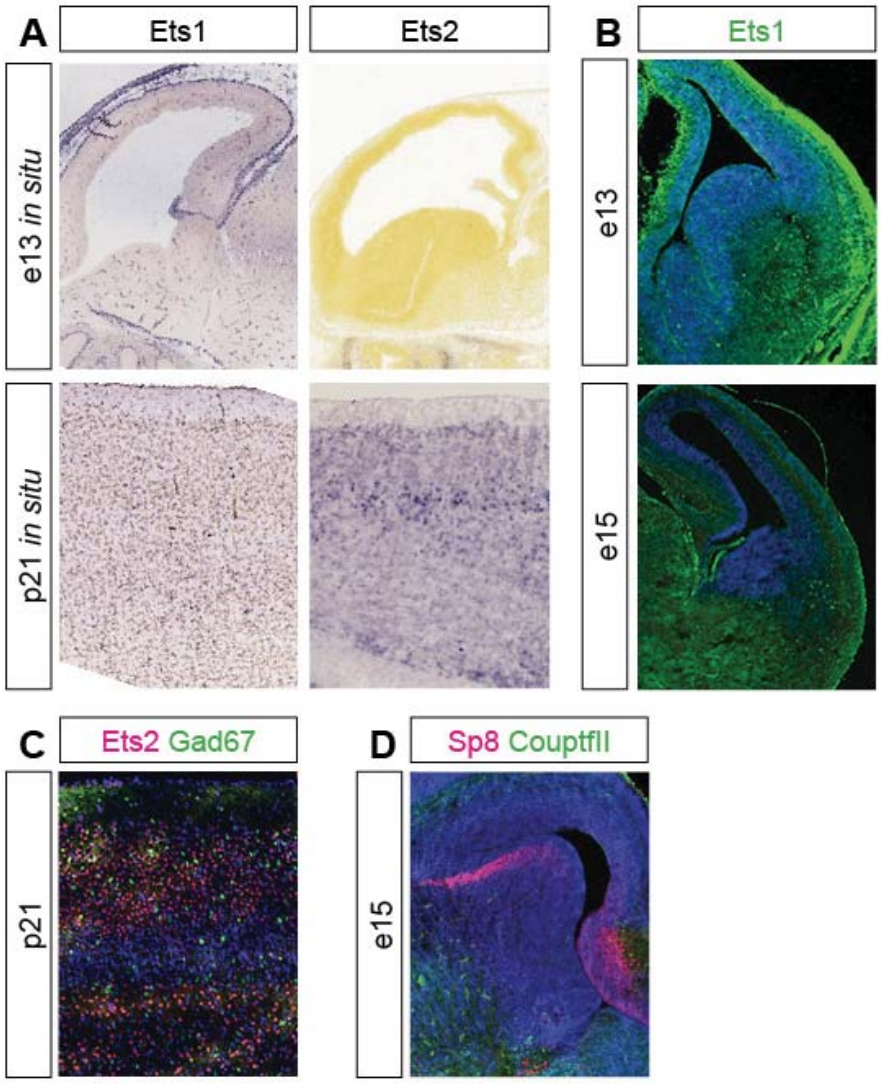

Figure 7 Ets1/2, Sp8 expression pattern

(A) In situ hybridization data for *Ets1 and 2* at e13 and p21 (Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org.) (B) Antibody staining for ETS1 in coronal sections of e13 and e15 brains showing isolated cells in the mantle (C) Protein expression of ETS2 using

newly developed antibody in multiple cell types in the adult cortex. (D) Sp8 is expressed at the lateral edge of the LGE/CGE in the e15 embryo, some overlap with the marker CouptfII.

Ets1 *null,* Ets2 *interneuron specific single and compound knock-outs show no specification defect*

While *Ets1* null mice are viable (Barton et al., 1998), *Ets2* nulls are embryonic lethal due to a defect in the trophoblast. Tetraploid trophoblast rescued *Ets2* null mice have an interesting hair formation phenotype reminiscent of EGF mutants (Yamamoto et al., 1998) consistent with its known role as an effector of EGF and FGF signaling.

We made use of an *Ets1* null allele (Barton et al., 1998) and *Ets2* floxed allele (Wen et al., 2007) to generate general and interneuron specific single and double knockouts.

We began our assessment of *Ets1* and *Ets2* by specifically knocking out *Ets2* in interneurons using the interneuron specific *CRE* driver, *Dlx5/6 CRE* (Stenman et al., 2003), in conjunction with the *ets2* floxed allele we acquired from Dr. Oshima's lab at the Burnham Institute. Previous work in the Fishell lab has found that five basic immunohistochemical markers; parvalbumin (PV), somatostatin (SST), calretinin (CR), vasointestinal

peptide (VIP) and reelin can together account for the majority of cortical interneurons (Miyoshi et al., 2010). Cell density and distribution across layers appeared to be largely normal for every marker analyzed by immunohistochemistry (Figure 8). The ability of a given *CRE* driver to recombine a pair of loxP sites varies significantly. To determine if the *Dlx5/6 CRE* driver successfully removed *Ets2* we stained for ETS2 protein expression using the antibody we generated. We were still able to detect ETS2 in cKO mice (data not shown). However, this staining could reflect a truncated protein produced by the recombined *Ets2* allele, as the epitope recognized is not part of the floxed region. To partially address the issue of incomplete removal, we also analyzed *Dlx5/6 CRE Ets2*$^{f/-}$ animals. The density and distribution of cortical interneuron marker expression in these mice was likewise indistinguishable from control littermates (data not shown).

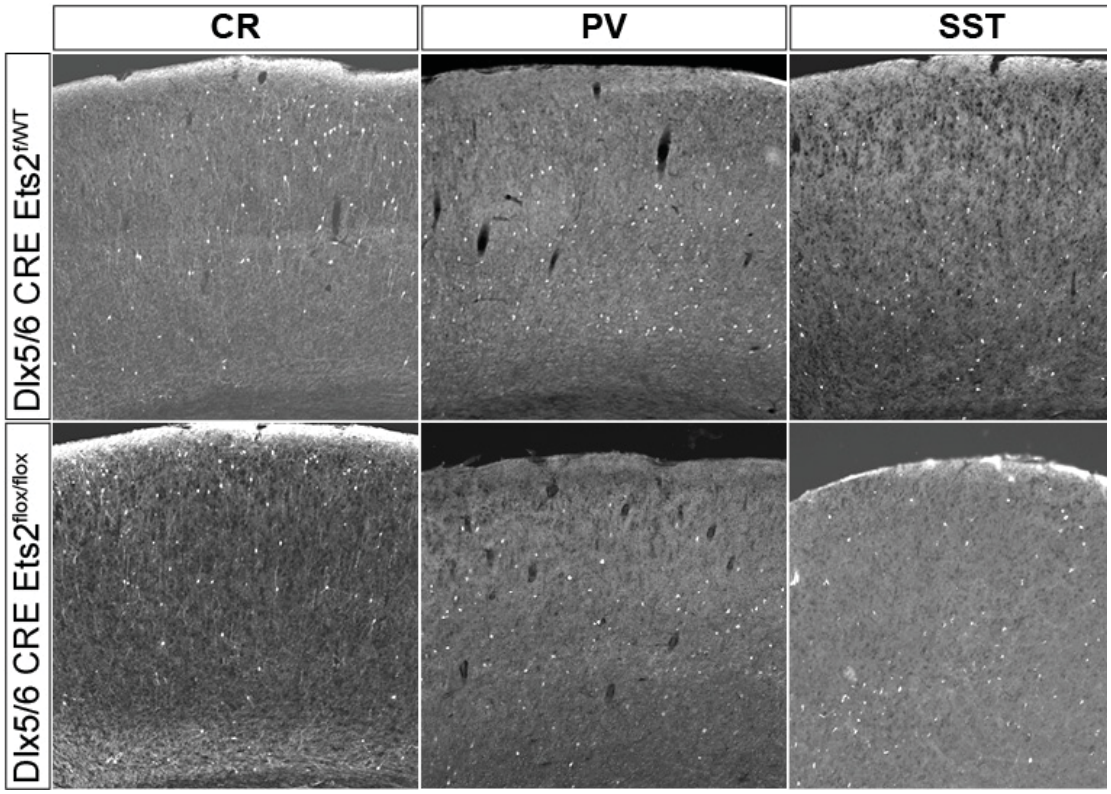

Figure 8 *Ets2* conditional knockout interneuron marker expression

Expression and density of calretinin (CR), parvalbumin (PV) and somatostatin (SST) in heterozygous (top row) and homozygous (bottom row) *Dlx5/6 CRE; Ets2 floxed* somatosensory cortex.

Preliminary cortical interneuron marker expression analysis of *Ets1* null animals revealed slight differences in some interneuron subtypes. CR positive bipolar cells were decreased and disorganized in the mutant and the heterozygous cortex. Staining for the pyramidal cell marker *Cux1* was

normal, suggesting that cortical organization was intact in the *Ets1* mutants (Figure 9). However, this phenotype was attenuated when the strain, on a C57/B6 inbred background was bred onto swiss-webster (an outbred strain), suggesting the phenotype may be strain dependent.

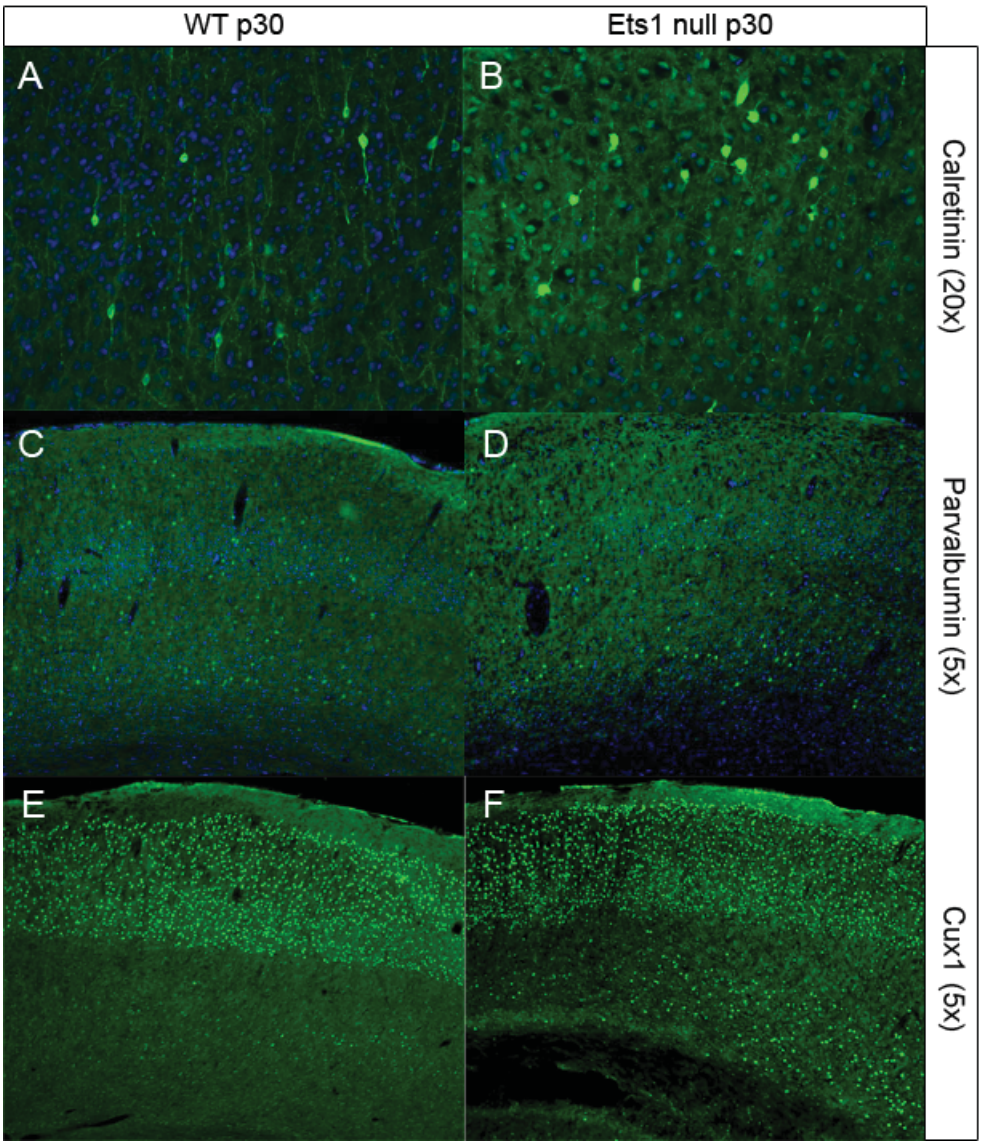

Figure 9 *Ets1* mutant shows partially penetrant interneuron phenotype in a normal cortex

A, B) 20x image of interneurons labeled with calretinin in wild-type and mutant, mutant shows disorganization of calretinin neurons that is lost after out-crossing. C,D) Parvalbumin staining in WT and mutant are similar. E,F) *Cux1* expression is similar in wild-type and mutant, despite decrease in

cortical thickness in *Ets1* mutants

Given the similarity between members of the ETS family, it was also possible that these proteins would be able to compensate for each other in the single mutants. To address possible redundancy in these alleles, we analyzed *Ets1* and *Ets2* double mutants using the Dlx5/6 cre driver previously mentioned. Double knockouts (*Dlx5/6 CRE; Ets2*$^{f/-}$*; Ets1*$^{-/-}$) similarly showed normal interneuron marker expression, and did not exhibit an exacerbation of the *Ets1*$^{-/-}$ partially penetrant phenotype (data not shown).

Removal of adaptor protein FRS2α in cIN populations

Effector molecules theoretically represent only a fraction of the signaling that occurs downstream of receptor activation. Needless to say, it stands to reason that a removal of all FGF signaling could exert an effect where loss of an effector molecule had failed. Importantly, as redundancy had been a concern in the manipulations of the effector molecules, it was also a concern when targeting the receptors. To circumvent this difficulty, we devised a strategy targeting the adaptor protein *FRS2a*, which has been found *in vitro* to be important for FGF signaling through canonical pathways

(Hadari et al., 2001). We found *FRS2α* is broadly expressed in the adult mouse cortex by *in situ* hybridization. FRS2α protein was localized in puncta adjacent to pyramidal cell axons (Figure 11).

We first aimed to verify the ability of *FRS2α* removal to mimic loss of all *FGFRs*. Previous studies have indicated that the telencephalic progenitors require FGF signaling for their survival (Paek et al., 2009). We reasoned that a loss of *FRS2α,* if it similarly resulted in a loss of FGF signaling, should phenocopy the triple *FGF receptor* knockout using the early, pan-telencephalic *CRE* driver *FoxG1 CRE*. Surprisingly, the *FOXG1 CRE, FRS2α*$^{f/f}$ embryo was indistinguishable from its wild-type littermates. We observed a complete loss of the FRS2α protein by immunohistochemistry and no gross morphologic defects in coronal sections of the mutant embryos (Figure 10).

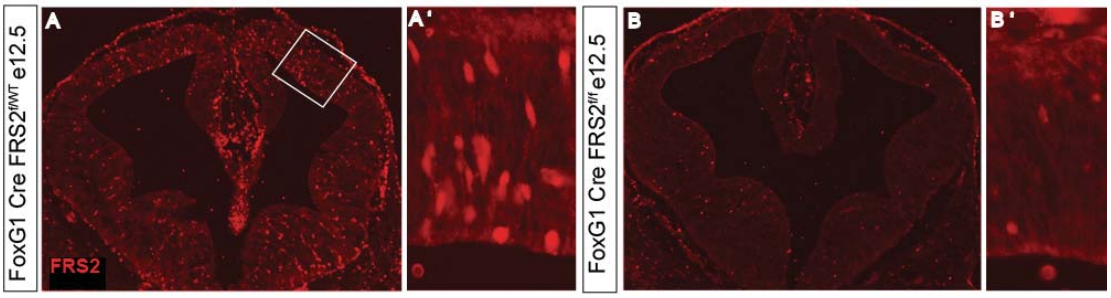

Figure 10 *FRS2α* removal by FOXG1 CRE

(A) FRS2α protein expression in coronal sections through e13.5 brains. conditional knockout (B) and wild-type littermates (A). Close-up image of the cortex (A',B') shows complete loss of expression in the knockout (B')

Though these results suggested that a *FRS2α* KO was not equivalent to a triple *FGFR* KO, the possibility remained that it still accomplished a partial loss-of function. With this possibility in mind, we generated an interneuron specific *FRS2α* conditional loss of function, again using the postmitotic *Dlx5/6 CRE* driver. Cortical expression of interneuron markers in these mutants resembled their wild-type littermates in numbers, density and distribution (Figure 11).

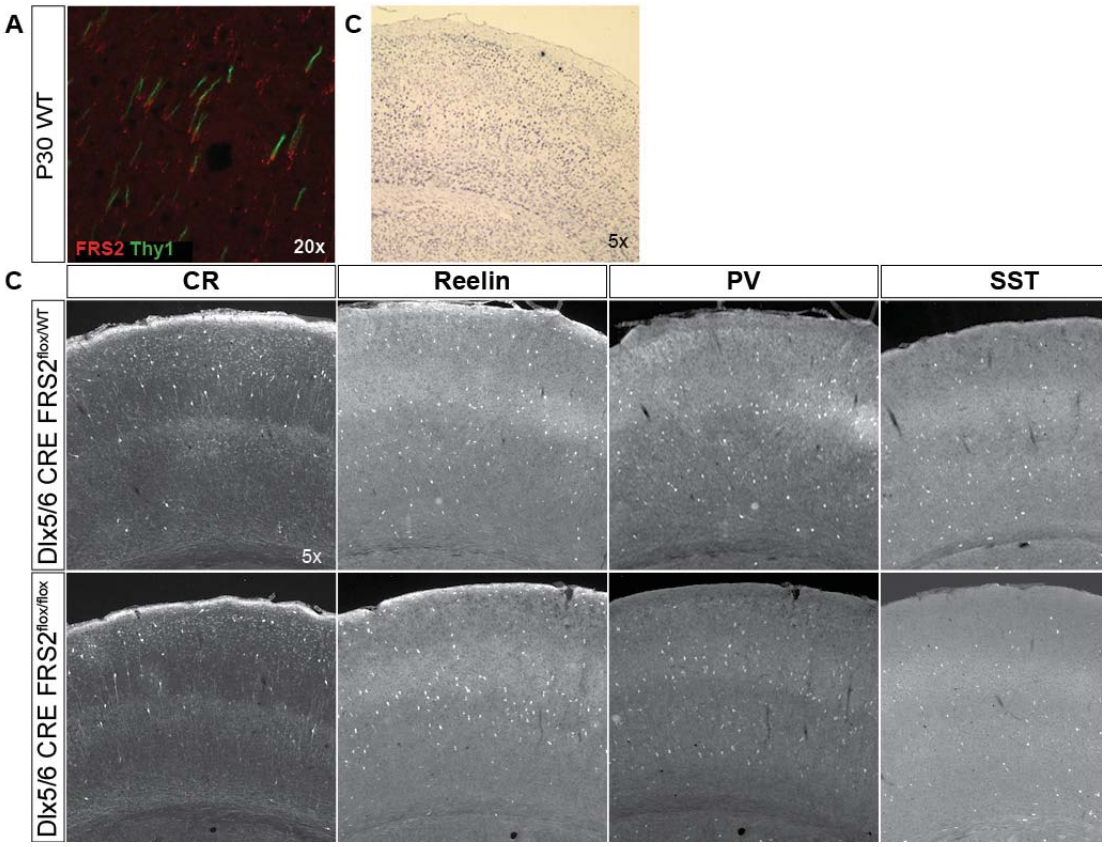

Figure 11 *FRS2α* is broadly expressed and interneuron specific knockout doesn't effect cortical interneuron specification

(A) WT FRS2α cortical protein (red) localization Thy1 transgenic (green) labels a subset of pyramidal neuron dendrites. (B) Widespread WT cortical *FRS2α* mRNA expression. Adult cortices (p21) of mutant and control mice immunostained for the interneuron markers, calretinin (CR), Reelin, Parvalbumim (PV) and Somatostatin (SST) show similar expression patterns.

Loss of FGF receptors

With an aim to avoid difficult genetic crosses and to knock out FGF in a more broad way than can be accomplished by targeting downstream effectors, we designed additional strategies to assess directly the role of FGF in interneuron development. We have employed *in utero* electroporation to specifically eliminate FGF signaling in both interneuron progenitors and postmitotic cells in the process of migrating to the cortex. By using plasmid vectors encoding interneuron specific *CRE* drivers in embryos homozygous for floxed or null alleles for each of the three FGF receptors expressed in the brain, we hoped to eliminate the problem of redundancy in FGF signaling and bypass the difficulty of multiallelic genetic crossing schemes to assess the cell autonomous role of FGF in postmitotic cortical interneurons. After over 10 electroporations of triple floxed homozygous timed pregnant females, (approximately 60 embryos) we were unable to obtain any electroporated triple mutants. The technical difficulty of this experiment is complicated by the necessity of crossing the mice as heterozygotes for the *FGFR3* receptor null mutation; so only 25% of the electroporated embryos are homozygous mutant. Mutant embryos may also be more sensitive to the *in utero* manipulation procedure, though many more electroporations would be required to test this hypothesis.

Though we attempted to circumvent the tedious and challenging traditional crosses through the use of the tricks above, in the end, those crosses provide the most definitive verdict on the role of FGF in this system. We again used the *Dlx5/6 CRE* driver in conjunction with a reporter and a floxed allele for *FGFRs 1and2* on the *FGFR3* null background. After 96 offspring from 9 litters, the cross yielded one mutant which lived to p21 (when it was euthanized for analysis). Staining for interneuron markers in this mutant revealed a similar pattern of staining to heterozygous and wild-type controls (Figure 13). Single and double conditional *FGFR* mutants similarly showed no phenotype (data not shown). Additionally, chi-square analysis of the offspring indicated that the compound mutants were each born in the expected Mendelian ratios (Figure 12).

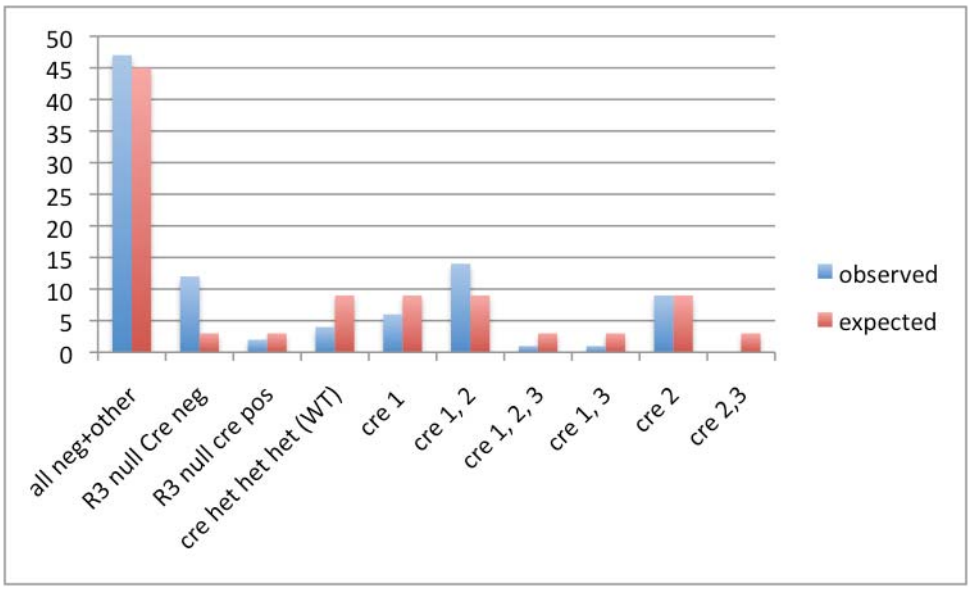

Figure 12 *FGFR* triple knockout is produced in roughly Medelian ratios

Expected (based on total offspring) and observed numbers for each genotype from the productive cross. Chi square p >.05.

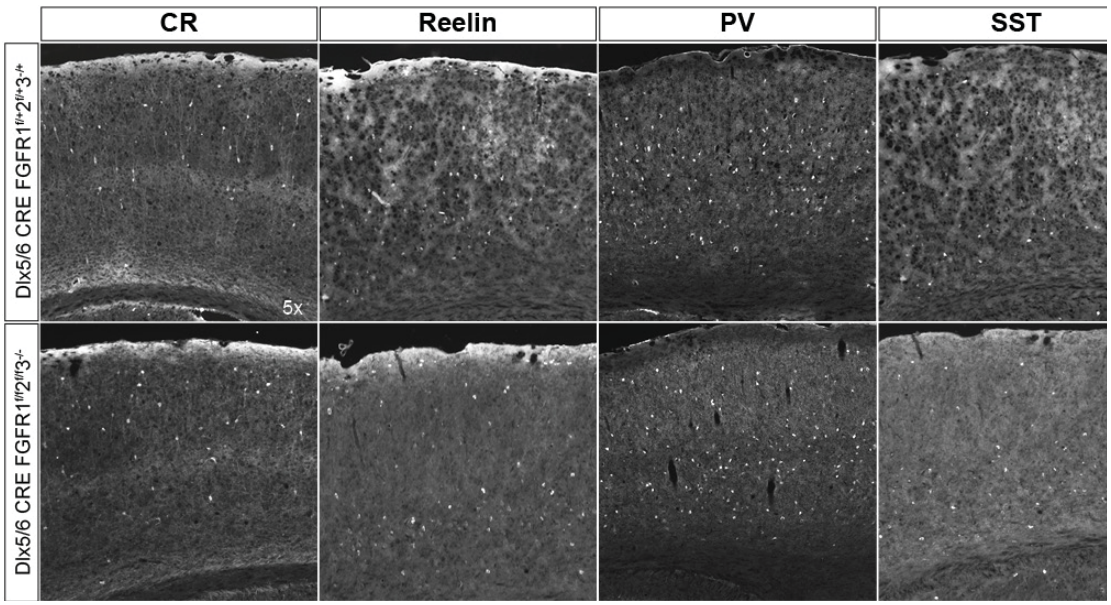

Figure 13 Interneuron specific *FGFR* triple knockout has normal interneuron specification

Interneuron marker expression was roughly normal in p21 mutant cortex

Discussion:

Our study has aimed to disrupt a number of arms of FGF signaling in postmitotic interneurons, from the receptor level, to signaling intermediates and downstream effector molecules. Regardless of the manipulation, cortical interneuron specification remained largely normal despite perturbed FGF signaling. This is in marked contrast to the essential role for FGF signaling in the establishment and survival of interneuron progenitor zones (Gutin et al., 2006; Paek et al., 2009). Despite the ramification of FGF ligand complexity in the developing telencephalon, it appears that FGF signaling influences on interneuron specification are largely restricted to the pre-mitotic neural progenitor. As will be discussed below, this leaves open the possibility for FGF signaling to have other effects on interneuron maturation and development that our analysis is blind to.

Functional redundancy

There are a number of technical challenges in studying FGF signaling, not the least of which is the problem of functional redundancy. As mentioned above, there are numerous FGF ligands with similar structure and binding affinities. Many of these ligands are expressed in the nervous

system, and single *FGF* mutants rarely show a significant phenotype (Itoh and Ornitz, 2011). This is also true of FGF receptors, that are, by comparison, not as numerous (a total of four). Though only three receptors are expressed in the nervous system; those three can similarly compensate for one another. Removal of all three receptors with the pan-telencephalic *CRE* driver *FOXG1 CRE* results in a complete loss of the telencephalon, suggesting that FGF signaling is absolutely required for the development and survival of the progenitors of this structure. However, single or double mutants of any individual or pair of receptors results only in a decrease in the telencephalon's overall size, and no specific loss of a particular substructure. It is the latter point that is most suggestive of compensation between the different receptors. FGF receptors have regionally specific expression patterns in the forebrain. It seems parsimonious that each FGF receptor would have a particular 'specialty' and responsibility in the development of the telencephalon. Obviously, that the loss of a single receptor doesn't result in the catastrophic failure of its specialty region argues against this common sense assumption. However, the combined evidence of multiple different combinations of FGF signaling knockouts in a patterning context indicate that there are regions of differential sensitivity to FGF signaling loss and manipulation; anterior ventral pallium is the most sensitive and posterior dorsal is the least (Gutin et al., 2006; Storm, 2006).

Again, this notion is consistent with the varied expression of different FGF ligands - which also are able to compensate for one another, and each have a very specific distribution within the brain during development. Their ability to compensate for one another might lead one to believe that one FGF is equivalent to another. If so, what is the purpose of the highly specific expression patterns of each of these ligands? One simple explanation is that the early development of the organism is a highly important evolutionary event, and is thus heavily selected for. More than any other later biological event, save, perhaps reproduction, formation of the embryo is critical to be considered “the fittest”. One might imagine a better-something-than-nothing approach to brain development could be advantageous. Perhaps they are able to replace one another to perform the most basic, essential requirements of the cells to create a serviceable entity, but are unable to compensate for more subtle functions. As a substrate for evolution, a brain development strategy with this sort of flexibility is optimal. How much individual variety could be generated by introducing changes in broad signaling factors that, because of functional redundancy, are unlikely to devastate the developmental process? Whether each of these receptor/ligand pairs is entirely equivalent will require a very sensitive analysis that can detect even subtle defects.

Restricted Analysis

The Paek *et al* study, looked simply at the survival of cells, and we have looked only at the expression of basic cell markers, density and localization. There are many other levels of specification and differentiation that we have not assessed in this study. In the hippocampus, specific FGF ligands are able to guide either the development of excitatory or inhibitory synapses (Terauchi et al., 2010). It is entirely possible that all of the mutants we have generated above had a subtler defect that was invisible to our methods. The localization of FRS2α to the axons of pyramidal neurons suggests a potential synaptic role for FGF signaling. To this end, we recorded miniature IPSCs in pyramidal neurons in *DLX5/6 CRE FRS2*$^{f/f}$ cortical slices. Consistently, our preliminary results found a trend toward a decrease in miniature IPSC frequency, suggesting a change in synapse number (data not shown).

Timing

In this study we have focused our efforts on the postmitotic effects of FGF signaling on cortical interneuron specification. However, there is abundant evidence suggesting that much of an interneuron's identity is specified before its final mitosis (Butt et al., 2005; Inan et al., 2012; Miyoshi et al., 2010; Nery et al., 2002; Wichterle et al., 2001). Our results are

consistent with the idea that much of an interneuron's identity is resistant to modification after becoming postmitotic. Indeed, most of the genetic manipulations performed were dependent on the postmitotic *CRE* driver *Dlx5/6 CRE.* Any version of our manipulations, from the most restricted removal of a single transcription factor to the broad removal of three FGF receptors in postmitotic interneurons showed no effect on interneuron specification. A removal of FGF signaling in cortical interneuron progenitors might have had a more significant effect. However, the challenge would be to disentangle survival and patterning effects from specification. It is already well known that subpallial progenitors depend on FGF signaling for their survival (Gutin et al., 2006; Paek et al., 2009; Storm, 2006). Unfortunately, progenitor specific CRE drivers like the MGE specific *Nkx2.1* are also expressed early during development, making any manipulations also necessarily effect patterning and progenitor survival as well. Though survival would still be an issue, the best way to circumvent these challenges with current technology will be mosaic, temporally controlled removals, as with virally encoded *CREs* or electroporation. We attempted the latter, though the difficulty of doing embryonic manipulations in complex genetic (and partially mutant) backgrounds proved to be prohibitive. Even electroporation will not eliminate the survival issue, leaving open the possibility that no electroporated cells will ever make it to the cortex.

Another problem with any manipulation in a complex genetic background is that of incomplete removal. Independent of the timing of the *CRE* expression, there is a significant chance that not all of the alleles will be promptly removed concurrent with the *CRE* expression, not to mention any produrance of remaining protein. The best technological development that could help address these issues would be a late-progenitor driver that is active in progenitors as they transition from a cycling stage to producing a daughter cell combined with a quick-acting dominant negative. Traditional dominant negative receptors are problematic due to their requirement for a significant accumulation of properly trafficked 'dummy receptors' to out-compete endogenous receptors.

While this study suggests that FGF signaling has little role in interneuron specification after mitosis, due to the aforementioned caveats, this is far from definitive. Future studies in this field will benefit from an increasing availability of specific *CRE* drivers and viral techniques to continue to unravel the function of FGF signaling throughout development.

A Novel Non-Canonical WNT gradient specifies cortical interneuron subtypes produced in the Medial Ganglionic Eminence

Melissa McKenzie Chang, Gord Fishell and Edmund Au

Contributions: EA cloned the *RykICD* construct; MMC and EA performed all other experiments co-operatively.

Abstract

Cortical GABAergic interneurons display an incredible range of phenotypes in their molecular marker expression, layer organization, cellular connectivity pattern and morphology. The means through which this diversity is achieved is still largely unknown. Because of their numerous and divergent roles in circuits, interneuron malfunction has been implicated in many neurologic and psychiatric disorders including epilepsy, schizophrenia, and autism. Using ultrasound guided *in utero* transplantation, we have discovered a novel rostral-caudal WNT gradient within the medial ganglionic eminence (MGE) that delineates the specification of the two main classes of cortical interneuron subtypes. Caudally located MGE progenitors receiving high levels of WNT signalling give rise to cortical interneurons labeled by somatostatin (SST). Parvalbumin expressing basket cells, by contrast, originate mostly from the

most rostral region of the MGE, and do not signal highly through WNT pathways. Interestingly, canonical WNT signaling through *β-catenin* is not required for this process. WNT signals transmitted via cleavage of the intracellular domain of the non-canonical receptor RYK, however, are sufficient to drive interneuron progenitors to a *SST* fate.

Introduction

From the very first divisions of embryogenesis, identically potent progenitors must differentiate and produce a variety of cell types. In many cases, the actions of secreted growth factors generate unique gradients across these progenitor zones to instruct and guide cell fate decisions. The generation of diverse neuronal cell types during development is of paramount importance in the brain. Cortical GABAergic interneurons (cINs) display an incredible range of phenotypes in their molecular marker expression, layer organization, cellular connectivity pattern and morphology, among other characteristics (Defelipe et al., 2013; Fishell and Rudy, 2011). Interneurons play divergent roles in circuits and perhaps because of this variety, their malfunction has been implicated in numerous neurologic and psychiatric disorders including epilepsy, schizophrenia, and autism (Chao et al., 2010; Gabis et al., 2005; Lewis, 2013; Marín, 2012; Stoner et al., 2014). Previous work in the field has shown that many

aspects of an interneuron's identity are determined by the place and time of its final mitosis (Butt et al., 2005; Inan et al., 2012; Miyoshi et al., 2010; Nery et al., 2002; Taniguchi et al., 2013; Wichterle et al., 2001).

The majority of interneurons (approximately 60%) arise from the medial ganglionic eminence (MGE) and become parvalbumin (PV) positive basket cells or somatostatin (SST) positive Martinotti cells (Miyoshi et al., 2007). Other interneuron cell types, positive for VIP, CR and Reelin molecular markers arise from the caudal ganglionic eminence (CGE) (Ma et al., 2011; Miyoshi et al., 2010; Nery et al., 2002; Rudy et al., 2011). Additionally, a small proportion of interneurons are generated in the lateral ganglionic eminence (LGE) and the pre-optic area (POA)(Gelman et al., 2009; O'Leary and Borngasser, 2006). The MGE's production of PV and SST positive cells follows a temporal timecourse; early in development proportionally more SST cells are produced compared to PV cells, shifting more to a preponderance of PV as development proceeds (Miyoshi et al., 2007).

One strategy to produce a number of different cell types from a single field of progenitors is through a morphogen gradient. The relationship between secreted morphogens, gradients and the production of neurons is probably best represented in the temporally and spatially defined effects of sonic hedgehog protein (SHH) secreted from the floor plate on the

specification of different neuronal populations of the spinal cord (Briscoe and Ericson, 2001; Dessaud et al., 2007; Ericson et al., 1997; Roelink et al., 1995). Similarly, *Shh* is expressed in the most ventral region of the telencephalon and, in coordination with fibroblast growth factor (FGF) originating from the anterior neural ridge, is critical for inducing the expression of Nkx2.1 and the MGE (Gutin et al., 2006; Shimamura and Rubenstein, 1997). This knowledge has led to the hypothesis that cells arising from the MGE might obey SHH based regional gradient cues (Flames et al., 2007; Xu et al., 2010). However, despite all we know about this important relationship and it's similarities to the well known paradigm of SHH in the spinal cord, establishing a strong causal relationship between exposure to SHH and the specification of interneurons within the MGE has thus far proven difficult. Work in the field has discovered many examples of regional variability within the progenitor zone of the ventral eminences as well as biases in the cell types produced within these subdomains (Flames et al., 2007; Inan et al., 2012; Puelles and Rubenstein, 2003; Rubenstein et al., 1994; Taniguchi et al., 2013; Wonders et al., 2008). Despite this, a clear gradient-based rule underlying the difference between the production of PV or SST cells from the MGE remains elusive.

The observation of regional differences in the cells produced in the progenitor zone of the MGE, suggested a role for gradients in the

generation of cellular diversity there. This coupled with the observation that WNT is important for the production of MGE interneurons (Gulacsi and Anderson, 2008), led us to investigate a potential WNT based gradient. In fact, the graded expression of the WNT responsive transcription factor TCF4 was particularly suggestive of a previously uninvestigated gradient oriented rostro-caudally. WNT ligands are expressed at the hem and in the developing thalamus, both adjacent or abutting the caudal MGE. To investigate whether this gradient is important for the specification of cortical interneuron cell types we used ultrasound guided *in utero* transplantation experiments combined with careful dissection along the putative axis to show that, indeed, this axis is can delineate regions producing PV and SST expressing interneurons (rostral and caudal respectively). Fate-mapping cells signaling highly through WNT using a transgenic WNT reporter mouse (Ferrer-Vaquer et al., 2010) combined with transplantation techniques recapitulated a caudal MGE fate and conversely inhibiting WNT through a small molecule inhibitor of WNT signaling was able to shift cells away from a caudal (SST) fate.

Interestingly, we found that removal of β-catenin thereby disrupting canonical WNT signaling pathway in transplanted interneurons did not greatly influence cell fate decisions. Instead, we found differential levels of non-canonical WNT signaling through the RYK receptor between rostral

and caudal MGE. Further, blocking the activity of the non-canonical WNT receptor RYK disrupted SST production and biased toward PV production. Transplanted RYK null MGEs similarly produced fewer SST cells, as well as a large number of non-PV, non-SST expressing neurons. Dominant active forms of the cleaved intracellular domain of the RYK receptor were able to bias mouse embryonic stem cells (ES cells) toward a SST fate.

We therefore propose, for the first time, a rostral-caudal oriented WNT gradient that signals through the non-canonical RYK receptor to specify SST or PV fates derived from the MGE.

Materials and Methods

Protein Analysis

Tissue samples for protein analysis were dissected into rMGE, cMGE or wMGE fractions as described in Rostral/caudal MGE dissections, and processed as below. For cell cultures protein analysis, Embryoid bodies were collected at 11-13 days after differentiation, rinsed with PBS and resuspended in 100 µl of lysis buffer (95mM NaCl, 25mM Tris-HCl pH 7.5, 10mM EDTA and 2% SDS, final pH=8.0 including protease inhibitor mix (cOmplete Ultra Roche)), and homogenized with a brief pulse using an ultrasonicator with microtip (Misonix S-4000, amplitude=1, 5s pulse). Samples destined for fractionation were processed as described (Suzuki et

al., 2010). Protein samples were quantified and lanes to be compared loaded with equal amounts of total protein. Blots were probed using an antibody mix and both channels imaged simultaneously with fluorescent secondary antibodies (Li-Cor). The following primary antibodies were used: Rabbit monoclonal anti Ryk (AbCam, 1:50,000), Rabbit anti-Ryk (Thermo 1:1000), mouse anti Cyclophilin A (Abcam 1µg/ml), Rb anti-histone H3 (Cell Signaling, 1:2000), mouse anti β-actin (1:2000). Blots were imaged using Odyssey CLx Infared Imager and analyzed using ImageStudio software (LiCor). Protein fluorescent signal was normalized to beta actin loading control intensity in whole cell or cytoplasmic fractions, and to histone in nuclear fractions.

Immunohistochemistry

Embryos aged e15.5 or later and adult mice were transcardially perfused with 4% paraformaldehyde in PBS (4%PFA). Tissue was prepared for either cryosectioning and slide staining or vibratome sectioning followed by free-floating immunohistochemistry. For cryosectioning, brains were dissected and post-fixed in 4%PFA one hour at 4°C, then cryoprotected in 30% sucrose W/V in PBS overnight at 4°C. Prepared brains were mounted in Tissue-Tek (Takara) frozen at -80°C for storage until cryo-sectioned at -20°C in 12µm sections for embryonic tissue and 20µm sections for adult tissue. Brains for cryosectioning were transcardially perfused as above and

post-fixed in 4%PFA overnight at 4°C. Brains were embedded in 4% low melt agarose in PBS and sectioned to 50μm on a Leica VT1000S vibratome. Sections were stored in a propylene glycol:glycerol:PBS solution (3:3:4) at -20°C until used. For immunohistochemistry, cryosections were allowed to dry for 1-2 hours then frozen at -20°C until they were used. Frozen sections were defrosted and dried at room temperature for 1 hour then rehydrated in blocking solution (4% normal serum, 0.1% TritonX-100 in PBS) for 1-4 hours. Primary antibody incubation followed in blocking solution overnight at 4°C. Primary antibodies were used at the following concentrations: Rat anti-SST (Millipore 1:500), mouse monoclonal anti-Parvalbumin (Sigma 1:1000). Sections were washed repeatedly in PBS then incubated in secondary antibodies conjugated to Alexafluor 488, 594, or 647, host matching serum used for blocking, for 45 minutes at room temperature. Free-floating sections were incubated in blocking solution (10% serum, 0.3% TritonX-100 in PBS) overnight at 4°C then incubated in primary antibody overnight at 4°C using the antibodies listed above. Free-floating washing steps in PBS were performed overnight or for 4 hours at room temperature. When nuclear labeling was desired, DAPI was applied after secondary antibody at 1:10,000 in PBS for 5 minutes. Slides (free-floating and cryosectioned) were promptly mounted with Fluoromount-G (Southern Biotech) and coverslipped.

Cell Counts and statistical analysis

Transplanted cells in cortical areas from sections containing the hippocampus were identified by their expression of the appropriate ubiquitous label (PKH, eGFP or TdTomato) and assessed for their co-expression of SST or PV. Cells were categorized as SST+, PV+ or 'unidentified'. Slides with unidentified %'s reaching above 30% due to poor staining or tissue quality were discounted. A minimum of 200 cells were counted per brain, from areas distributed across multiple anterior-posterior sections. Ratios of PV or SST were calculated out of the 'indentified' cells (PV+ or SST+). Ratios in each brain were considered a single n, and compared across conditions through un-paired student's t-test in GraphPad prism software.

Ultrasound guided in utero *transplantation*

Ultrasound guided *in utero* transplantation was performed as previously described except where noted below (Au et al., 2013; Liu et al., 1998; Nery et al., 2002; Wichterle et al., 2001). For transplantation of *β-catenin* mutant, plugs were generated from a cross of *Nkx2.1 CRE*; *β-catenin*$^{f/+}$ males with *β-catenin*$^{f/f}$*; RCE*$^{f/f}$ females. Homozygous mutant embryos were identified after dissection by midline fusion at the base of the MGE, and tissue samples from the embryo were retained for post-hoc genotyping confirmation. Mutant MGE's were dissected and pooled for transplants as

above. Ryk$^{-/-}$ embryos could not be identified visually, so pregnant females were deeply anethestized, and the uterus gently cut to reveal the amniotic sacs. Tail samples were carefully removed from each embryo and rapidly processed for PCR genotyping (as described (Halford et al., 2000)), leaving the embryos in place. MGEs were dissected from the identified mutant embryos and processed for transplantation. E13.5 wild-type, unlabeled MGE or LGE tissue was mixed with mutant MGE and used as carrier when little mutant tissue (2 MGEs- 1 null embryo) was available for transplantation.

IWP2

Timed pregnant Swiss Webster females mated to homozygous Ai9 germline recombined males were deeply anesthetized with isofluorane and an incision was made to expose the uterus. IWP2 (sigma) was dissolved in DMSO and frontloaded into a beveled glass pipette (as above). 25nls were injected into the ventricles of e12.5 Swiss Webster embryos, and the uterus was replaced and the incision stitched, allowing the pups to be born normally or sacrificed 1 day later for MGE collection and transplantation or analysis.

Rostral/caudal MGE dissections

Animals expressing a ubiquitous fluorescent marker were generated by crossing either *Ai9* (Madisen et al., 2010) or *RCE* (Sousa et al., 2009)

reporter mice with the germline *CRE* driver *TK CRE* (Bai et al., 2002). Pilot experiments were performed using unlabeled SW embryos, dissected MGEs were labeled using a PKH26 Red Fluorescent Cell Linker Mini Kit for General Cell Membrane Labeling (Sigma) following manufacturer's instructions. Ubiquitous fluorescent males were crossed with SW females, and pregnant females were collected at e12.5, morning of vaginal plug discovery counted as e0.5. Embryos were collected and brains were dissected into ice-cold PBS. Cortices were removed and rostral MGE or cMGE was dissected and treated as previously described (Butt et al., 2005).

TCF4 fate-mapping

Transgenic embryos from timed pregnant females were identified under a fluorescent macroscope. Ai9 positive, eGFP negative MGEs and positive MGEs were collected and processed for FACS sorting. Negative MGEs were used for gating (Figure 22). eGFP; Ai9 positive MGEs were dissociated and prepared for FACS sorting following normal cell dissociation method used for transplantation as described above. Cells were sorted according to expression of eGFP using an iCyt reflection sorter with a 100uM nozzle by the NYU Cytometry and Cell sorting core facility (supported by Perlmutter Cancer Center grant P30CA016087). Collected

cells (approximately 9% of cells sorted- Figure 22) were then transplanted into e13 embryos as above.

Cell culture

Mouse ES cells were maintained using standard protocols (Joyner, 2000). Low passage cell lines were differentiated using protocols described previously (Au et al., 2013; Watanabe et al., 2005). Briefly, ES cells in late log phase growth were dissociated into single cells and plated into non-TC treated wells at a density of 35 000 cells/well in order to establish floating embryoid body (EB) cultures. EBs were successively treated with Dikkopf-1 (2.5 ng/ml), and Sonic Hedgehog (40 ng/ml) for 11-13 days, after which they were gently dissociated (Accutase (Invitrogen), 15', 37°C) for subsequent experiments. The *RykICD* was generated by PCR using full length human *Ryk* cDNA with a C-terminus myc tag (provided by Stephen Stacker, Peter MacCallum Cancer Centre, Melbourne, Australia) as a template and cloning a nuclear localization signal in frame at the N-terminus (Lyu et al., 2008). This construct was introduced into a bi-directional tet-responsive element driving Dlx2 in one direction and RykICD in the other (Au et al., 2013). This assembly was then nucleiofected into a Dlx6a-cre; Ai9 reporter ES cell line along with Nestin-Nkx2-1-IRES-tTA (Lonza). Individual clones were isolated, expanded, genotyped and verified by *in vitro* differentiation.

Animal husbandry

All animal handling and maintenance were performed according to the regulations of the Institutional Animal Care and Use Committee of the NYU School of Medicine. The following lines were maintained on a mixed background with Swiss Webster (SW- Taconic) and genotyped as previously described: *NKX2.1 CRE*(Xu et al., 2008), *Ryk* (Halford et al., 2000), *β-catenin* (Brault et al., 2001), *WNT reporter* (Ferrer-Vaquer et al., 2010), *Ai9* (Madisen et al., 2010), *RCE* (Sousa et al., 2009),*TK CRE*(Bai et al., 2002), *RCE* (Sousa et al., 2009). Wild-type, Swiss Webster timed pregnant females for transplant recipients, IWP2 or RYK function blocking antibodies were ordered from Taconic. All embryonic time points were counted from discovery of vaginal plug (e0.5).

Results

WNT is oriented rostro-caudally across the MGE

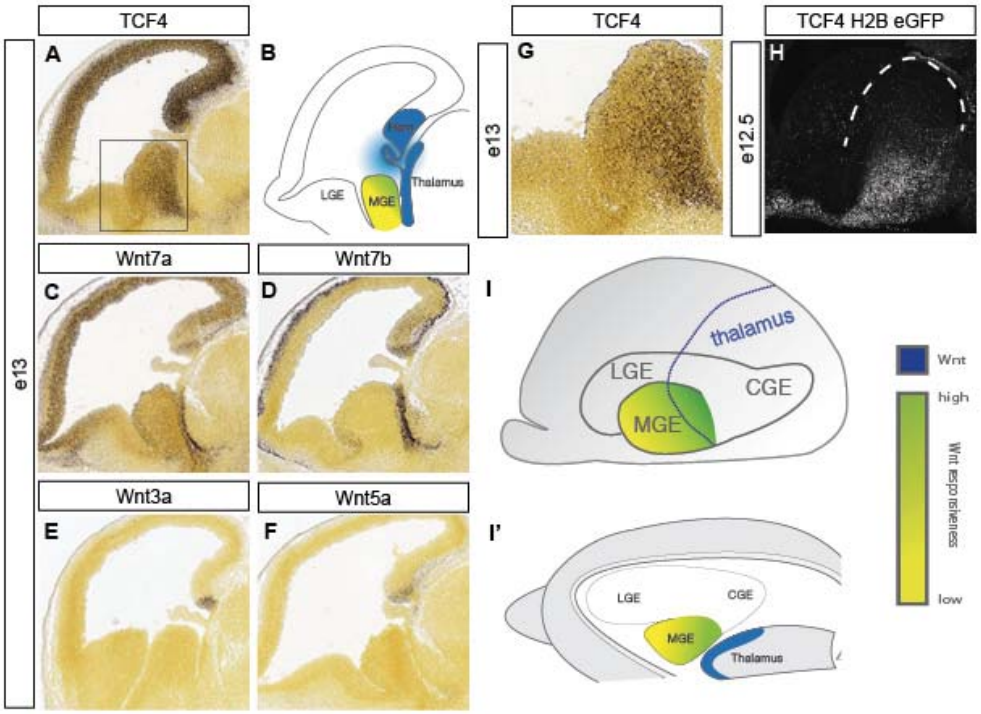

Figure 14 WNT **signaling is oriented in a caudal-rostral gradient across the MGE**

(A) Parasaggittal section through the mouse brain at e13, WNT responsive transcription factor *Tcf4* expression is enriched in the caudal MGE. (B) Scheme of a parasaggital brain section summarizing *Wnt* expression (blue) in the hem and developing thalamus as it influences WNT signaling levels (green) in the MGE (yellow). (C,D) *Wnt7a* and *7b* are expressed in the prethalamic eminence and (E,F) *Wnt3a*, and *5a* are expressed in the

cortical hem. (G) Close up of caudally enriched *Tcf4* expression in the MGE (box in A) as mirrored by *Wnt reporter* eGFP expression (H), MGE denoted by dashed line. (I) Scheme denoting relationship between *Wnt* expression (blue), MGE (yellow) and TCF (green) in view of ganglionic eminences from the midline, and (I') in horizontal section. (A,C,D,E,F,G) *in situ* hybridization data (Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org.)

Significant advancement has been made in the availability of gene expression data in the central nervous system to the general public via online databases such as the Allen Brain Atlas (Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas [Internet]. Available from: http://developingmouse.brain-map.org.) Using this tool, we were able to identify a pattern of gene expression suggestive of a rostal-caudally oriented WNT gradient across the MGE. *Tcf4*, a transcription factor traditionally downstream of canonical WNT signaling is enriched in the caudal MGE (Figure 14) (reviewed in (Cadigan and Waterman, 2012)). A review of WNT ligands present in the developing embryo revealed a number of ligands are present in close proximity to the MGE. Of particular interest was *Wnt7a* and *Wnt7b*, both highly expressed in the superficial

stratum of the prethalamic eminence as well as in the hem, where *Wnt3a* and *Wnt5a* are also expressed (Figure 14). All of these regions are likely in intermittent or constant contact with the caudal aspect of the MGE, where we observe an enrichment of *Tcf4* expression. Interestingly, the ventricular zone along the rostral aspect of the MGE expresses the WNT antagonist *Sfrp1 (Secreted frizzled receptor protein1).* To further explore this observation, we made use of a WNT reporter mouse expressing histone bound eGFP under the control of general TCF/LEF promoter elements(Ferrer-Vaquer et al., 2010) (*WNT reporter*). Reporter activity was also enriched in the caudal aspect of the MGE (Figure 14). With this pattern as our guide, we hypothesized that WNT signaling could be differentially active in the MGE, generating a rostral-lateral high to caudal-medial low gradient (scheme in Figure 14).

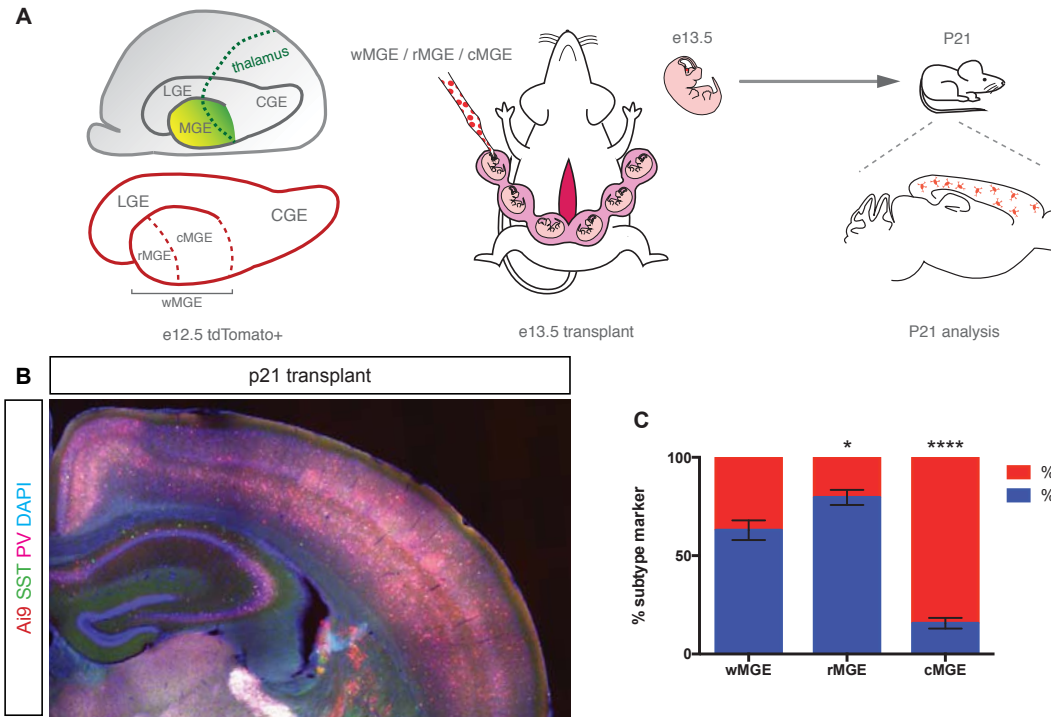

Figure 15 rMGE and cMGE give rise to PV-positive and SST-positive interneurons

(A) Experimental design. Ubiquitously labeled eminences were dissected into rostral, caudal or whole fractions based on their relationship to the hypothesized WNT gradient. Cells were dissociated and injected into unlabeled e13 host embryos. Host embryos were birthed normally and sacrificed at p21 for mature subtype analysis. (B) Stitched image of a transplant showing density and distribution of labeled interneurons. (C) %subtype marker out of identified cells n=4-7, p=.02, 0.0001

To test the hypothesis that this gradient could result in cell type production biases from these two regions, we developed an *in utero* transplantation scheme. We generated e12.5 embryos labeled ubiquitously with a fluorescent marker. Then, using our hypothesized gradient of WNT originating from the thalamus as a guide, we dissected either rostral MGE (rMGE) or caudal MGE for embryonic transplantation into e13.5 host embryos. To allow for the proper expression of mature interneuron markers we waited until host animals were p21 for analysis (scheme Figure 15). Compared to control transplants of the whole MGE (wMGE), rMGE and cMGE transplants were remarkably biased for PV and SST respectively (n=4-7, p value=.02, 0.0001, Figure 15).

Fate-mapped WNT responsive cells are biased toward SST production

We next aimed to determine if this rostral-PV caudal-SST bias was indeed based on a WNT gradient, as we hypothesized. To do this, we first devised a transplant scheme to allow us to fate-map those cells signaling highly through WNT. We first crossed the *WNT reporter* mice onto a ubiquitous Td-tomato background, and generated e12.5 embryos. We dissected wMGE and dissociated the cells for fluorescent cell sorting (FACS) to collect only those cells that are highly eGFP+, reflecting WNT reporter activity (Figure 22). Those cells, when transplanted into e13.5 hosts and analyzed at p21, as above, are enriched for SST expressing

interneurons (n=2-7, compared to wMGE p=.0003, student's unpaired t-test Figure 16 A, E). To further investigate the ability of WNT to affect the cell types produced by the MGE, we next employed the use of IWP2, a small molecule inhibitor of the membrane bound O-acyl transferase (MBOAT) protein Porcupine (PORCN) (Chen et al., 2009). Porcupine is essential for post-translational acylation and secretion of all secreted WNTs (Barrott et al., 2011; Proffitt and Virshup, 2012; Willert et al., 2003). Indeed, IWP2 injection into the ventricle of e12.5 *WNT reporter* mice was able to reduce eGFP reporter expression significantly 24 hours later (Figure 14). Perhaps as a testament to its effectiveness, IWP2 injection prior to e12 was remarkably lethal at even low doses. We reasoned that a reduction in WNT signaling would mimic an expansion of the low-WNT rostral MGE and result in a proportional decrease of SST cells. To test this, we injected the ventricles of fluorescently labeled mice with IWP2 at e12.5 and collected both MGEs for transplantation at e13.5 into e13.5 host embryos. When analyzed at p21, we found that a reduction in WNT signaling had significantly shifted the population produced toward a SST fate compared to wMGE transplants (n=2-7 p=.03 student's unpaired t-test Figure 16).

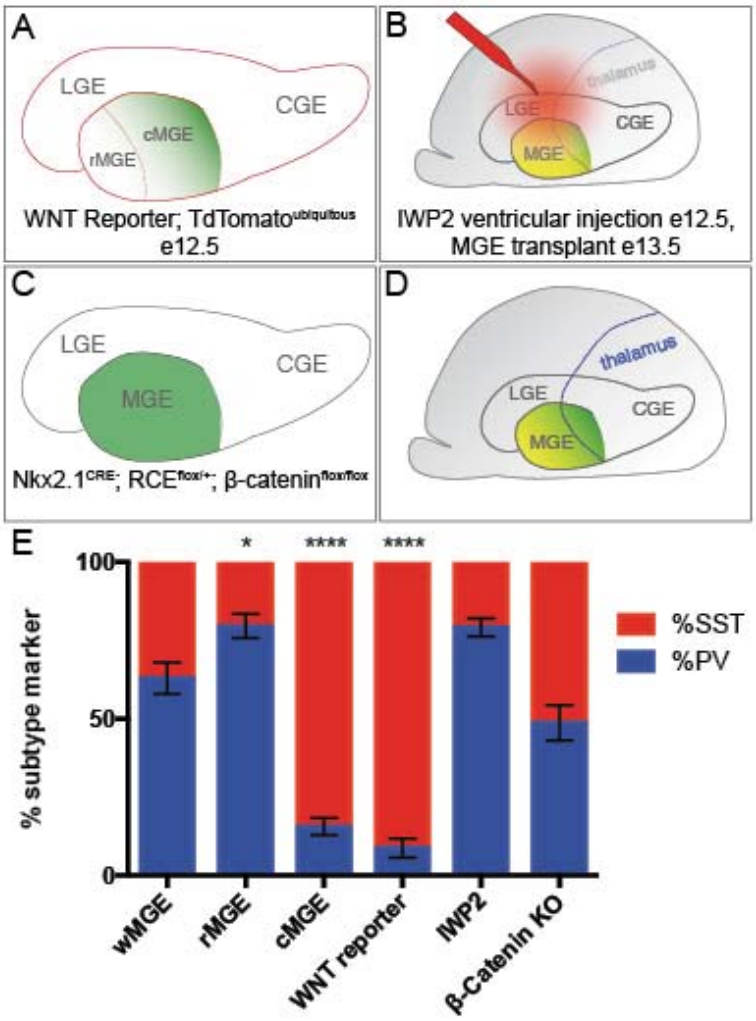

Figure 16 WNT signaling directs interneuron specification, but not through canonical pathways

(A-C) Scheme of transplanted tissue (A) *Ai9*^{ubiquitous};*WNT reporter* MGEs were dissected and eGFP positive cells collected by FACS (B) *Ai9*^{ubiquitous} embryos were injected with IWP2 at e12.5 and whole MGEs were transplanted at e13.5 (C) MGE specific knockouts of β-catenin were

generated and whole reporter labeled MGEs were transplanted. (D) reference scheme (E) %subtype marker out of identified cells. N=2-7, *WNT reporter* p=.0003 IWP2 p=.03, β-catenin=.1 student's unpaired t-test

Canonical WNT signaling doesn't influence cell fate

Canonical WNT signaling requires the intracellular signaling molecule β-catenin. Under basal conditions, β-catenin is sequestered in the cytoplasm and targeted for degradation by GSK3-β. In the presence of bound WNT ligand, Frizzled receptors recruit members of this destruction complex, de-repressing β-catenin and allowing its accumulation and translocation to the nucleus where it interacts with transcription factors such as the TCF/LEFs to modulate gene expression (reviewed in (Willert and Nusse, 2012)). Previous studies have shown that β-catenin is important for the proper formation of the MGE (Gulacsi and Anderson, 2008). Unfortunately, MGE specific knockout of β-catenin is late-embryonic lethal, preventing an accounting of mature cell types produced from the mutant MGE (Gulacsi and Anderson, 2008). To circumvent this lethality and to assess whether disrupting WNT signaling through β-catenin shifts cell type specification in the MGE, we generated e12.5 MGE specific knockouts of β-catenin for transplantation using a floxed β-catenin (Brault et al., 2001) and MGE specific *CRE* driver *Nkx2.1 CRE* labeled with the eGFP based CRE

reporter *RCE* (Sousa et al., 2009; Xu et al., 2008). Embryonic mutants were identified by a midline fusion defect in the MGEs, which were collected and transplanted into embryonic hosts. Interestingly, analysis of this population at p21 showed little change in the proportion of PV positive to SST positive cells compared to wMGE (n=2-7, p=.1 Figure 16). In fact, the PV production was more effected than SST in this population, leading us to consider the role of other, non-canonical WNT signaling pathways.

Non-Canonical WNT receptor RYK is preferentially active in cMGE

WNT can signal through a number of β-catenin independent pathways including the planar cell polarity (PCP) and convergent extension pathways (CE). Additionally, WNT can signal through a number of non-FZD receptors including RYK and ROR1 and 2 (Green et al., 2014). RYK drew our particular interest because, like β-catenin, RYK has been shown to have a role in cortical neurogenesis and in MGE cell production (Lyu et al., 2008; Zhong et al., 2011). RYK signaling in the cortex also can occur via the cleavage and nuclear translocation of the intracellular portion of the RYK receptor (Lyu et al., 2008). As with previous studies on the β-catenin knock-out, *Ryk* nulls don't survive post-natally, preventing an analysis of their interneuron marker expression. To investigate the possibility that RYK signaling might have a role in distinguishing rMGE from cMGE, we collected protein from pooled samples of rostral and caudal MGE for comparison.

The cleavage of the RYK receptor through γ-secretase interestingly appears to be independent of WNT signaling, though the translocation of the RYK intracellular domain (RYK ICD) is responsive to WNT activation. With this in mind, we separated the protein samples into whole, cytoplasmic and nuclear fractions. Interestingly, we found the RYK ICD to be enriched in the nuclear fraction of the cMGE compared to the rMGE (Figure 17); supporting the hypothesis that RYK ICD signaling downstream of WNT is highest in the cMGE.

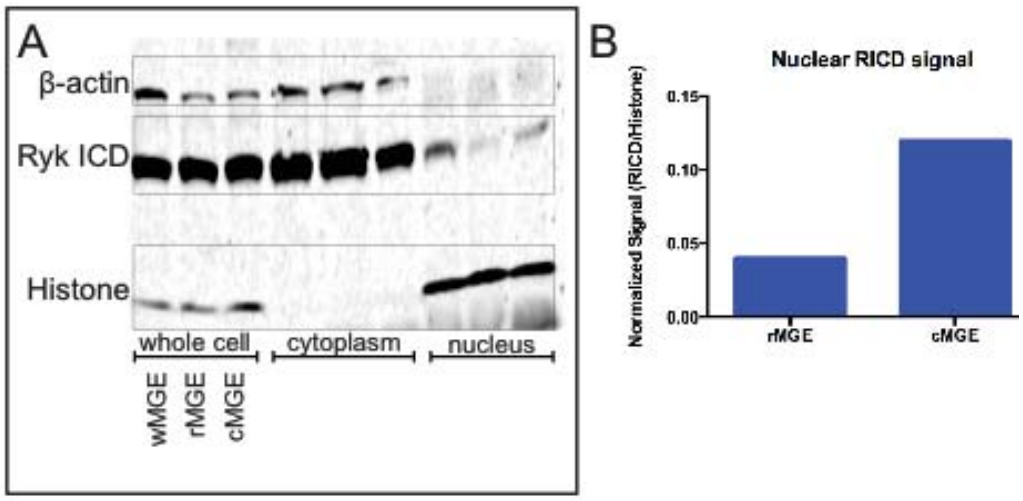

Figure 17 RYK ICD is enriched in the cMGE nucleus

(A) Western Blot of fractionated protein taken from wMGE, rMGE, and cMGE stained for RYK ICD with histone and β-actin controls. (B) Signal of RYK ICD in the rMGE and cMGE nuclear fractions, normalized to histone signal intensity.

Decrease of SST production with Ryk *loss*

As discussed above, RYK has been shown to decrease interneuron production and increase oligodendrocyte production in the MGE, however the mutants die post-natally, preventing an analysis of the mature interneuron subtypes produced from this population. To address this issue,

we again employed a transplantation scheme similar to that of the *β-catenin* loss of function. We crossed *Ryk*$^{+/-}$ animals onto a germline recombined Ai9 reporter: (ubiquitous) TdTomato background. We then crossed *Ryk*$^{+/-}$ *;Ai9*^{ubiquitous} and identified *Ryk*$^{-/-}$ embryos for dissection by PCR genotyping. We transplanted these cells into wild-type e13 host embryos for post-natal analysis. Interestingly, we found the loss of *Ryk* to greatly decrease the number of marker expressing cells in the transplanted group (from a maximum of 30% to 50%, n=2 Figure 18).

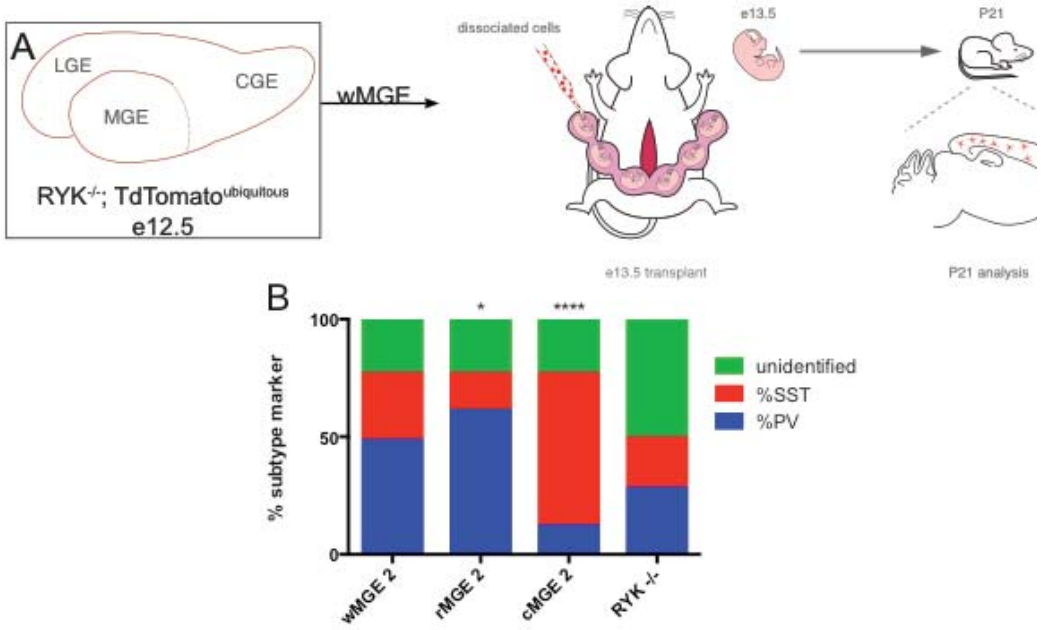

Figure 18 *Ryk*$^{-/-}$ interneurons lose substantial portion of mature marker expression

(A) Scheme of experimental design, e12.5 *Ryk*$^{-/-}$ *; Ai9* MGEs were transplanted into e13.5 recipients for post-natal analysis at p21. (B) preliminary results revealed a substantial decrease in subtype marker expressing cells (maximum of 30% to 50%) n=2 p value pending increased n.

Transplanted *Ryk*$^{-/-}$ cells may lack marker expression due to a later role of RYK signaling in interneuron specification or maturation. To partially address this concern we used a function-blocking antibody to perturb RYK function in the MGE while preserving later RYK functionality. We accomplished this by injecting the ventricles of e12 embryos with a human

function blocking antibody to the RYK extracellular domain (Halford et al., 2013). In this case, decreased SST with normal levels of PV would suggest a later role for RYK in PV cell maturation, whereas an increase in PV would suggest a fate switch. When analyzed at p21, animals treated with the function blocking antibody showed a 30% increase in PV and a 30% decrease in SST numbers in the cortex (Figure 19), suggesting RYK was able to induce a fate switch. Of course, these experiments cannot exclude the possibility of a later postmitotic role for RYK in PV cell maturation.

Given these data, we propose that RYK signaling, while not strictly necessary, is permissive and important for the production of SST interneurons.

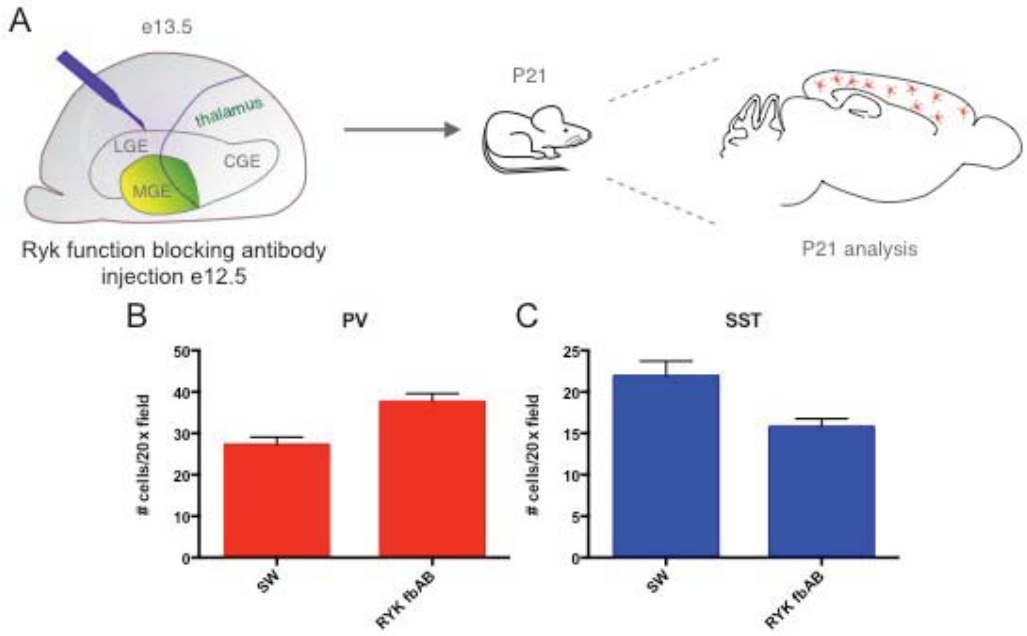

Figure 19 Blocking RYK activity causes a fate switch of SST cells to PV fate

(A) Experimental design. Preliminary results find that injection of RYK function blocking antibody increases PV cells by (B) ~30% and decreases (C) SST cells by ~30% n=2 p value pending increased n

Activated RYK gain-of-function in embryonic stem cells biases cells toward SST *fate*

RYK signaling appears to be permissive for the production of a large number of SST interneurons, but the question remains whether RYK signaling is sufficient to drive interneurons toward a SST fate. To address this, we made use of a recently published strategy to generate interneurons in large numbers in mouse embryonic stem cells (Au et al., 2013). An

additional advantage to this system is it's ready-made cloning site for gain of function testing of candidate genes. To apply this strategy to RYK signaling, we generated a RYK intracellular domain construct with a nuclear localization signal, as has been used previously (Lyu et al., 2008)(*RykICD*). This construct is able to act as a dominant active because the nuclear localization signal immediately shuttles the RYK ICD to the nucleus, allowing it to bypass the requirement of activation by WNT signaling, thus rendering it WNT independent and constitutively active. We introduced this construct into mouse embryonic stem reporter line (*Dlx6a-CRE; Ai9*) as well as a sequence of interneuron specification genes including *Nkx2.1* and *Dlx2.* This line also contains a *Nestin* promoter driving a tetracycline transactivator element (*tTA*). *RykICD* was placed downstream of a tetracycline responsive element (*TRE*). After the ES cells differentiate to neurons, the *Nestin* promoter becomes activated, causing tTA to accumulate in the cells, bind the *TRE,* and drive the expression of *RykICD*. ES cells grown as embryoid bodies under these conditions for 13days in vitro (DIV) generate about 6% cortical interneurons as measured by *Dlx5/6* reporter allele expression (Au et al., 2013). When these cells are dissociated and transplanted in e13 embryonic hosts, they behave as MGE transplants do by migrating to the cortex and integrating into the circuit (Au et al., 2013). Strikingly, gain of function of *RykICD* was able to drive ES

cells toward a SST fate (Figure 20), providing further support for our model that WNT activated RYK signaling through the RYK intracellular domain drives cells toward a SST and cMGE fate.

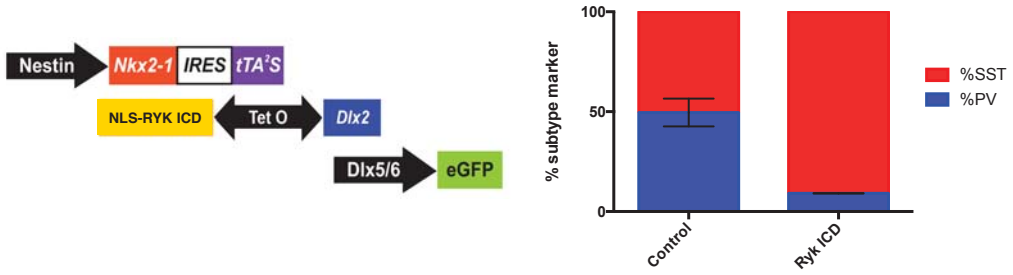

Figure 20 *RykICD* Gain of function in ES cells

Transgenic constructs introduced into mouse ES cells as has been shown previously (Au et al., 2013) with the addition of *RYKICD* fused to nuclear localization signal. Compared to ES cells without the *RYKICD,* the constructs drive cells to an increase in SST fate. n=2, p value pending increased n.

Discussion

During development, simple epithelial sheets of progenitor cells must generate thousands of neurons of subtypes in reliable ratios. Gradients of secreted morphogens are a classic strategy to achieve this goal. Cortical arealization and motor neuron generation in the spinal cord are good examples of this (Briscoe and Ericson, 2001; O'Leary et al., 2007). In this

study, we have uncovered a novel, non-canonical WNT based gradient responsible for generating different interneuron cell types in the subpallium. We observed that caudally located MGE cells closest to the thalamic eminence and hem expressing Wnt are enriched for the activated form of the non-canonical WNT receptor RYK

Is RYK acting as a morphogen gradient?

We've shown that RYK ICD gain of function is able to drive cells towards a SST fate. One important question remaining is if levels of RYK signaling have any functional repercussions in a graded, morphogen-like fashion. Transplant of *Ryk*$^{-/-}$ cells result in a decrease in all cell types expressing a mature subtype marker. This is consistent with a model in which RYK acts as a morphogen. Low levels of RYK signaling (RYK^{low}) direct cells to a PV fate, and high levels of RYK (RYK^{high}) yield SST. No RYK signaling at all generates problems in forming either cell type. One way to address this possibility is using our ES cell gain of function system, in which the *RykICD* is under the control of a Tetracycline (TET) responsive element. By repressing this TET element with doxycycline, we can titrate the levels of RYK gain of function along a continuum. Transplanting these cells would allow the determination of either an inflection point or a sliding scale of PV and SST production dependent on the amount of RYK signaling. An explant culture of MGE exposed to WNT soaked beads might

also allow a more careful analysis of the relationship between WNT, RYK, and MGE specification.

Our preliminary findings blocking RYK function by injecting a RYK antibody into the embryonic ventricle demonstrate an increase in PV and a decrease in SST. It's likely that this manipulation approximates a RYK hypomorph, while the cells are mitotically active, while sparing RYK function postmitotically. This diminished activity might bring more cells to a RYK^{low} level of signaling; thus the increase in PV we've observed is consistent with a morphogen model.

Another possibility that would account for our decrease in marker expressing cells in the *Ryk*$^{-/-}$ transplants is that RYK has a later role in regulating the maturation of PV cells. In this case, cells without RYK are theoretically specified, but do not express mature PV markers. Future experiments to address this might include the generation of *Ryk*$^{-/-}$ ES cells into which we could introduce a late expressing *Ryk* rescue construct. A rescue of PV expressing neurons would suggest a secondary role for RYK in PV maturation. Additionally, the electrophysiological characterization of unlabeled transplanted *Ryk*$^{-/-}$ cells in the cortex might allow us to identify immature fast-spiking cells that fail to express PV.

MGE Heterogeneity

Our discovery of a caudal-medial to rostro-lateral WNT gradient within the MGE reflects a novel way of considering spatial heterogeneity within the MGE. Previous studies have consistently found slight biases in both gene expression and cell type production within the MGE along the dorsal-ventral axis (Flames et al., 2007; Inan et al., 2012; Wonders et al., 2008). Our results fit well with these previous observations. If one takes a coronal section through the middle of the MGE, the dorsal aspect is part of our cMGE dissections. More ventrally localized progenitors, by contrast, are part of what we've termed rMGE. One explanation for less robust biases when looking along the dorsal ventral axis is the variability of this delineation, especially when coronal sections range along the anterior-posterior axis. Importantly, these findings highlight the importance of continuing to consider the subpallium in three dimensional space as it relates to the neighboring substructures.

The MGE gives rise to many cell types beyond PV and SST positive cortical interneuron populations, including cells destined for the striatum and globus pallidus (Flandin et al., 2010; Wichterle et al., 2001). Our analysis of cortically bound cells necessarily precluded any ventrally destined neurons or glia, though many cells do remain close to the site of injection well into mature stages. Previous studies have shown a role for

RYK signaling in determining the fate choice between cortical interneurons and oligodendrocytes. Though our mode of analysis is mostly blind to changes in oligodendrocyte production, it is possible that ventral fate choices are also intertwined in this RYK based WNT gradient. Future experiments focusing on ventral cell types either through transplants of smaller numbers of cells at different ages to facilitate the analysis of ventral cells, or through ES cell directed differentiation could further elucidate this relationship.

Taken together, these results greatly advance our understanding and appreciation of the processes that guide the generation of cellular diversity from the ventral pallium. Future experiments to shed light on the interaction of WNT with other signaling pathways and known transcriptional regulators will continue to untangle this complex and important process.

Supplemental figures

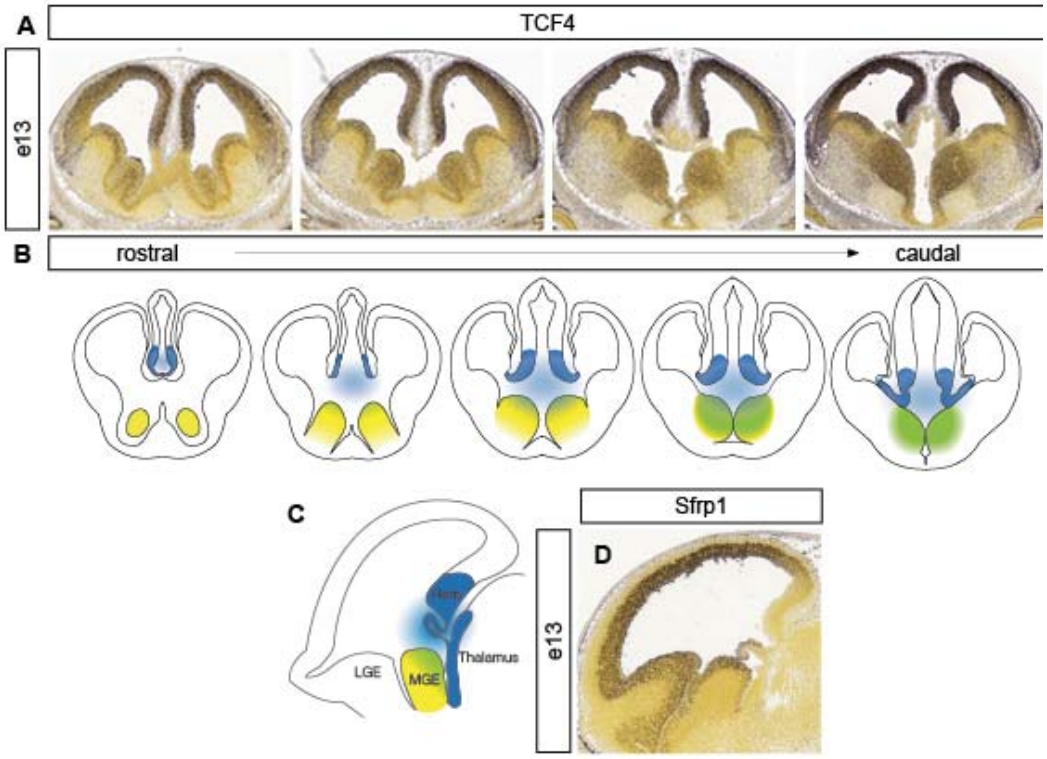

Figure 21 Supplemental TCF gradient

A) Serial coronal sections through the mouse brain at e13 showing *in situ* hybridization data of WNT responsive transcription factor *Tcf4* expression is enriched in the caudal MGE. (B) Scheme summarizing *Wnt* expression (blue) in the hem and developing thalamus as it influences WNT signaling levels (green) in the MGE (yellow). (C) Parasagittal scheme. (D) WNT antagonist *Sfrp1* is expressed in the rostral MGE VZ. *In situ* images (Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse

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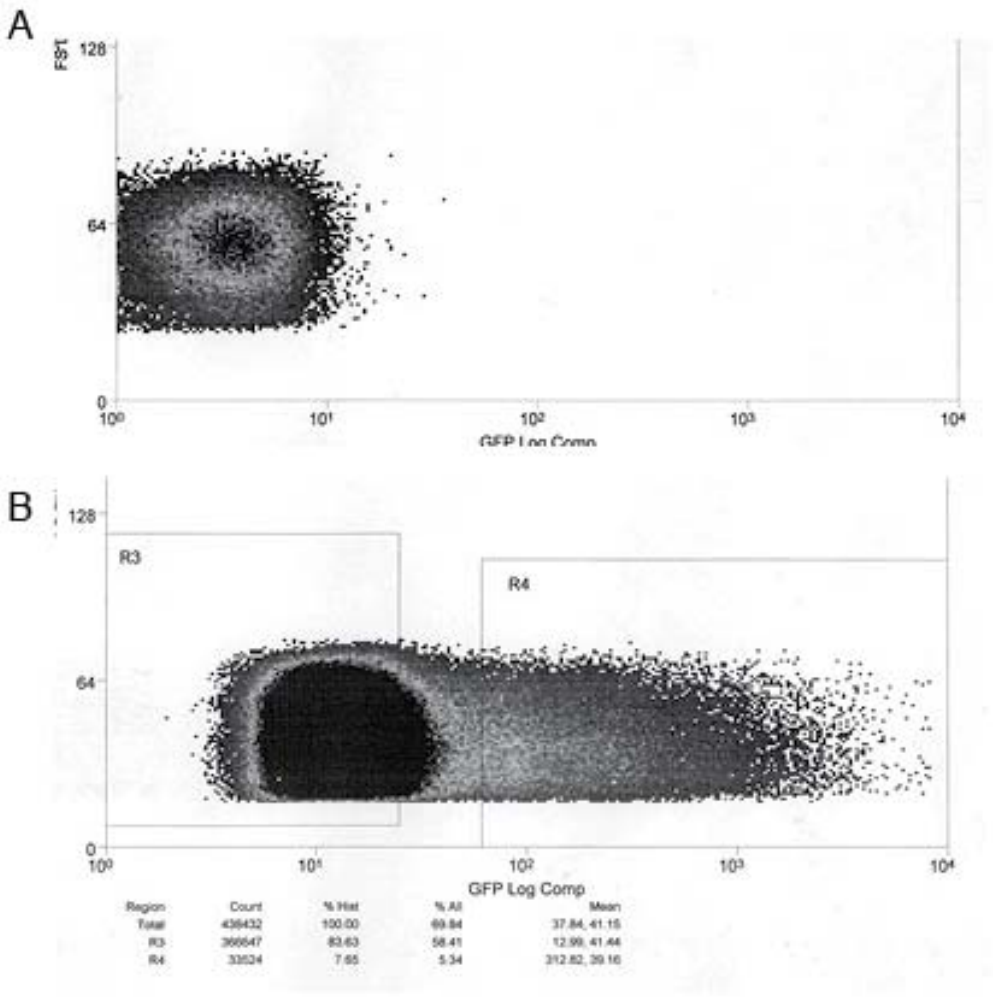

Figure 22 TCF Gating

FACS plot showing eGFP expression on the x-axis. (A) eGFP negative reference cells (B) whole MGE from WNT reporter mice. Left box (R3) is “negative” gate. R4 box is cells considered eGFP positive and selected for transplantation.

Discussion

Developmental processes must always be considered as events in time and space. Forebrain patterning signals are fairly well understood at early stages. In this work, I have aimed to investigate how the influence of the well-characterized patterning signals FGF and WNT evolve as the forebrain begins producing interneurons and it's three-dimensional landscape matures. I removed FGF signaling from interneurons through multiple avenues. Whether through the loss of downstream effector transcription factors *Ets1*/2, the signaling cascade mediating adaptor protein *FRS2*α or the broad removal of multiple *FGF receptors*, I found interneurons were not sensitive to FGF signaling loss after becoming postmitotic. This is in marked contrast with the dramatic effect of FGF signaling loss on forebrain progenitors shown in previous studies (Paek et al., 2009; Storm, 2006). Strikingly, with respect to WNT signaling, I discovered a novel WNT gradient that delineates the production of the two most prevalent interneuron subtypes. We have demonstrated that the non-canonical WNT receptor RYK promotes a somatostatin fate through the cleavage and translocation of its intracellular domain to the nucleus. This represents a significant advancement in our understanding of how interneuron diversity is generated. In the next section, I will discuss both

how these findings fit into what is already known about the interneuron specification and what open questions remain.

Time

At e8 in the mouse forebrain, FGF and WNT are inductive, survival, and proliferative factors. At this early timepoint, the brain is in the process of building progenitor zones. In this first stage, the primary task is to generate enough stem cells to meet the demand for mature neurons. Indeed, with early loss of many growth factors in the telencephalon, a prominent phenotype is reduction in size, reflecting the early importance of proliferation in establishing the stem cell niche (Chiang et al., 1996; Gulacsi and Anderson, 2008; Paek et al., 2009; 2011). Later, these progenitors must then produce a wide array of neurons to outfit circuits with the full complement of neural subtypes. As development proceeds, the effect of signaling molecules are refined to fit the changing needs of the tissue. For example, in the telencephalon, SHH signaling shifts from a patterning to a proliferative influence through the changing competence of the recipient cells ((Kohtz et al., 1998) reviewed in (Sousa and Fishell, 2010)). In the spinal cord, this later role includes cell specification as well (Briscoe and Ericson, 2001). My studies have focused on specific time points, well after the initial patterning of the telencephalon is established, through dissection

or by using *Dlx* based *CRE* drivers to remove or manipulate FGF or WNT at around e12.5 (Liu et al., 1997; Stenman et al., 2003).

FGF signaling is critical for the survival of forebrain progenitors at early stages, as evidenced by the drastic loss of forebrain in *FOXG1 CRE* removals of various *FGFR*s (Gutin et al., 2006; Paek et al., 2009). At these early stages, even loss of a single FGF ligand, FGF8, is particularly devastating (Storm, 2006). This is also true in the developing midbrain, where FGF8 is required for the expression of other growth factors and maintenance of progenitors (Chi et al., 2003). I found a lack of phenotype with a later removal of *FGFRs* or FGF signaling through *FRS2α* with *Dlx5/6 CRE*. Much could account for this difference, from simple technical difference between the efficiencies of *CRE* drivers to more biologically interesting explanations. In order to speculate and discuss these possibilities, let's assume both *Dlx5/6 CRE* and *FoxG1 CRE* are equally capable of removing each of the *FGFRs* or *FRS2α* alleles. All things being equal, the effect of FGF loss appears to become gradually less significant with time. This could be a reflection of the change in the types of cells that are present in the population at later ages, or it could reflect a more general change in the significance of FGF signals in all cell types. At early stages, the progenitor zone must first expand itself and hasn't yet assumed neuronal production as a major mode of cell division. Even a small change

in the proliferation rate of stem cells during the early period of exponential growth would severely impact the size of the entire tissue. Early stem cell proliferation eventually transitions to a mode of neuronal production shifting the predominant cell type from symmetrically dividing progenitors to a mix of stem cells and postmitotic neurons. Unfortunately, removal of *FGFRs* with *Dlx5/6 CRE* is a both a 'later' removal and targets postmitotic cells, preventing us from distinguishing between a general temporal effect from a stage specific or cell type specific one. As a result, one question that remains is whether progenitors continue to require FGF signaling as they shift from symmetric to asymmetric divisions. Our results suggest that postmitotic cells do not require FGF signaling for their survival or basic maturation. A removal of *FGFRs* in MGE specific progenitors, for example, would begin to address this question. FGF signaling is required in early progenitors to actively counter a pro-apoptotic signal downstream of TGFβ signaling (Paek et al., 2011), so the devastating effects of early FGF loss isn't simply due to an under-proliferation of progenitor cells. It isn't clear over what temporal window this active repression is important, but it will be interesting to understand when and how cells become insensitive to these signals. If TGFβ signaling is simply stopped, what positive influences of FGF remain in its absence? Alternatively, if FGF signaling is still important

for cell survival in MGE progenitors, what changes occur in postmitotic neurons to ensure their survival in the absence of FGF signaling?

Interestingly, WNT in the early embryo is also important for this same process- canonical WNT signaling through β-catenin promotes the FGF expression that inhibits the TGFβ apoptotic signal (Paek et al., 2011). Later, a knock-out of *β-catenin* specifically in the MGE progenitor zone leads to a severe reduction of the size of the MGE through a change in proliferation rates (Gulacsi and Anderson, 2008). This alone presents some evidence that the specific role of WNT has changed from its earlier promotion of FGF signaling and prevention of apoptosis, suggesting that MGE progenitors present a different context for the action of WNT and FGF signaling. Akin to our FGF results, postmitotic removal of *β-catenin* by *Dlx5/6 CRE* has no influence on interneuron numbers or fates in adult mice (Vitor Sousa, personal communication; (Gulacsi and Anderson, 2008)). SHH signaling is also important for the growth and proliferation of the MGE (Chiang et al., 1996; Fuccillo et al., 2004; Machold et al., 2003; Xu et al., 2005). If FGF is indeed important in the MGE progenitor zone, it will be interesting to see how FGF, WNT, SHH and other signaling pathways impinge on one another. In particular, the interaction of proliferation and cell type production will be interesting to elucidate, especially in light of our findings that WNT

signaling through a non-canonical pathway can confer distinct cell fates onto interneuron progenitor cells and will be discussed more in detail later.

Neurogenesis isn't limited to developmental stages, and many developmental pathways that regulate this process early on are reiterated at later stages. For example, work in the Fishell lab has shown an important role for SHH in maintaining the stem cell niche in the adult SVZ (Ahn and Joyner, 2005; Balordi and Fishell, 2007a; 2007b; Machold et al., 2003). In addition to a number of growth factors, FGF signaling is also present and important in the maintenance of adult neural progenitors (Frinchi et al., 2008; Vaccarino et al., 2001). The interplay between these signaling factors at this late time point is a fruitful avenue of research that remains relatively unexplored.

WNT gradient over time

Clearly, the role of multiple patterning molecules changes drastically over the course of development. This is most clear, as discussed above, in the shift from early, inductive steps to later maintenance and survival signals. We have discovered a novel WNT based gradient acting through a non-canonical pathway that is important for determining cell fate. For simplicity's sake, we focused our analysis of this process on the peak of MGE cell production, e12.5 (Miyoshi et al., 2007), however, some MGE derived interneurons are postmitotic as early as e9.5 or as late as

e17(Miyoshi et al., 2007; Picardo et al., 2011; Taniguchi et al., 2013). Cortical interneuron production in the MGE shifts slightly from predominantly SST to predominantly PV over time (Miyoshi et al., 2007). While we have only dissected the gradient at e12.5, an interesting question is whether RYK signaling is a driving force to produce SST cells early on as well, or if the competence of the MGE to respond to WNT via RYK changes over time. Gradients provide a convenient way to dynamically shift the production of cells temporally. There are a couple of ways our model of RYK signaling could account for the temporal changes in cells produced by the MGE.

One way is through a change in competence. Perhaps progenitor cells exposed to the WNT signal gradually downregulate their response to WNT. Thus, at first, the MGE progenitor zone is mostly responsive to WNT, and the first cells produced are mostly SST. As more and more cells lose their responsiveness to WNT, they produce more and more PV cells. This model, however, doesn't really explain why WNT responsiveness is oriented caudal to rostral in the MGE. This would require either a movement of cells rostrally as they age or one might expect a front of non-WNT responsive cells in the caudal MGE.

Another possibility is a simple physical explanation. WNT ligands undergo extensive post-translational modification are thus greatly

hydrophobic, thereby limiting the distance over which they can diffuse (Logan and Nusse, 2004). As a result, one could imagine a scenario in which the rostral portion of the MGE grows away from the caudally located WNT source in the thalamus. As the MGE grows, the rostral portion, i.e. the fraction of the MGE farthest from the WNT source is increased disproportionally to the caudal, WNT exposed portion- accounting for the shift from SST to PV cells. This idea can also be combined with what we know about proliferation and canonical/non-canonical WNT signaling. We know from previous studies that canonical WNT via β-catenin is important for maintaining proliferation in the MGE (Gulacsi and Anderson, 2008). Our own fluorescent cell sorting results using the WNT reporter suggests that canonical WNT signaling is present and active throughout the MGE, though the levels are highest in the cMGE (Figure 22). A total loss of the MGE in a β-catenin mutant (Gulacsi and Anderson, 2008) is consistent with this, though the *Nkx2.1 CRE* removal of β-catenin could reflect a much earlier role of WNT, since *Nkx2.1* is expressed from the 3-4 somite stage (about e7). Low levels of WNT signaling, then, present in the rMGE could be required to maintain proliferation rates. There is some evidence that PV cells adopt a different strategy of proliferation than SST cells ((Glickstein et al., 2007)Tim Petros, personal communication). *CyclinD2* mutants have selective deficits in PV cell numbers and a significant decrease in SVZ

mitoses, suggesting that progenitors producing PV cells go through an additional transit-amplification step before producing neurons. SST cells instead favor a direct neurogenesis path, contributing to their lower number in the cortex. Low levels of WNT signaling might therefore maintain a transit amplifying based progenitor proliferation strategy in the rMGE, but not reach levels of WNT ligand availability high enough to activate RYK. In the cMGE, high levels of WNT allow the activation of RYK, and RYK activity overrides the proliferative effects of β-catenin, favoring the direct neurogenesis path and the formation of SST positive interneurons. In the dorsal pallium, RYK intracellular domain activity drives neuronal progenitors toward direct neurogenesis in precisely this way (Lyu et al., 2008). In the absence of a transit amplifying cell marker in the ventral pallium, this hypothesis is particularly hard to test. However, in the meantime, a careful analysis of cell cycle markers including *cyclinD2* could begin to address this possibility.

At either extreme of the lifespan of the MGE, small populations of "special case" cells are born. The first, most early born cells are destined to be SST positive, highly connected hub neurons which are important for orchestrating early network dynamics in developing circuits in the hippocampus (Bonifazi et al., 2009; Picardo et al., 2011). At the other extreme, the latest born cells are predominantly the PV expressing, axon-

initial segment targeting chandelier cells (Taniguchi et al., 2013). These special cases, simply because of their unique temporal bias, suggest that they have a secondary mechanism to delineate their specification. The extent to which they are sensitive to RYK will be an interesting way to evaluate how the WNT gradient grows and shifts over time. Early removal of *Ryk* through the use of a conditional allele might be one way to see if hub cell generation is inhibited. If not, the mechanism that modulating MGE progenitor zone competence to respond to RYK might reflect a shift in production mode from pioneer neurons to more typical cells. That early cells hub are SST and late born chandelier cells are PV suggests that whatever spurs their development might also be related to the normal temporal dynamics of MGE cell production.

Previous work in the lab using ES cells has uncovered that the transcription factor *LMO3* acts to increase PV cell differentiation both *in vitro* and *in vivo* (Au et al., 2013). Given the relative dearth of known factors that influence this cell fate decision, we have began looking into a possible connection between RYK signaling and *LMO3*. ES cells overexpressing *LMO3* have a decrease in nuclear RYK ICD localization, suggesting *LMO3* might act as a brake on RYK signaling (Data not shown). A broad inhibition like this could increase the dynamic range, amplifying the difference between RYK signaling in the rostral versus caudal MGE. A change of

LMO3 levels over time could also explain a changing production of PV or SST cells if combined with a RYK based gradient, thereby changing the limits of the gradient without necessarily changing the gradient itself.

Many labs have leveraged the power of transcriptional profiling to characterize factors present in developing interneurons (Batista-Brito et al., 2008; Dumitriu et al., 2006; Okaty et al., 2009; 2011), though almost exclusively in mature cells. It will be important to expand this into the progenitor zone to uncover further factors that could contribute to this process. This has been particularly hard in the past due to the difficultly of distinguishing between cells destined for the cortex and those destined to remain ventrally. Another angle that will be important to explore here is that of non-transcriptionally regulated molecules/targets. In our case, we were able to identify the WNT gradient by observing the expression pattern of TCF4, which is transcriptionally regulated by canonical WNT signaling. However, since canonical WNT signaling appears to be dispensable for cell fate decisions in the MGE, TCF4 isn't likely to be important or directly related to the rMGE/cMGE difference we observed. At some point, the transcriptional programs of SST and PV cells must diverge to differentiate between the complex array of channels and other proteins that make these cells who and what they are. These particular changes need not be determined by single "master regulator" style transcriptional regulation.

Signaling pathways often perform regulation at the protein level- through phosphorylation, sequestration by binding or other modifications. The binding partners and targets of RYK are largely unknown. In the future, it will be interesting to see what those targets are and how they impinge on this system to regulate interneuron differentiation.

We've discussed changing competence of the progenitor zone and differential growth as mechanisms for the temporal change in MGE output. Another related means to conceptualize this is by considering the origin of the WNT signal. Much as individual interneuron cell types are important for proper brain function, the ratio of different interneuron cell types to their circuit partners is thought to be necessary for proper brain function. This notion is exemplified for interneurons in the context of "excitatory-inhibitory" balance, and has been hypothesized to be involved in diseases ranging from autism to epilepsy; although in truth, the term remains poorly-defined, imprecise and controversial (Connors, 2011; Marín, 2012). Nevertheless, some studies have shown that while interneuron cell numbers change over development, their ratio to excitatory cells remain constant (Sahara et al., 2012). Early rabies based viral tracing of presynaptic inputs onto SST cells revealed a transiently biased connection with the thalamus at very early timepoints (Sebnem Tucdemir, personal communication). That the production of SST cells is dependent on a factor secreted by the thalamus

could represent a functionally relevant relationship. This relationship could provide a substrate for temporal or scale matching between the MGE and thalamus. Indeed, balancing between different brain structures in the same general region is often achieved through a coordinated patterning system; through the cross-repressive actions of ventralizing and dorsalizing factors in the forebrain, for example. As a general idea, multiple impinging pathways of this sort establish a complex network that can internally manage the relationships between developing brain regions, much like the brain later manages homeostasis. In particular, it might be interesting to see how (developmental) thalamic mutants might have defects in interneuron subtype production.

Regional heterogeneity within the MGE has long been proposed in the literature (Flames et al., 2007; Inan et al., 2012; Wonders et al., 2008). To what extent this represents the biased, perhaps gradient-influenced, output of equally potent progenitors or subdomains of restricted progenitors is still unclear. Though it is still debated in the literature, there is some evidence in the cortex for progenitor cell heterogeneity: notably, the recent findings of *Cux2* positive radial glia subtypes which produce only upper layer of cortical neurons (Franco et al., 2012). Additionally in the retina, studies have shown marked heterogeneity among neural progenitors (Blackshaw et al., 2004; Hafler et al., 2012; Mizeracka et al., 2013). The

WNT based gradient induced heterogeneity described in this study is compatible with fate restricted or equally potent progenitors. As discussed above, temporal shifts in cell type production might suggest an equally potent progenitor zone, with a shifting influence of WNT washing over it, each progenitor producing a particular cell type based on it's position within the WNT gradient. So far, conclusive clonal lineage tracing of MGE progenitors has been impossible. Though initial tracings sought to use statistical probabilities to classify clustered cells as clones, later work by other labs has indicated that many clones in fact, disperse over a broad area ((Brown et al., 2011), Corey Harwell, Christian Mayer, personal communication). Careful viral tracing with a unique sequence tagged viral library revealed that progenitors give rise to multiple cells in a single clone; and, interestingly, of these multicellular clones, many are of mixed cell types, suggesting a more dynamic system (Christian Mayer, personal communication). Unfortunately, though clonal analysis with viral libraries is a vast technical improvement over statistical clustering, it appears that viral expression is suppressed in many cells, limiting the analysis to a few clones that are not all complete (Christian Mayer, personal communication). As a result, it is still unclear the average size of a clone resulting from a single progenitor in the MGE. This information, combined with long term imaging experiments to observe the amount of active movement within progenitors

and the amount of time over which a single progenitor gives rise to multiple neurons will alone elucidate much of the mystery about how the gradient is interpreted by progenitors.

One strength of a signaling pathway to determine a binary fate choice is flexibility. Evidence suggests that cell fate is chosen close to the time of a cell's final mitosis (Sussel et al., 1999). Signaling gradients have the potential to be more dynamic than linear transcriptional cascades. As signaling components, acting on many independent protein intermediaries and even transcriptional targets, accumulate in cells they can shift the balance toward one cell fate or another based on what is active at a particular time-point. Beside *Nkx2.1* and *Lhx6* (both are important for PV and SST cell fate (Du et al., 2008)), it has been particularly challenging to identify a single transcription factor important for specifying either PV or SST (rather than their mature function (Batista-Brito et al., 2009; Close et al., 2012)). In our case, considering this in the context of a gradient, one can imagine a group of equally potent progenitors in intermediate levels of WNT that hover around a PV/SST set point. Final cell fate decisions of these progenitors might reflect the status of dynamically shifting intracellular RYK signaling at the time of mitosis. This is also a potential explanation for the mixed cell fates of clonal related neurons. In the cortex, radial glial progenitor cell bodies move up and down as well as laterally within the

ventricular zone (Fishell et al., 1993; Pilz et al., 2013). Cortical progenitor cell bodies have also been suggested to move along their radial fibers during cell cycling (Dehay and Kennedy, 2007). Staining for the glutamate transporter, GLAST, which marks radial glial fibers, in the ventral eminences suggests that the fibers are oriented posteriorly and downward (Shibata et al., 1997)(Data not shown). Progenitor cell bodies moving within the WNT gradient could in this way generate mixed clones. Live imaging and characterization of transcriptional and proteomic dynamics in the ventral progenitor zones will aid in further elucidating the mechanisms regulating cell fate choice in the MGE. Proteomic and transcriptional characterization of multiple individual progenitor cells is rapidly becoming possible with current technology, and will be particularly enlightening.

Regionality

Our results found a clear distinction between cells born in the rostral compared to the caudal MGE. Regional biases of cell types produced in the MGE with respect to SST and PV cells have been shown previously (Wonders et al., 2008). Additionally, previous studies have suggested that gene expression varies across these regions to generate multiple subdomains (Flames et al., 2007). These previous studies have focused extensively on the dorsal-ventral axis. The induction of *Nkx2.1* by SHH, located ventrally, was particularly suggestive of a spinal-cord style gradient

(Goodrich et al., 1997; Sussel et al., 1999). Our results reflect a significant advancement in our knowledge by simply changing the perspective by which to consider ventral forebrain patterning. Our findings are entirely compatible with what has been seen previously and reiterate the discovery of heterogeneity in the progenitor zone of the MGE. Importantly, our results highlight the importance of considering the forebrain in three-dimensional space, and its constantly evolving spatial relationship with surrounding structures. I think it will be particularly important for future studies to consider the interaction of multiple factors and gradients to completely understand how diversity from the MGE is generated. RYK signaling seems to be important for SST specification, but alone cannot explain fate decisions in the MGE. One potential way a ventral to dorsal SHH gradient might interact with caudal to rostral WNT is by influencing cell proliferation rates or modes of neurogenesis. As discussed briefly above, PV cells are thought to tend toward a transient amplifying mode over that of direct neurogenesis (Glickstein et al., 2007). The extent of this proposed phenomenon is still elusive and could certainly be mediated through the single or coordinated actions of multiple mitogenic factors, including SHH, FGF and WNT. In the interests of keeping the language simplified, we have referred to the WNT gradient as being oriented caudal to rostral, however, in truth, this gradient is truly based on proximity to the WNT source- the

thalamus- located at the caudal midline, and slightly dorsal. If one takes a mid-coronal section through the center of the MGE, there is the same dorsal SST, ventral PV gradient that has been observed in previous studies (Wonders et al., 2008). This area particularly is close to the midpoint of the gradient, where one is likely to see the most variability, perhaps accounting for less robust findings in coronal slice based studies. This overlap also highlights again how a ventral to dorsal gradient of a factor like SHH could easily be overlaid onto our observed gradient to produce further refinement.

Other MGE derived cell types

One key advantage of *in utero* transplantation analysis when looking at cortical interneuron lineages is that in order to be analyzed, healthy cells must migrate a long distance from the transplant site, providing a convenient biological prerequisite for assessment. A major downside, is our inability to assess our production of ventrally destined cell populations, including globus pallidus and striatal interneurons (Flandin et al., 2010). Though many cells remain close to the site of transplantation (Figure 15), their location there is no indication of their *destiny* for this location. Many studies have used direct transplantation of MGE derived cells into the early post-natal cortex to assess cortical interneuron fates (Southwell et al., 2010). Though this method has the caveat of potentially including ventrally

fated cells in a cortical analysis, it is much more accessible as a technique than *in utero* transplantation and is widely accepted (Vogt et al., 2014). It would be interesting to see if post-natal transplantation of cells into the striatum or globus palliudus would be similarly successful in allowing the characterization of cells fated to these regions. This approach, combined with directed ES cell differentiation or manipulated MGEs could provide a method to further delineate other cell types produced from the MGE. In light of previous results reporting a role for RYK in mediating the choice between the differentiation of oligodendroctyes and cortical interneurons (Zhong et al., 2011) it will be particularly interesting to see how else RYK signaling or other signaling factors aid in the production of non-cortical bound MGE derived cell types.

Gradients as a developmental strategy

As a path to generating cell diversity, gradients provide a number of advantages, many of which have been discussed previously, including potential to temporally vary cell production, to coordinate output of one structure to the growth of another or to simply generate diversity from a relatively homogeneous progenitor pool. Another, broader, advantage is an evolutionary one. Small, even transient, changes in ligand availability, binding specificity, or transport could yield inter-animal variability for natural selection. Much as we were able to transiently inhibit WNT signaling by

IWP2 or through a RYK function-blocking antibody, small mutations that impinge on this system are not likely to be devastating to the embryo. These small modifications introduce a sort of sliding scale to change the ratio of different cell types in a broad way without disrupting cell specification. Interneuron cell types vary between phyla (Raghanti et al., 2010), and it would be interesting to see how RYK signaling contributes to these differences. Large numbers of interneurons undergo cell death around post-natal day 7, determined by a cell-intrinsic mechanism (Southwell et al., 2012). The existence of this refining and pruning period suggests either that ultimately the ratio of PV and SST cells produced during development is unimportant or has some earlier significance. We were able to change the ratio of PV and SST cells in adult animals by varying WNT or RYK signaling, suggesting that any ratio refinement function performed by the cell death period is limited by the proportion of cells produced during development. A better understanding of early circuit dynamics, which we know are important for CGE interneuron development, will shed light on the importance of these early born interneurons, later fated to die. It might also be interesting to see what, if any, the functional consequences are to varying the ratio of different interneuron cell types. Of course, the excess cells produced during development could instead be functionally irrelevant, a remnant of an over-production strategy to ensure

sufficient population levels. Later, some secondary mechanisms determine which cells survive, and that the relative number of those cells is maintained. The former survival determination could potentially be through a Hebbian style weeding out of unconnected cells. The latter through some sort of population specific scalable signal, like a secreted autocrine survival factor (Gord Fishell, personal communication). If early born cells are of little functional consequence, it will nevertheless be of significant interest to determine how population ratios are maintained through the cell death period.

Interneurons have a long road to functional maturity and the full manifestation of their mature phenotype. Much of this is unlikely to depend on early specification, but will be refined as interneurons settle into a particular cortical area, layer and functional unit. A simple WNT gradient as we've described would be woefully insufficient to provide this type of diversity or level of specificity. However, as a cell type that migrates long distances to reach the cortex, there is little opportunity for feedback between the assembling cortical structure to communicate back to the interneuron progenitor zone, so it is particularly important that the MGE can provide basic neuronal substrates on which those later specializations can be layered. A temporal timecourse and regionally based characterization of the restriction of interneuron fates would be particularly illuminating.

Transplantation techniques might also provide some insight as to whether cells become more and more fate restricted as they reach new areas and start establishing connections. Much progress has been made in gently dissociating post-natal cortical cells for FAC sorting. Transplantation across regions at different time points have the potential to give some interesting insight, if difficulties with cell survival could be surmounted.

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