

Genetic Approaches to Understand the Timing of Gene Expression
and Lineage Allocation in the Mouse Auditory System

by

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Chapter 1

Introduction

Vital to the function of any organ system is the proper specification and distribution of cell types during development. This is especially true for the human nervous system, where hundreds of billions of cells are distributed throughout the body. Aside from generating correct cell types in developing tissues, the nervous system forms precise connections to coordinate and control a diverse array of systems. Deciphering the genetic and molecular events which mediate these processes is of fundamental importance in understanding how the complex interactions are established during development. Genetic approaches in the mouse model system have allowed for the investigation of developmental processes that link mechanisms of early gene expression and regulation with the emergence of unique cell types. The auditory system in mammals is an excellent example of multiple anatomical regions that work cooperatively to transduce and integrate information central to hearing. Notably, the gene *Wnt1* is expressed in the primordia of two critical auditory centers, the cochlear nucleus of the hindbrain and inferior colliculus of the midbrain. Through a genetic inducible fate mapping approach, we demonstrate the relationship between the timing of *Wnt1* expression in progenitors and the terminal fate and distribution of auditory neurons. In addition, we used mutant analysis to investigate the functional requirement of *Wnt1* in auditory system development. Our results provide evidence for two models of how *Wnt1* expression differentially affects the development of the cochlear nucleus and inferior colliculus, and that *Wnt1* is required for a subset of auditory domains. Finally, a novel transgenic strategy for the *in vivo* assessment of genetic lineages is presented.

Part I

The sense of hearing

Hearing is the ability to detect sound from the environment coupled with the extraction of meaningful information. Hearing is one of the five classical senses (including sight, touch, smell and taste) and is common to both vertebrates and arthropods (Boekhoff-Falk, 2005). Hearing is fundamentally important for the propagation of many species, as it plays central roles in survival (Hoy et al., 1989; Takahashi, 2010), communication (Vergne et al., 2009; Knudsen and Gentner, 2010), and courtship behaviors (Nowicki and Searcy, 2004; Hoikkala et al., 2005). Animals use auditory information to avoid predators, relying heavily on their ability to detect the approach or the warning calls of other members of a group. Conversely, predators use their sense of hearing in part to detect and locate prey. Acoustical information is also utilized by many species to attract and assess the reproductive fitness of potential mates. For humans, the sense of hearing has additional incalculable value, being the only species to have evolved the capacity for speech.

In vertebrates, sound is detected and perceived by a complex of spatially separated but functionally connected structures that comprise the auditory system. Sound itself is caused by vibrations that are transmitted through a medium such as air, and are first detected in the periphery by the ear. Specialized structures within the inner ear convert the physical properties of sound into nerve impulses perceived by the central auditory system of the brain. The central auditory system is comprised of interconnected auditory centers,

linked by neuronal projections that are required for normal hearing. Auditory processing is hierarchal, beginning in the brainstem and ascending through subcortical and cortical areas (Figure 1). The following sections of Part 1 provide a brief overview of common themes underlying organization and function of two auditory structures within the mammalian brain: the cochlear nucleus of the brainstem, and inferior colliculus of the midbrain.

Tonotopic organization of the auditory system starts with the ear

A central organizing principle underlying function throughout the central auditory system is the preservation of the tonotopic representation of sound first generated in the cochlea of the inner ear (Osen, 1970; Guinan et al., 1972; Aitkin and Moore, 1975; Morel et al., 1987; Schreiner, 1995). Within the the spiral-shaped cochlea is the organ of Corti (Figure 2), which contains hair cells that are responsible for the mechanosensation of fluid-borne vibrations of the cochlea created as sound is transduced within the ear (reviewed by Schwander et al., 2010). Because of the unique morphological properties of the cochlea and organ of Corti, the position of hair cells along the basilar membrane within the cochlear duct dictates the frequency of sound to which they respond; hair cells at the base are responsive to higher frequencies while those at the apex are responsive to the low end of frequencies (Figure 2) (Liberman, 1982; Müller, 1991). This topographic arrangement and consequential selectivity of frequency responses establishes the foundational mapping of sound in the auditory system. As this organization occurs outside the central nervous system, spiral ganglion neurons

are responsible for communicating the electrical representation of sound information from the hair cells to the brain (Rusznák and Szucs, 2009). The axons of these neurons form the eighth cranial nerve, which relays frequency specific information directly to the cochlear nucleus of the brain (Osen, 1970; Fekete et al., 1984). Spiral ganglion neurons innervate neurons of the cochlear nucleus in exactly the same order as their target hair cell. This organization ensures that the topography of frequency established by the hair cells and transmitted by the spiral ganglion neurons is recreated within the cochlear nucleus through the precise organization of spiral ganglion projections.

The cochlear nucleus

The cochlear nucleus of the brainstem is composed of three distinct divisions with partially overlapping function and organization (Figure 3): the anteroventral cochlear nucleus (AVCN), posteroventral cochlear nucleus (PVCN), and dorsal cochlear nucleus (DCN) (Osen, KK, 1988). As briefly described in the previous section, the cochlear nucleus is the initial site of convergence for auditory information in the brain (Osen, 1970). The neuronal and axonal organization within the cochlear nucleus creates the primary tonotopic representation of sound within the central auditory system (Figure 4) (Bourk et al., 1981; Luo et al., 2009). Within the cochlear nucleus the properties of sound are parceled apart prior to being sent to higher order auditory centers that are necessary for decoding information unique to pitch (frequency), intensity (amplitude), and localization of the sound source (Winer and Schreiner, 2005).

These functions are mediated by the multitude of cell types within the cochlear nucleus and the circuits they form.

Classically, the cell types of the cochlear nucleus were identified through general histological methods such as Nissl and Golgi staining (Harrison and Irving, 1965; Harrison and Irving, 1966; Osen, 1969). Nissl staining for example, revealed three general classes of cells based on morphology: small cells, large cells, and granule cells (Brawer et al., 1974). The granule cells are perhaps the most readily distinguished cell type, densely populating the microneuronal shell that surrounds the cochlear nucleus subdivisions dorsally and laterally, and separates the DCN from the AVCN and PVCN (Figure 3) (Brawer et al., 1974). Granule cells receive input from both descending auditory (Caicedo and Herbert, 1993; Saldaña et al., 1996) and non-auditory pathways (Weinberg and Rustioni, 1987; Wright and Ryugo, 1996). The granule cells synapse locally within the DCN (Mugnaini et al., 1980) and are believed to be involved in multimodal sensory integration (Ryugo et al., 2003).

Based on identifiable projection patterns, there are at least eight separate cell types in the cochlear nucleus encompassing the small and large cells, which mediate connections to other brain centers (reviewed by Cant and Benson, 2003; Malmierca, 2003). Of these, only one type does not mediate connections to other auditory centers, the cochlear root neurons (Lingenhöhl and Friauf, 1994). These neurons provide connections to brainstem premotor areas and are believed to mediate auditory evoked startle reflexes in rodents (Kandler and Herbert, 1991; López et al., 1999).

Spherical and globular bushy cells of the AVCN/PVCN innervate aspects of the superior olivary complex and ventral nucleus of the lateral lemniscus (VNLL) (Cant and Casseday, 1986; Smith et al., 1993; Glendenning et al., 1991). Similarly, octopus cells of the PVCN innervate the VNLL (Friauf and Ostwald, 1988). The connections to the superior olivary complex and VNLL are required of sound localization (Casseday and Neff, 1975; Jenkins and Masterton, 1982). These pathways relay binaural cues, such as the difference in time and levels of sound detected between each ear.

Multipolar cells are a large class of neurons in the AVCN/PVCN which are heterogenous in morphology and function (Osen, 1969; Feng et al., 1994; Ostapoff et al., 1994). These neurons can be divided into two classes (type I and type II) based on physiological properties and projections paths (Smith and Rhode, 1989). Type II multipolar neurons provide inhibitory input to the contralateral cochlear nucleus (Schofield and Cant, 1996; Alibardi, 1998), which likely modulates auditory information in the brainstem (Doucet and Ryugo, 2006). In contrast, the more abundant type I multipolar neurons constitute a substantial source of excitatory input to the contralateral inferior colliculus, relaying frequency-specific auditory information (Beyerl, 1978; Adams, 1979; Oliver, 1987; Alibardi, 1998; Oliver et al., 1999). Only a small population of type I neurons provide ipsilateral innervation of the inferior colliculus (Adams, 1979; Oliver, 1987). Interestingly, all type I multipolar neurons of rat, which project to the contralateral inferior colliculus, express the calcium-binding protein parvalbumin,

while all of those which provide ipsilateral innervation express calretinin (Fredrich et al., 2009).

Another source of axonal input to the inferior colliculus arises from the fusiform and giant cells of the DCN (Osen, 1972; Adams, 1979; Oliver, 1984). While the precise role of giant cells is unknown, fusiform cells may communicate the location of sound sources through direct innervation of the inferior colliculus (Sutherland et al., 1998; May, 2000). As was demonstrated for the AVCN/PVCN, neurons of the DCN which project to the contralateral inferior colliculus in rat express a unique calcium-binding protein, calbindin (Fredrich et al., 2009). The establishment of cochlear nucleus neurons differentially expressing calcium-binding proteins is a major point of analysis in Chapter 2.

The connections from the cochlear nucleus to the inferior colliculus terminate in laminar distributions, and function in part as a substrate for tonotopic organization in the midbrain (Malmierca et al., 2005; Malmierca et al., 2008; Loftus et al., 2010). Importantly, tonotopic preservation is conserved from brainstem to midbrain.

The inferior colliculus

The inferior colliculus of the dorsal midbrain is an obligatory processing hub for all ascending auditory information en route to higher order centers (Aitkin and Phillips, 1984). It is a bilateral structure classically defined as having a central nucleus surrounded by lateral and dorsal cortices (Figure 5) (Morest and Oliver, 1984). The central nucleus of the inferior colliculus (ICC) is the most

prominent subdivision, and is distinguishable from the cortices histologically. Molecularly, the differential expression of calcium-binding proteins segregates inferior colliculus subdivisions; the ICC stains broadly for parvalbumin, while calbindin is largely confined to external cortices (Coleman et al., 1992; Vater and Braun, 1994; Lohmann and Friauf, 1996; Sharma et al., 2009). The functions of the external cortices are not entirely clear, and neither contain a tonotopic map (Winer and Schreiner, 2005). The lateral cortex, however, is likely involved in multisensory integration, receiving substantial non-auditory input from the somatosensory system (Aitkin et al., 1978; Aitkin et al., 1981; Morest and Oliver, 1984). Projections to the dorsal cortex arise primarily from intrinsic and intercollicular neurons, as well as the auditory cortex (Aitkin and Phillips, 1984; Winer et al., 1998). The role of the dorsal cortex in hearing is largely unknown, though lesion studies in cat suggest that it is involved in attention to auditory stimuli (Jane et al., 1965). The ICC however, is solely involved in auditory integration (Aitkin et al., 1994) and will be the focus of this section as it pertains to Chapter 2.

The cytoarchitecture of the ICC underpins its integrative role in auditory processing. The dendritic fields of ICC neurons and afferents of every lower auditory center form laminae which correspond to distinct isofrequencies; individual lamina contain thousands of neurons tuned (responsive) to a characteristic frequency of sound (Clopton and Winfield, 1973; Merzenich and Reid, 1974; Malmierca et al., 1993; Brown et al., 1997; Schreiner and Langner, 1997). This organization establishes the tonotopic map of the ICC (Figure 6),

where neurons residing in the dorsolateral ICC are responsive to low frequencies, and neurons contained within the ventromedial aspects are responsive to high frequencies (Merzenich and Reid, 1974; Schreiner and Langner, 1997).

The ICC contains two major cell types, the disc-shaped neurons and stellate neurons. The disc-shaped neurons are the most abundant, forming rows with highly oriented, large dendritic fields yielding the laminar units which underpin tonotopic organization discussed above (reviewed by Oliver, 2005). The stellate neurons have spherical dendritic fields which may span across multiple laminae, allowing them to integrate ascending auditory input across a larger frequency spectrum (Winer and Schreiner, 2005).

The axonal outputs of ICC neurons are diverse. ICC neurons primarily innervate the medial geniculate body (MGB) in the thalamus, where auditory and non-auditory information is integrated and sent to higher order processing in the auditory cortex (Andersen et al., 1980). The ICC afferents terminate in the ventral division of the MGB (Calford and Aitkin, 1983), forming laminae that establish tonotopy in the thalamus (Morest, 1965; Cetas et al., 2001). ICC neurons also provide substantial innervation within and between each colliculi (Aitkin and Phillips, 1984; Oliver et al., 1991). The commissural connections are readily identified in coronal sections of the mouse brain (Figure 5, see Chapter 2). Finally, reciprocal descending innervation of the auditory brainstem nuclei (Huffman and Henson, 1990) allows for modulatory and feed back control of auditory processing.

Conclusion, Part I

Neuronal and axonal structure underlie the functional organization of the auditory system, and collectively provide the physical substrate for which the complexities of sound may be broken down into manageable pieces of information transmitted from one center to another. Frequency discrimination is one function of the auditory system, and is accomplished by orderly arrays of neurons and their processes at each anatomical level of the auditory system. While much has been learned regarding the structure and physiology of auditory centers, far less is known of how auditory centers develop. In particular, the genetic and molecular cues which guide the organization of auditory neurons and their connections are poorly understood. Part II of this introduction provides the background and rationale for the genetic experiments of Chapter 2, which aim to further our understanding of the developmental mechanisms which establish the cochlear nucleus and inferior colliculus.

Part II

Origins of the cochlear nucleus and inferior colliculus

The cochlear nucleus and inferior colliculus are derived from segregated neuroepithelia in the rhombencephalon (rhombomeres (r)2-5) (Pierce, 1967; Martin and Rickets, 1981; Tan and Le Douarin, 1991; Ivanova and Yuasa, 1998; Nichols and Bruce, 2006; Farago et al., 2006) and dorsal mesencephalon (mes) (Alvarado-Mallart and Sotelo, 1984; Hallonet and Le Douarin, 1993; Millet et al., 1996; Zervas et al., 2004), respectively (Figure 7). Classical and modern

histological approaches in the mouse embryo demonstrated that a germinal zone in the dorsal rhombencephalon, the rhombic lip, produces tangentially migrating progenitors which give rise to neurons of the cochlear nucleus in the auditory hindbrain (Pierce, 1967; Martin and Rickets, 1981; Ivanova and Yuasa, 1998). This was further confirmed by quail-chick chimera studies in which transplanted ventral (basal) domains of the rhombencephalon generated motor centers while dorsal (alar) domains formed sensory centers, including the cochlear nucleus (Tan and Le Douarin, 1991). Similarly, quail-chick chimera studies demonstrated that the midbrain tectum, inclusive of the inferior colliculus, is derived from the dorsal mes at the border between the presumptive midbrain and cerebellum (Alvarado-Mallart and Sotelo, 1984; Hallonet and Le Douarin, 1993; Millet et al., 1996).

The germinal zones of the cochlear nucleus and inferior colliculus can be further identified by unique gene expression patterns. The homeodomain containing transcription factors of the *Hox* family display rhombomeric specific gene expression patterns, and are important for maintaining segmental identity along the anterior-posterior axis of the hindbrain (Murphy et al., 1989; Wilkinson et al., 1989; Krumlauf et al., 1993; Lumsden and Krumlauf, 1996; Lumsden, 2004). For example, *Hoxa2* is the only *Hox* gene expressed in r2, while *Hoxb2* is expressed at the anterior r2/r3 boundary, *Hoxb3* at the r4/r5 anterior boundary, and *Hoxb4* is present at the r6/r7 boundary (Wilkinson et al., 1989; Gavalas et al., 1997). One function of the differential and overlapping domains of *Hox* genes is to regulate the expression of cell adhesion molecules that keep adjacent

rhombomeres from mixing (Jones et al., 1992; Wizenmann and Lumsden, 1997; Tümpel et al., 2009).

Homeobox transcription factors are expressed in the dorsal mes as well. *En1/2* and *Otx1/2* are required, in part, for the induction of a signaling center, the isthmus organizer (IsO), which is essential for patterning the midbrain and cerebellum through the actions of *Fgf8* (Joyner and Martin, 1987; Millet et al., 1996; Acampora et al., 1997; Millet et al., 1999; Broccoli et al., 1999). Another gene that is required for the maintenance of this signaling center and the development of the midbrain is *Wnt1* (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992; Adams et al., 2000; Matsunaga et al., 2002). At midgestation, *Wnt1* in the dorsal mes is confined to a midline stripe and a posterior ring of expression at the interface between the presumptive midbrain and hindbrain (Figure 7) (Wilkinson et al., 1987; Gavin et al., 1990; Zervas et al., 2004; Ellisor et al., 2009). *Wnt1* is also expressed throughout the germinal zone of the cochlear nucleus, the rhombic lip of r2-r5, more commonly referred to as the lower rhombic lip (LRL) (Figure 7) (Wilkinson et al., 1987; Nichols and Bruce, 2006; Farago et al., 2006). In the absence of *Wnt1*, the midbrain and cerebellum fail to develop (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). In addition, mice homozygous for the *swaying* (*sw*) allele, a naturally occurring mutation in *Wnt1* believed to result in hypomorphic function, display malformed midbrain and cerebellum (Lane, 1967; Thomas et al., 1991; Bronson and Higgins, 1967; Bally-Cuif et al., 1995).

The following section describes the significance of the *Wnt* family of genes, with emphasis on *Wnt1* and canonical WNT signaling.

Wnt1 and the effects of canonical Wnt signaling

Wnt1 belongs to a large, highly conserved family of genes that encode secreted glycoproteins important for many developmental processes in all animals (van Amerongen and Nusse, 2009). *Wnt1* was the first of the *Wnts* to be cloned, originally identified as the proto-oncogene *Int1* (*Integration 1*), which was shown to be dysregulated in mouse mammary tumors following a viral integration within the *Int1* locus (Nusse and Varmus, 1982). Once the *Drosophila wingless* (*wg*) gene was isolated and shown to be homologous to *Int1* (Rijsewijk et al., 1987), the names merged to identify the class of these genes: *Wnts*. To date, 19 *Wnt* genes have been identified in mammals, mediating diverse roles in development including cell proliferation and fate, cell/tissue polarity, cell movement, stem cell maintenance, and axon guidance (Ciani and Salinas, 2005; van Amerongen and Nusse, 2009). Wnt signaling is notoriously complex, and is mediated through the interaction of WNTs and Frizzled (Fz) receptors, of which 10 have been identified in mammals (Bhanot et al., 1996; van Amerongen and Nusse, 2009). Downstream of the combinatorial effects between WNTs and Fz receptors there are at least three separate pathways controlling cellular responses: the canonical Wnt/ β -catenin pathway, planar cell polarity (PCP) pathway, and the Wnt/calcium pathway (Ciani and Salinas, 2005). WNT1 signals through the canonical pathway, as the inactivation of β -catenin in *Wnt1*-

expressing cells phenocopies the *Wnt1* null mutant phenotype (Brault et al., 2001). In the canonical pathway (Figure 8), the interaction of WNT/Fz activates Dishevelled (Dsh), which then induces the disassembly of a complex consisting of AXIN, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β), and β -catenin (Logan and Nusse, 2004). In the absence of WNT signal, β -catenin is phosphorylated by GSK3 β , targeting it for degradation (Figure 8A). Thus, activated Dsh results in elevated levels of β -catenin, allowing it to translocate to the nucleus where it forms a complex with lymphoid enhancer-binding factor 1/T-cell specific transcription factor (LEF/TCF) to mediate transcription of target genes (Figure 8B). Many of the components of canonical Wnt signaling are targets for transcriptional regulation downstream of WNT/Fz interactions, providing feedback that may be used to modulate signaling (Logan and Nusse, 2004). However, it is the target genes outside of the canonical signaling cascade that are likely involved in cell proliferation and cell fate decisions (Logan and Nusse, 2004; van Amerongen and Nusse, 2009). WNT1 signaling through β -catenin directly influences sensory fate specification in neural crest stem cells (Lee et al., 2004). By constitutively activating the canonical pathway, sensory neurons are specified at the expense of other neural crest derivatives. Thus, canonical WNT signaling through WNT1 is able to specify cell fate in the developing nervous system. In the absence of canonical WNTs, phenotypes may be explained by a lack of cell fate specification or cell proliferation. This is evident by limb bud outgrowth failure in *Wnt5A* (Yamaguchi et al., 1999) mutants, and compromised expansion of the mes/r1 in *Wnt1* nulls

(McMahon and Bradley, 1990; Megason and McMahon, 2002). In addition, a role for Wnt signaling in axon remodeling (Lucas and Salinas, 1997; Hall et al., 2000) and guidance has emerged (Lyuksyutova et al., 2003). Axon remodeling occurs through a divergent canonical pathway which effects microtubule stability (Ciani et al., 2004). Though guidance of commissural spinal cord axons was shown to be mediated through Wnt/Fz (*Wnt4a/Fz3*) interactions (Lyuksyutova et al., 2003), whether this occurred through canonical signaling/ β -catenin is not certain. However, in testing the effects of several secreted molecules, it was demonstrated that WNT1 stimulated the extension of post-crossing commissural axons, suggesting that WNT1 could act as an attractant cue (Lyuksyutova et al., 2003).

Given the multiple and overlapping roles *Wnts* play in development, it is likely that cell proliferation, cell fate, and possibly axon guidance are affected by *Wnt1* expression in the development of the cochlear nucleus and inferior colliculus. Of importance is discerning how *Wnt1* expression is related to these processes. One approach to understand the role of *Wnt1* in development is to determine how the timing of *Wnt1* expression in progenitors is related to the establishment of mature structures.

The following section describes genetic approaches in the mouse which have begun to elucidate the role of *Wnt1* in auditory system development. Limitations and gaps of these studies are discussed, which provide the rationale for the research described in Chapter 2.

Genetic fate mapping in the mouse: a model approach to auditory system development

The mouse has emerged as an important species for genetic approaches in developmental studies, because genetic manipulations of the germ line have been perfected and exploited for over three decades (reviewed by Nagy, 2003). Discovering that targeted mutations (knock-in/knock-out) could be made in mouse ES cells provided the groundwork for generating germ line mutations in chimeric animals (Schwartzberg et al., 1989; Thompson et al., 1989; Zijlstra et al., 1989). This has allowed investigators to probe the functions of genes in the context of development, either through their disruption or modification.

Wnt1 was one of the first genes to be knocked out in mice through targeted disruption with a neomycin selection cassette (Thomas and Capecchi, 1990). Mice homozygous for the loss of *Wnt1* fail to develop midbrain and cerebellum, and die perinatally (Thomas and Capecchi, 1990; McMahon and Bradley, 1990). Because the generation of null alleles may lead to vast developmental perturbations or embryonic lethality, conditional genetic manipulations were developed to provide cell or tissue specificity in gene modifications, enabling context dependent analysis of gene function (Nagy, 2000; Nagy et al., 2009). This same technology has provided the means for genetic fate mapping approaches; through conditionally activating a heritable reporter gene in genetically-defined progenitors, one may follow the contribution of genetic lineages across development (Chapters 2, 3).

Classically, cell lineage analysis in vertebrates has been assessed in avian systems through heterotopic transplantations in quail-chick chimera studies (Le Douarin et al., 1975), or through the injection of vital dyes in progenitor domains (Bronner-Fraser and Fraser, 1988). These approaches are physically invasive and are not easily amenable in the context of mammalian development. Additionally, chimera studies may lack specificity, and vital dyes may be diluted beyond detection following expansion of the marked progenitor population. In either case, the progenitor population to be followed lacks any genetic pretext. Genetic fate mapping has revolutionized the way fate mapping studies are conducted, because it provides noninvasive, heritable marking of progenitors defined by unique gene expression. This approach is accomplished through the activity of a site-specific recombinase, which catalyzes the removal or inversion of DNA sequences flanked by the consensus sites to which the recombinase binds (reviewed by Dymecki, 2000; Joyner and Zervas, 2006). Identified and isolated from bacteriophage P1 (Sternberg, 1979), Cre recombinase mediates the recombination between 34 bp *loxP* sequences (Sauer, 1998). Transgenic (Novak et al., 2000; Awatramani et al., 2001) or targeted (Soriano, 1999; Srinivas et al., 2001; Hippenmeyer et al., 2005) reporter alleles containing *loxP*-flanked stop cassettes have been generated which remain quiescent in the absence of Cre-mediated recombination. Upon cell-type specific Cre expression in a genetically-defined population of interest, the reporter is turned on following the recombination and excision of the stop cassette. Constitutive and heritable expression of the reporter then allows the population of interest and all cells

descendant from it to be assessed later in development or in the mature animal. This provides a cumulative fate map, where every cell with a history of Cre expression and all its descendants are marked through the indelible expression of the reporter.

This cumulative approach was used to fate map the *Wnt1* lineage in the cochlear nucleus (Figure 9) (Farago et al., 2006). By heritably marking every cell with a history of *Wnt1* expression, Farago et al. determined that each of the cochlear nucleus subdivisions were largely derived from *Wnt1*-expressing progenitors in the LRL. Additionally, they determined the axial level from which the progenitors came using intersectional genetic fate mapping. By marking progenitors based on their combined expression of *Wnt1* and rhombomeric-specific genes, it was shown that the cochlear nucleus is derived from r2 through r5. However, with cumulative approaches, the relationship between timing of gene expression in progenitors and the distribution and terminal fate of cochlear nucleus neurons was unresolved. In addition, the molecular profile of *Wnt1*-derived neurons was not determined. As *Wnt1* expression defines the germinal zone of the cochlear nucleus, understanding the temporal expression of *Wnt1* in progenitors and how its expression relates to the functional organization of the mature tissue could further our understanding of how the cochlear nucleus is put together. In particular, deciphering how neurons of the cochlear nucleus are distributed may provide insight for the mechanisms underlying tonotopic organization. To address, these concerns, we employed genetic inducible fate

mapping (GIFM; reviewed by Joyner and Zervas, 2006) in the studies described in Chapter 2.

GIFM is an advance in genetic marking methods because it provides temporal control of reporter gene activation in addition to the spatial control that is derived from the Cre expression domain of the population to be followed (Figure 10). GIFM takes advantage of a ligand-activated form of Cre recombinase, CreER (Feil et al., 1996). This recombinase is a fusion protein of Cre, and a modified form of the estrogen receptor ligand-binding domain (ER), which is selectively activated by the drug tamoxifen (Feil et al., 1996). Upon expression, CreER remains sequestered in the cytoplasm through its interaction with heat shock protein 90 (hsp90), which binds the ER domain (Figure 10A). When tamoxifen is introduced to the cell, it displaces hsp90 and allows CreER to translocate to the nucleus to mediate recombination and activation of the reporter allele (Figure 10C). By expressing CreER through transgenic or targeted alleles unique to progenitors of interest, the investigator gains temporal control of reporter gene activation by administering tamoxifen *in utero* at developmental stages of their choosing. As apposed to a cumulative fate map, this approach enables the dissection of genetic lineages across development. In particular, GIFM provides a powerful tool in deciphering how complex structures with diverse cell types and functional organization (such as auditory centers) are established over time. As not every cell type which comprises a tissue is generated in synchrony, understanding the relationship between the timing of

gene expression in progenitors and the emergence and distribution of distinct cell types is of fundamental importance in developmental biology.

GIFM has demonstrated that aside from maintaining the IsO, *Wnt1*-expressing progenitors contribute to adult midbrain structures (Zervas et al., 2004). This study utilized mice that were engineered to express CreER under the control of *Wnt1* regulatory elements (*Wnt1-CreER*). By combining this transgene with a conditional reporter allele in doubly transgenic mice and administering tamoxifen at midgestation, the fate of *Wnt1*-expressing progenitors was followed during development and in the adult brain. Specifically, Zervas et al. determined that *Wnt1*-expressing progenitors gave rise to both ventral and dorsal tissues of the midbrain, including the inferior colliculus (Figure 11). Marking *Wnt1*-expressing progenitors in the posterior mes at E9.5 and assessing the lineage contribution at E15.5 demonstrated that these progenitors expanded rostrally, and were distributed throughout the posterior inferior colliculus primordium. Yet, the terminal contribution from this marking stage was not assessed. However, by marking *Wnt1*-expressing progenitors at E10.5 and determining the adult contribution, Zervas et al. demonstrated that the E10.5 *Wnt1* lineage modestly contributed to the posterior inferior colliculus (Figure 11). Of importance, this study did not fully address how the timing of *Wnt1* expression in progenitors affected the distribution of neurons within the inferior colliculus. Additionally, how *Wnt1*-derived neurons are allocated between subdivisions of the auditory midbrain was not assessed. These gaps are addressed through the experiments described in Chapter 2.

Conclusion, Part II

The germinal zones of two critical auditory centers are defined by the expression of *Wnt1*, an essential gene that influences key developmental mechanisms. Genetic studies have demonstrated that *Wnt1* directly affects both proliferation and cell fate in the embryonic nervous system (Panhuysen et al., 2004; Lee et al., 2004). However, there is little understanding of how and when *Wnt1* may be important for these presumed processes in the development of the cochlear nucleus and inferior colliculus. The study described in Chapter 2 uses GIFM to test the hypothesis that the timing of *Wnt1* in auditory progenitors is related to the functional organization and cell fate of auditory neurons. By assessing temporally defined *Wnt1* lineage fate maps in mature structures, evidence for *Wnt1*-mediated developmental mechanisms are uncovered.

Part III

Limitations of current fate mapping reporter alleles

The genetic fate mapping experiments described in Chapter 1, Part II, and performed in Chapter 2, described the use of alleles that conditionally expressed enzymatic or fluorescent gene products, including β -galactosidase (β -gal) (Zervas et al., 2004; Farago et al., 2006) or green fluorescent protein (GFP) (Farago et al., 2006). β -gal-expressing cells can be identified by either a substrate dependent enzymatic assay (X-gal histochemistry, see Chapter 3) or through immunological detection using antibodies specific to β -gal (Chapters 2,

3). GFP-expressing cells may be visualized directly by fluorescence microscopy, or through immunological detection (Chapters 2, 3). Typically, genetic lineage contribution is assessed post-mortem, especially with regard to deep tissues that are not accessible to fluorescence microscopy. A limitation then, is that these methods do not allow for the longitudinal assessment of a genetic lineage across time in a living animal. In the context of development, the morphometric changes that occur as progenitors establish tissues are largely analyzed through static evaluations of embryological tissue sections at multiple stages. In the context of disease models, the changes in an affected population are also often assessed in static, post-mortem tissue. An advancement in genetic fate mapping approaches that would allow the repeated, longitudinal visualization of lineage contribution *in vivo* could greatly change the way developmental genetics and disease-based studies are conducted.

Genetic manipulations for contrast-enhanced magnetic resonance imaging (MRI)

One platform that is appropriate for repeated, longitudinal *in vivo* analyses is magnetic resonance imaging (MRI). MRI allows non-invasive three-dimensional imaging of soft tissues, and is used in medical settings to assess tissue damage, tumor progression, and neurological abnormalities (Golder, 2004; Rosen and Lenkinski, 2007). More recently, the use of contrast agents having paramagnetic and supraparamagnetic properties such as manganese and iron, has allowed activity-dependent imaging in the brain (Silva and Bock, 2008) and cellular resolution (Shapiro et al., 2006; Shapiro and Koretsky, 2008) in small

animal MRI. Manganese-enhanced MRI (MEMRI) provides contrast to metabolically active parts of the brain, allowing for example, the detection of frequency specific responses in the inferior colliculus following pure-tone stimulation (see for example Figure 6B) (Yu et al., 2007). Iron oxide has also been successfully used to track the migration of transplanted cells, yet these approaches typically require the loading of iron oxide in culture (Bulte and Kraitchman, 2004).

Genetic approaches to load cells with iron have included expressing iron metabolic enzymes in cell lines which can be imaged by MRI (Deans et al., 2006). In this study, 2,500 cells transfected with transferrin receptor and ferritin expressing constructs were transplanted into the striatum of adult mice, and the graft could be imaged *in vivo* by MRI. In a separate study, ferritin was conditionally expressed in hepatocytes, allowing for *in vivo* MRI detection of the liver in developing and adult mice (Cohen et al., 2007). A potential problem with these strategies is that the accumulation of iron, which resulted from iron metabolic enzyme overexpression increased reactive oxygen species (ROS), which can be detrimental to cell health. A genetic approach that would allow the specific, transient accumulation of contrast agents in genetic lineages has not been reported.

A novel transgenic approach for in vivo assessment of genetic lineage contribution

Chapter 3 of this thesis describes an approach to couple GIFM with a novel transgenic reporter designed to provide a cytoplasmic molecular scaffold for the accumulation of biochemically tagged compounds. Specifically, we were interested in the delivery of contrast agents for MRI. Previously, we had successfully imaged both midgestation embryos and adult brains by MRI (Figure 12A,B). We determined a resolution capacity of 120-150 μm^3 using a three tesla (3T) magnet and gradient insert for small animal imaging. This experiment demonstrated the principle that small populations of cells could be resolved following successful contrast enhancement. We were subsequently awarded a pilot study grant from the Institute for Brain Sciences at Brown University to develop our transgenic reporter. We engineered a Cre-responsive reporter, *Z/StrepG*, designed to express *LacZ* (β -gal) in all cell types. Following recombination, *Z/StrepG* would be reconfigured to express *streptavidin-IRES-eGFP* only in lineage marked (Cre-expressing cells)(Figure 13). Thus, cells which recombine the transgene would express two reporter proteins from a bicistronic message encoding *streptavidin* and *GFP*. Streptavidin is a bacterial protein from *Streptomyces avidinii* which binds the small molecule biotin (vitamin B7) with an affinity of K_d 10^{-15} M, ranking amongst the strongest of non-covalent interactions observed in molecular biology (Argarana et al., 1986). Biotin is readily coupled to other compounds chemically, and because of their unusually strong affinity, streptavidin-biotin interactions have been used in a wide array of

biochemical assays and purification procedures (Bayer and Wilchek, 1980; Wilchek and Bayer, 1988; Diamandis and Christopoulos, 1991). We hypothesized that the constitutive expression of streptavidin would create a molecular sink for biotinylated-compounds, thereby allowing their transient enrichment in cells targeted by Cre-mediated recombination. By coupling this allele with *Cre/CreER* mouse lines of interest, three-dimensional fate maps may be generated through MRI following exogenous delivery of biotinylated contrast agents either *in utero* or the post-natal animal. Because of the dual expression of streptavidin and GFP, the MRI fate map could be verified at the cellular level through fluorescence microscopy. Other advantages of concurrent GFP expression are discussed in Chapter 3, which involve the specific isolation and transplantation of progenitors expressing *Z/StrepG* in disease models. The engraftment of progenitors expressing the streptavidin reporter in cell replacement therapy studies could, in theory, be monitored over time by MRI following delivery of biotinylated iron-based contrast agents. This would provide the transient accumulation of iron in the transplanted population and may reduce the risk of ROS production.

Conclusion, Part III

Determining genetic lineage contribution *in vivo* by MRI could provide a great tool in assessing how organs and systems are assembled in three dimensions during the course of development. It could also be used to monitor the progression of disease, or the maintenance of a transplanted population.

Chapter 3 describes one approach with these goals in mind, and provides analysis of problems areas and alternative tactics.

Research aims

This thesis aims to examine the involvement of *Wnt1* in the development of the cochlear nucleus and inferior colliculus using genetic inducible fate mapping approaches in mouse. We test the hypothesis that the temporal expression of *Wnt1* in progenitors is important for specifying the distribution and fate of auditory neurons in the cochlear nucleus and inferior colliculus. By dissecting *Wnt1* lineage contribution across time, we uncovered two models which differentially explain how *Wnt1*-expressing progenitors establish these auditory centers. Additionally, the design, engineering, and testing of a novel fate mapping reporter allele is presented. This reporter was designed to provide three-dimensional lineage assessment by MRI.

The specific aims are to determine:

- 1) how *Wnt1*-expressing progenitors at distinct developmental stages, contribute to the cochlear nucleus and inferior colliculus.
- 2) the requirement of *Wnt1* in the development of the cochlear nucleus and inferior colliculus.
- 3) the efficacy of streptavidin as a conditional fate mapping reporter allele capable of providing the molecular capacity to accumulate biotinylated compounds.

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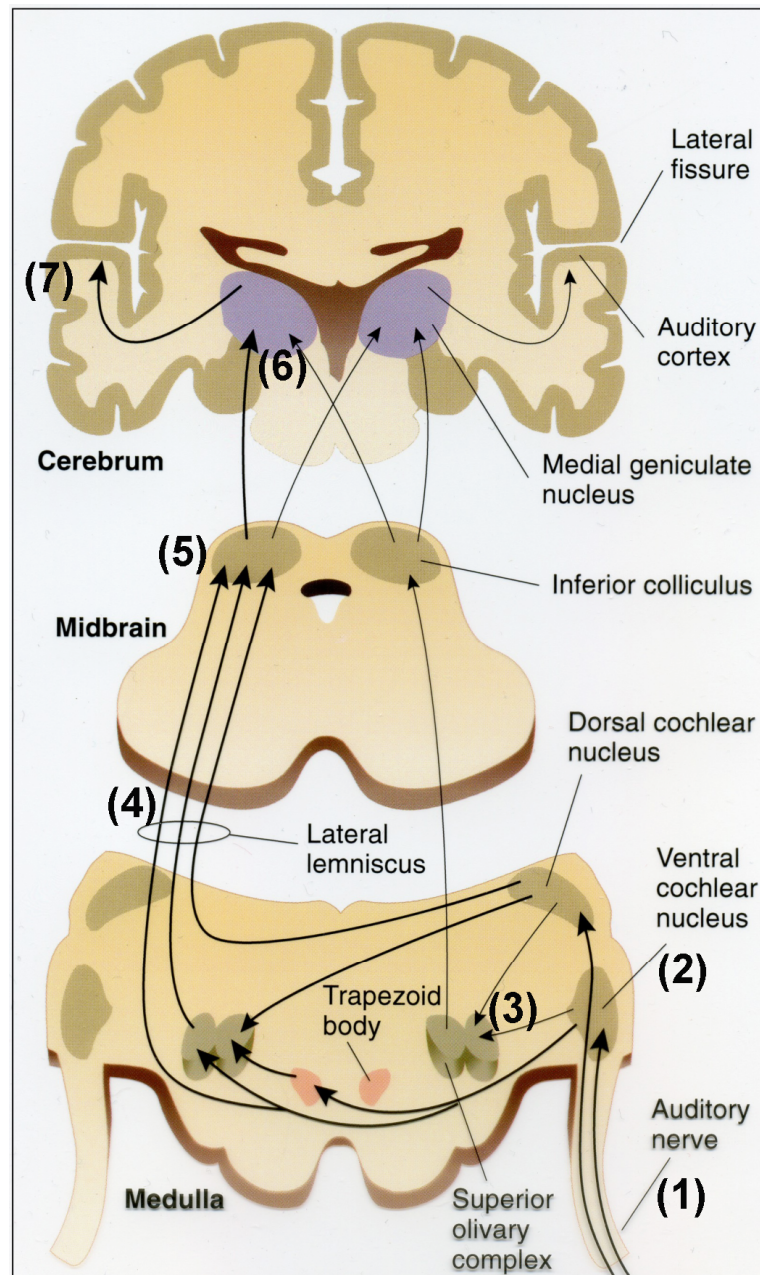


Figure 1. Pathways of the central auditory system. Sound information from the periphery enters the brain via the the auditory nerve (1) at the cochlear nucleus in the brainstem (2). The cochlear nucleus projects to other brainstem nuclei including the superior olivary complex (3), and provides substantial contralateral innervation (4) of the inferior colliculus in the midbrain. All ascending auditory information enters the inferior colliculus (5), where it is integrated prior to being sent to higher order processing centers including the medial geniculate body of the thalamus (6) and auditory cortex (7). Image adapted from Martin and Clark, 2006.

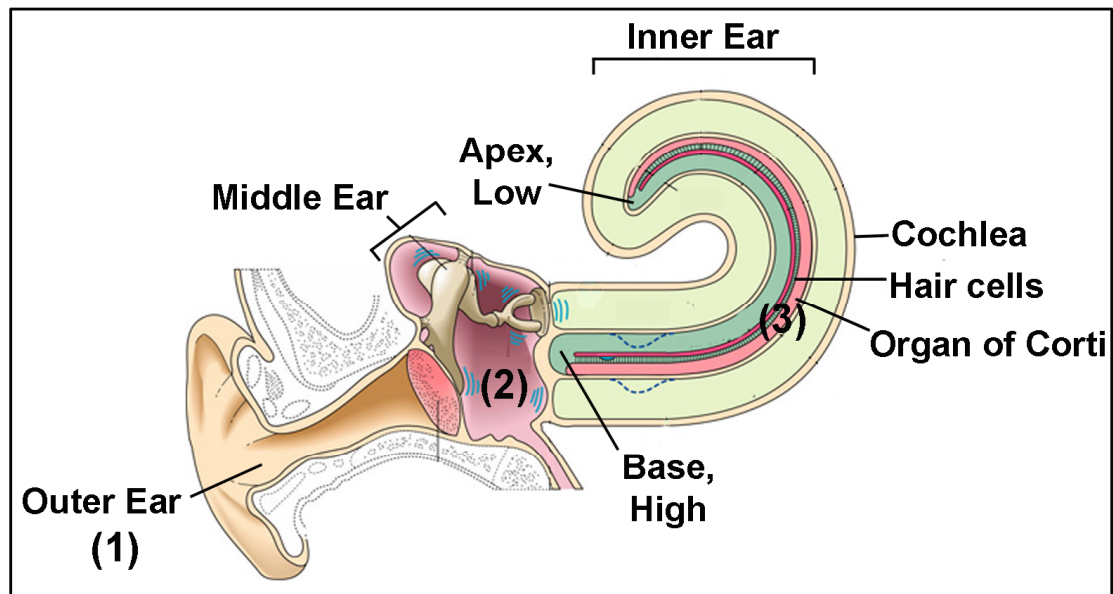


Figure 2. The cochlea of the inner ear establishes the topography of frequency preserved throughout the central auditory system. Sound travels through the air and enters the outer ear (1). Sound vibrations are transferred to a fluid medium within the inner ear through the mechanics of the middle ear (2). Hair cells within the organ of Corti (3) are topographically arranged and respond to fluid vibrations of characteristic frequencies from high to low (base to apex, respectively). Image adapted from Marieb, 2005.

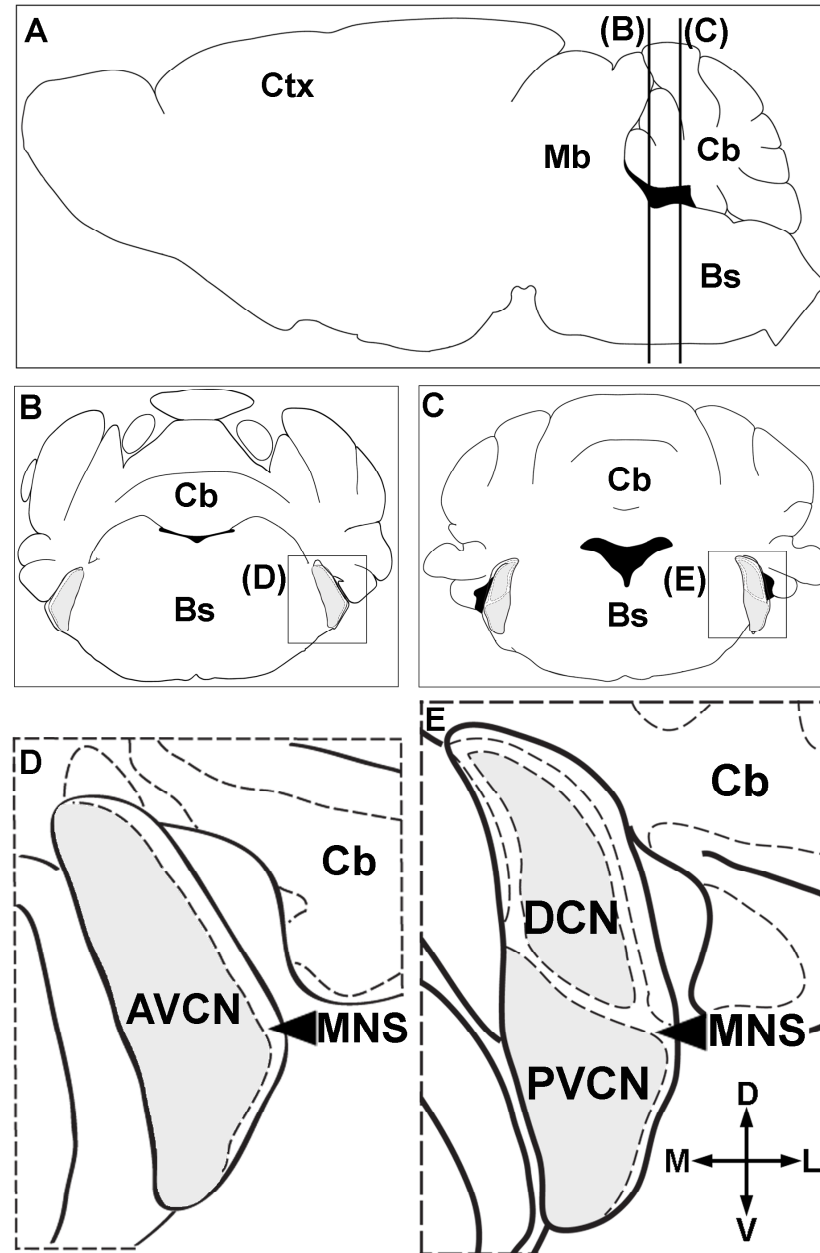


Figure 3. The cochlear nuclei of the mouse brain. (A) Sagittal schematic of the mouse brain depicting coronal planes of section containing the cochlear nuclei shown in (B, C). (B) Coronal section through the cerebellum (Cb) and anterior brainstem (Bs) depicting the boxed location of the AVCN in (D). (C) Coronal section posterior to (B), depicting the boxed location of the DCN/PVCN in (E). (D, E) The principal AVCN, DCN, and PVCN subdivisions, highlighting the magnocellular cores in grey, and the microneuronal shell (MNS) containing granule cells is represented by dashed lines. The dorsal-ventral (D-V) and medial-lateral (M-L) axes are depicted for orientation in (D, E). Other abbreviations: cortex (Ctx), midbrain (Mb). Image adapted from Brown and Zervas, 2011, submitted.

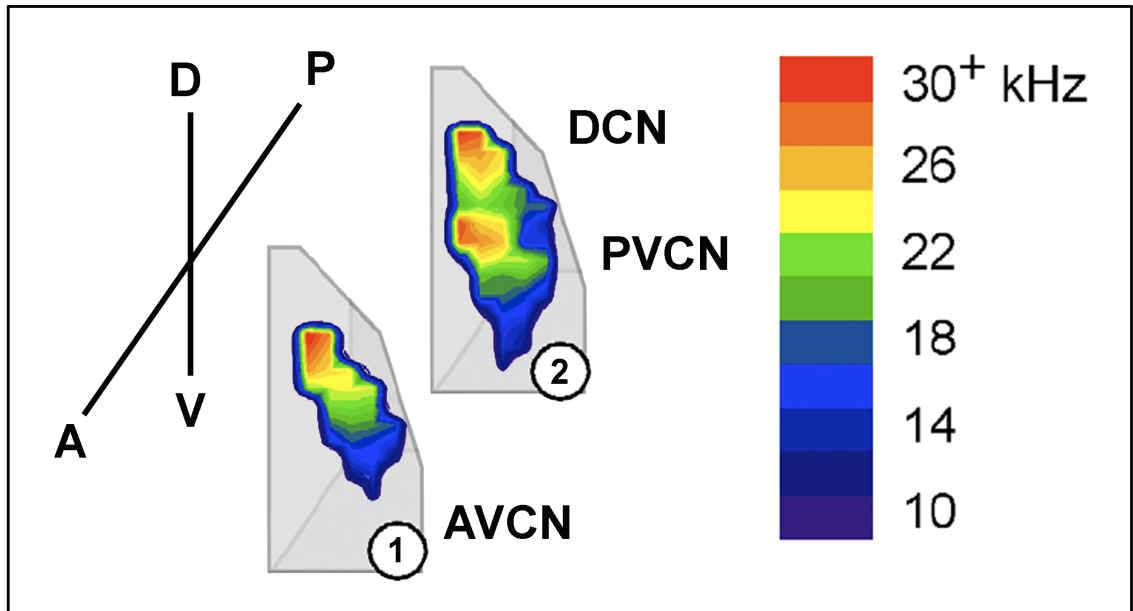


Figure 4. Tonotopic organization of the cochlear nucleus. Electrophysiological mapping of the mouse cochlear nucleus demonstrated the gradients of frequency response correlated from high to low across the dorsal-ventral (D-V) axis of each subdivision. The principal subdivisions are depicted: AVCN (1), DCN/PVCN (2). The anterior-posterior (A-P) and dorsal-ventral (D-V) axes are depicted for orientation. Image adapted from Luo et al., 2009.

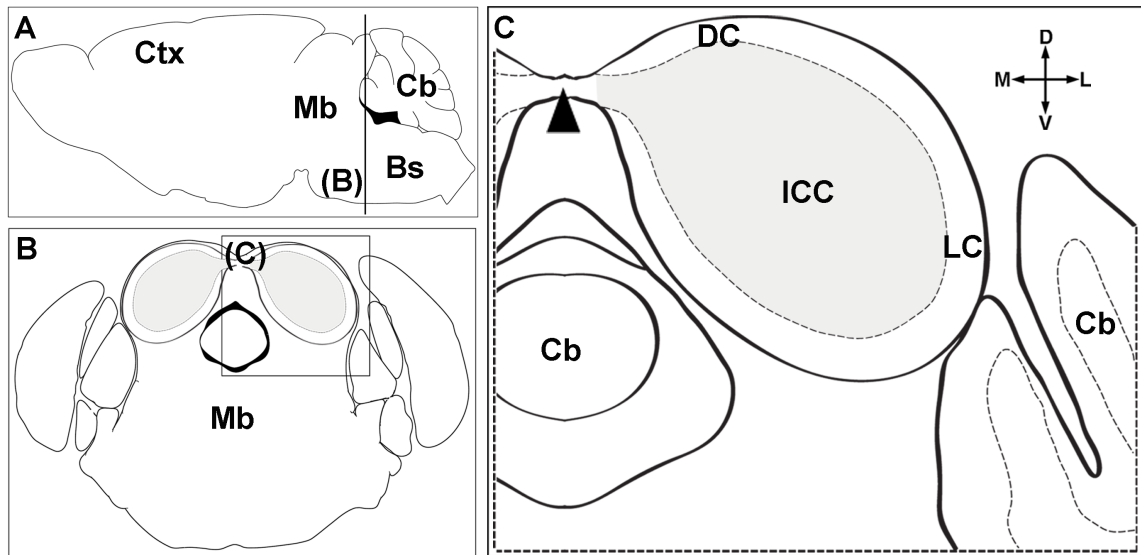


Figure 5. The inferior colliculus of the mouse brain. (A) Sagittal schematic of the mouse brain depicting the coronal plane of section containing the inferior colliculus in (B, C). (B) Coronal section through the midbrain (Mb) delineating the boxed (unilateral) inferior colliculus in (C). (C) The central nucleus of the inferior colliculus (ICC) in grey is surrounded by a dorsal (DC) and lateral cortex (LC). Arrowhead identifies the commissural connections between the bilateral inferior colliculi. The dorsal-ventral (D-V) and medial-lateral (M-L) axes are depicted for orientation in (C). Other abbreviations: brainstem (Bs), cortex (Ctx), cerebellum (Cb).

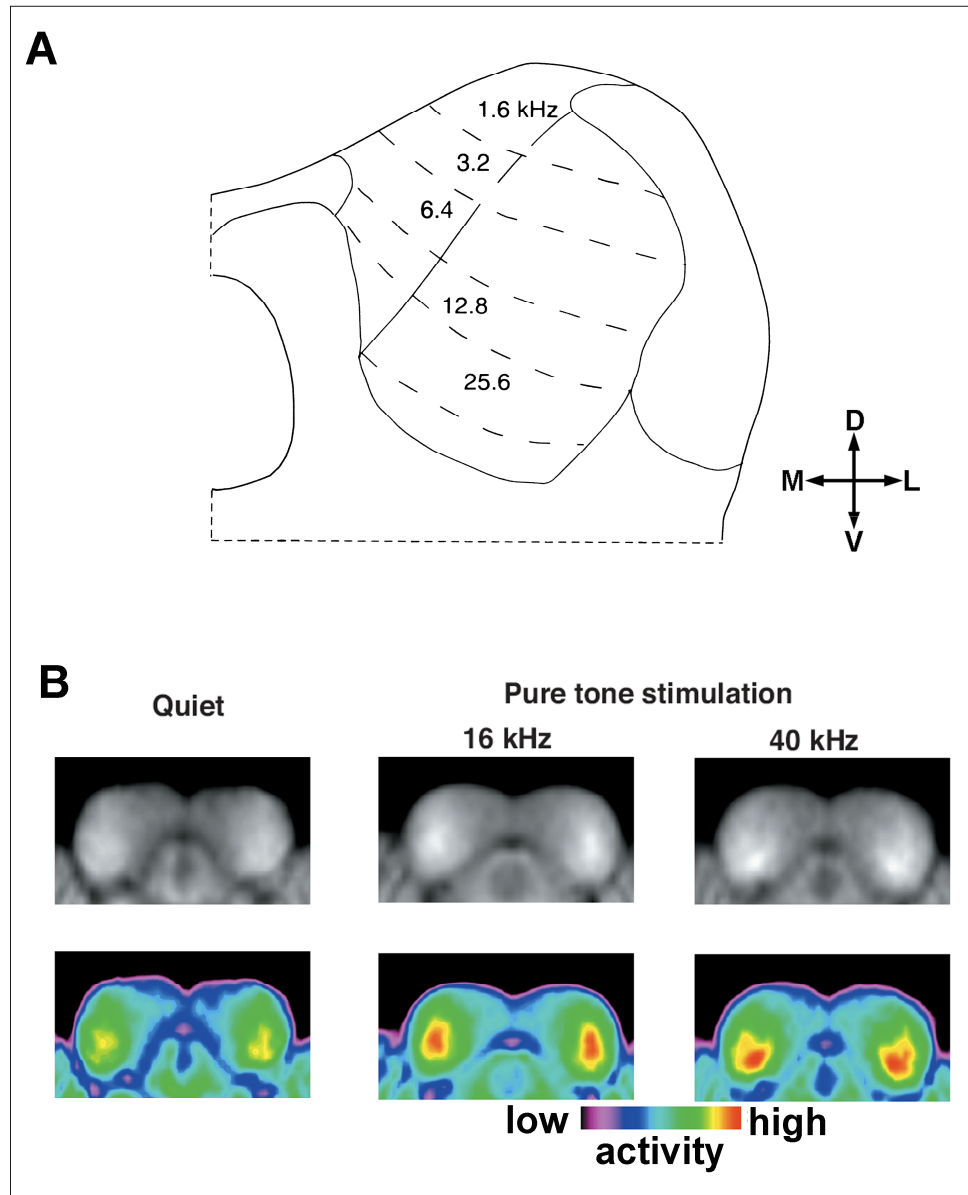


Figure 6. Tonotopic organization of the central nucleus of the inferior colliculus (ICC). (A) Classically, electrophysiological methods have defined the characteristic frequencies of lamina in the ICC of the cat brain. The representation of frequency in the ICC is low to high, dorsolaterally to ventromedially. (B) Activity-dependent, manganese-enhanced magnetic resonance imaging (MEMRI) of the mouse brain following exposure to pure tone acoustic stimulation shows the shift in activity to more ventral aspects of the ICC upon high frequency (40 kHz) stimulation. The dorsal-ventral (D-V) and medial-lateral (M-L) axes are depicted for orientation. Images adapted from (A) Winer and Schreiner, 2005 (Originally from Merzenich and Reid, 1974) and (B) Yu et al., 2007.

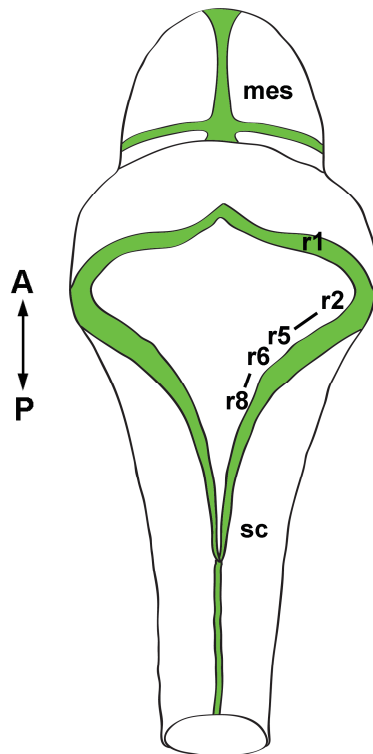


Figure 7. *Wnt1* expression in auditory primordia. A top-down, dorsal schematic view of a midgestation mouse embryo delineating dorsal *Wnt1* expression domains (green). *Wnt1* expression in the dorsal midbrain primordia (mesencephalon, mes) is confined to a midline stripe and ring of expression at the posterior limit of the mes, which encompasses the inferior colliculus progenitor domain. *Wnt1* expression in the hindbrain is found within the rhombic lip of rhombomeres (r)1-8. The upper rhombic lip defines the dorsal germinal zone of r1, the cerebellar primordium. The lower rhombic lip spans the dorsal tiers of r2-r8, encompassing the germinal zones of the cochlear nucleus (r2-r5) and pre-cerebellar system (r6-r8). Caudal to r8 is the spinal cord neuroepithelium (sc). The anterior-posterior (A-P) axis is indicated.

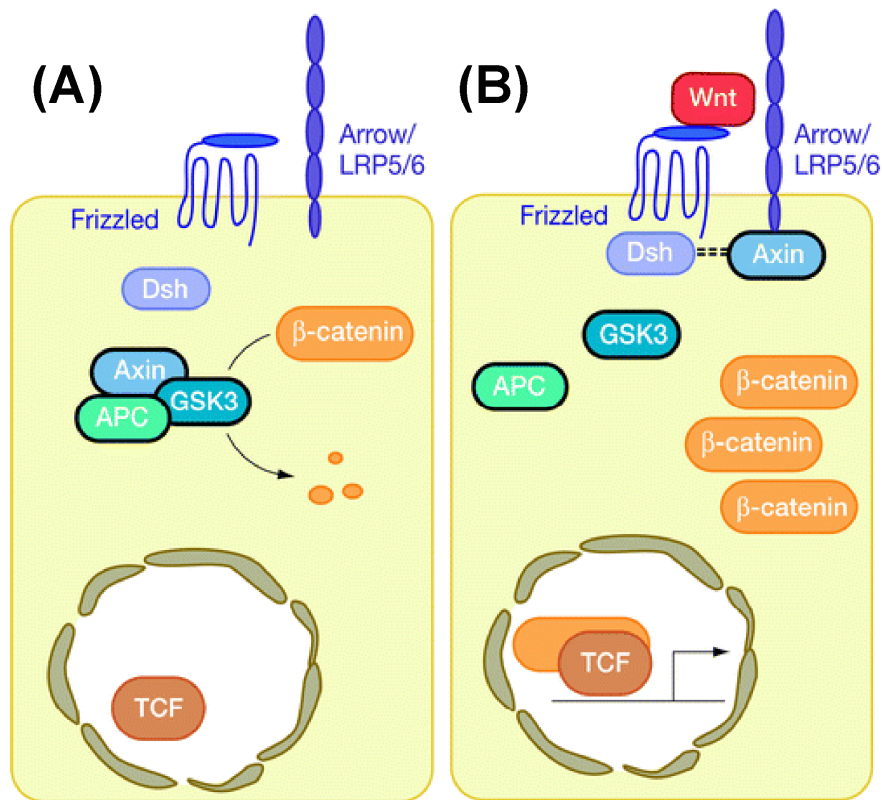


Figure 8. Canonical Wnt/β-catenin signalling. (A) In the absence of WNT ligand, AXIN/APC/GSK3β target β-catenin for degradation. (B) WNT ligand binds Frizzled, and activates Dsh. Dsh induces AXIN/APC/GSK3β complex disassembly, and β-catenin accumulates, allowing it to enter the nucleus to mediate transcription of target genes through a complex containing TCF. Image adapted from Logan and Nusse, 2004.

Cumulative *Wnt1* fate mapping

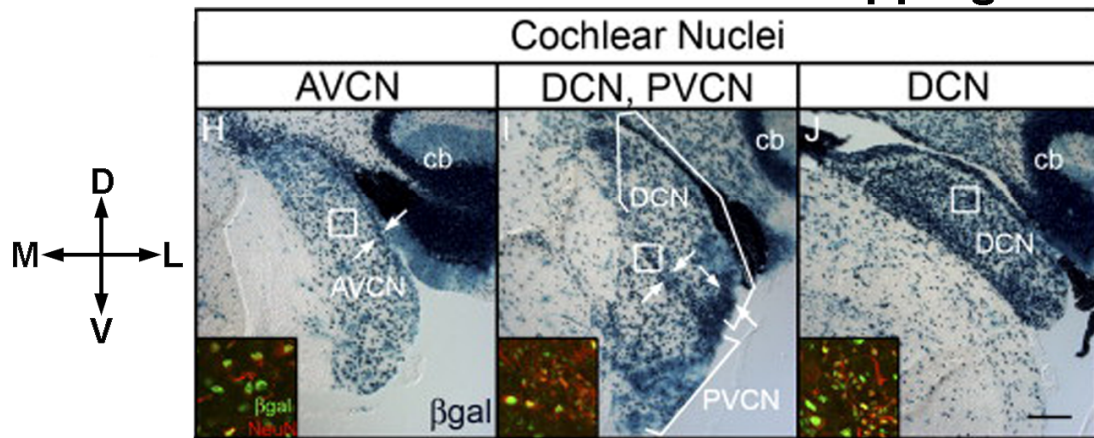


Figure 9. *Wnt1*-expressing progenitors contribute to the cochlear nucleus. Cells derived from *Wnt1*-expressing progenitors are labeled by β -galactosidase activity in blue. Cumulative fate mapping demonstrated that each of the cochlear nuclei are derived from *Wnt1*-expressing progenitors through the dorsal-ventral (D-V) and medial-lateral extent (M-L) in adult coronal sections. Image adapted from Farago et al., 2006.

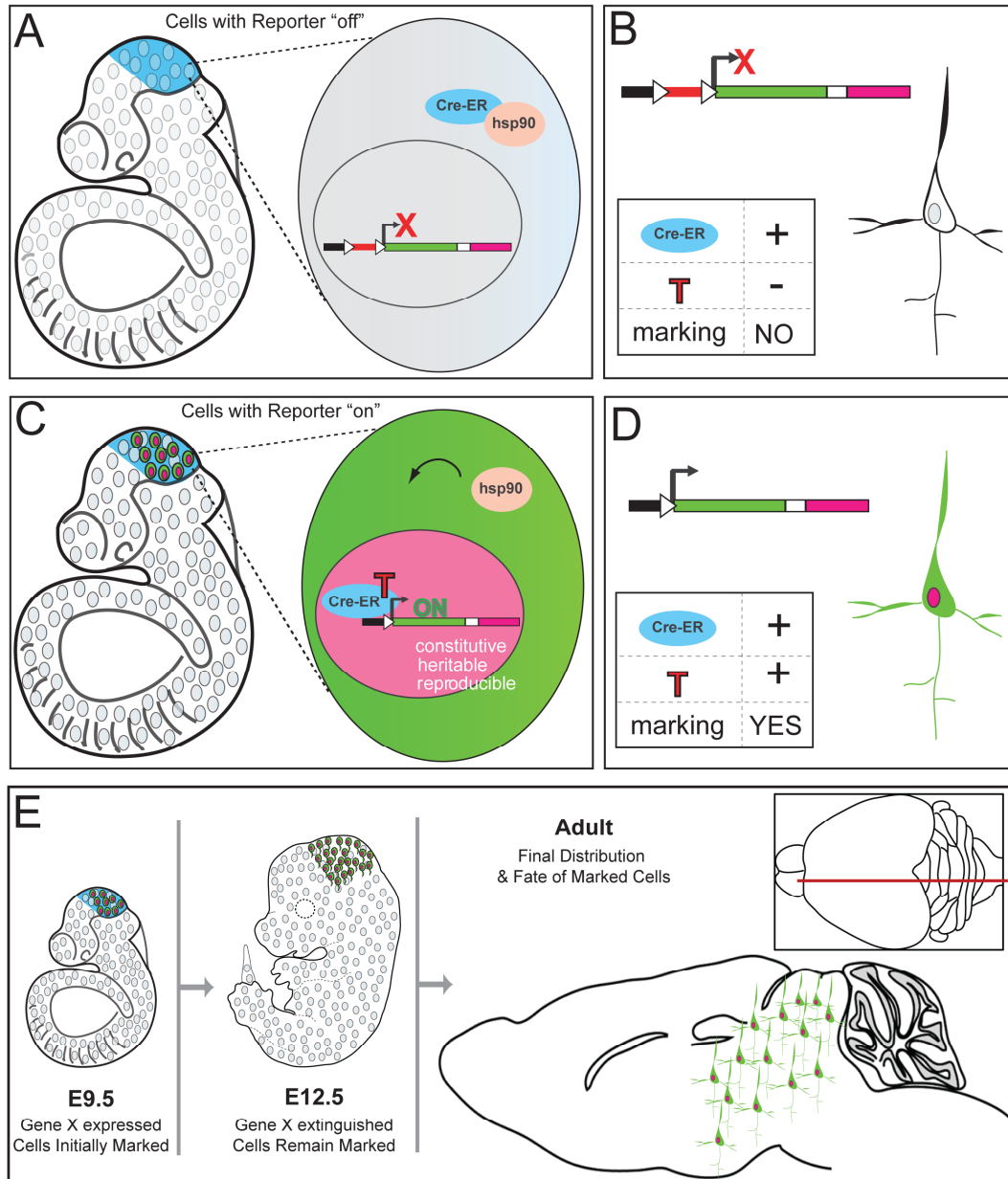


Figure 10. Genetic Inducible Fate Mapping (GIFM).

GIFM permanently and heritably marks cells of a specific genetic lineage. GIFM requires three components: CreER, a conditional reporter allele, and tamoxifen. Mice are engineered to express CreER in a unique progenitor domain (blue). Once expressed (A), CreER is sequestered in the cytoplasm by Heat Shock Protein 90 (hsp90), and the reporter allele remains quiescent (A,B). Once introduced *in utero*, tamoxifen binds to CreER (C), releases it from hsp90, and allows CreER to enter the nucleus. In this example, CreER excises the floxed STOP sequence (C) in the *mGFP* reporter allele (Hippenmeyer et al., 2005). This results in the constitutive and heritable expression of GFP (green) and nuclear β -galactosidase (pink) (C,D), which can be detected by endogenous fluorescence or immunocytochemistry. Temporal control of marking is achieved by tamoxifen, which can be administered at specific time points. The contribution of marked progenitors can be assessed later in development or in the adult brain (E). Brown et al., 2009

Inducible *Wnt1* fate mapping, E10.5 marking

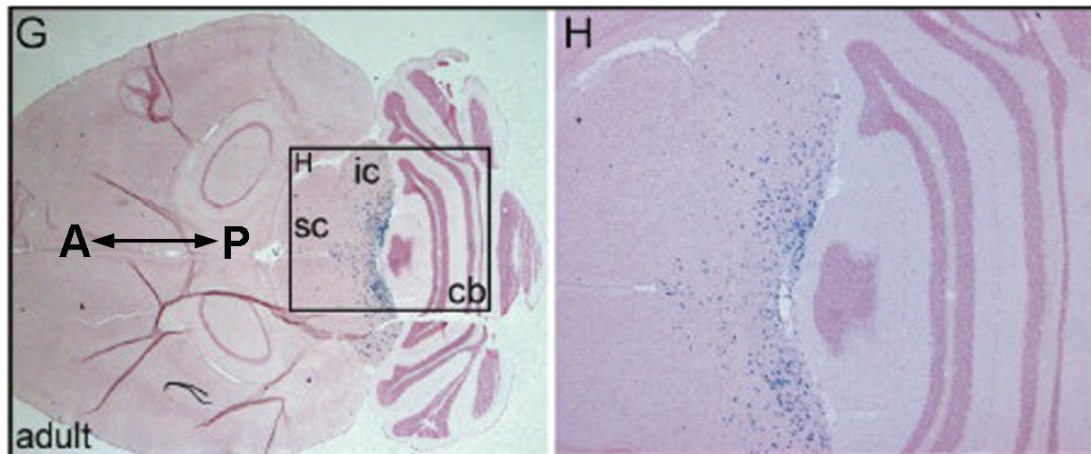


Figure 11. *Wnt1*-expressing progenitors contribute to the inferior colliculus. Cells derived from *Wnt1*-expressing progenitors are labeled by β -galactosidase activity in blue. Marking *Wnt1*-expressing progenitors at E10.5 through GIFM resulted in posterior contribution to the inferior colliculus. The anterior-posterior (A-P) axis of the adult horizontal section is indicated. Image adapted from Zervas et al., 2004.

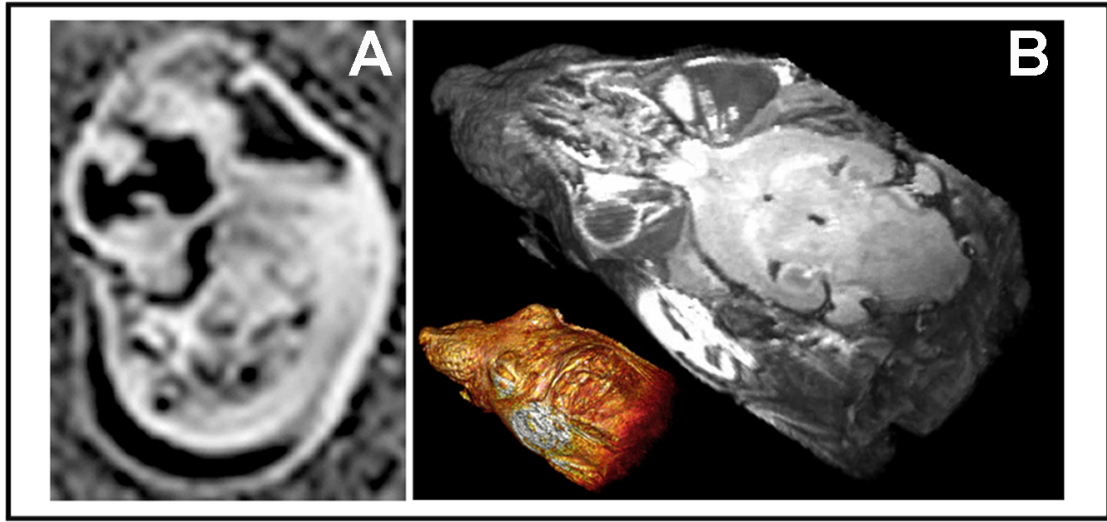


Figure 12. MRI of embryos and adult brains of mice. Using a 3T magnet, we imaged E11.5 mouse embryos (A) and adult brains (B) at $150\ \mu\text{m}^3$ resolution. Brown and Zervas, unpublished.

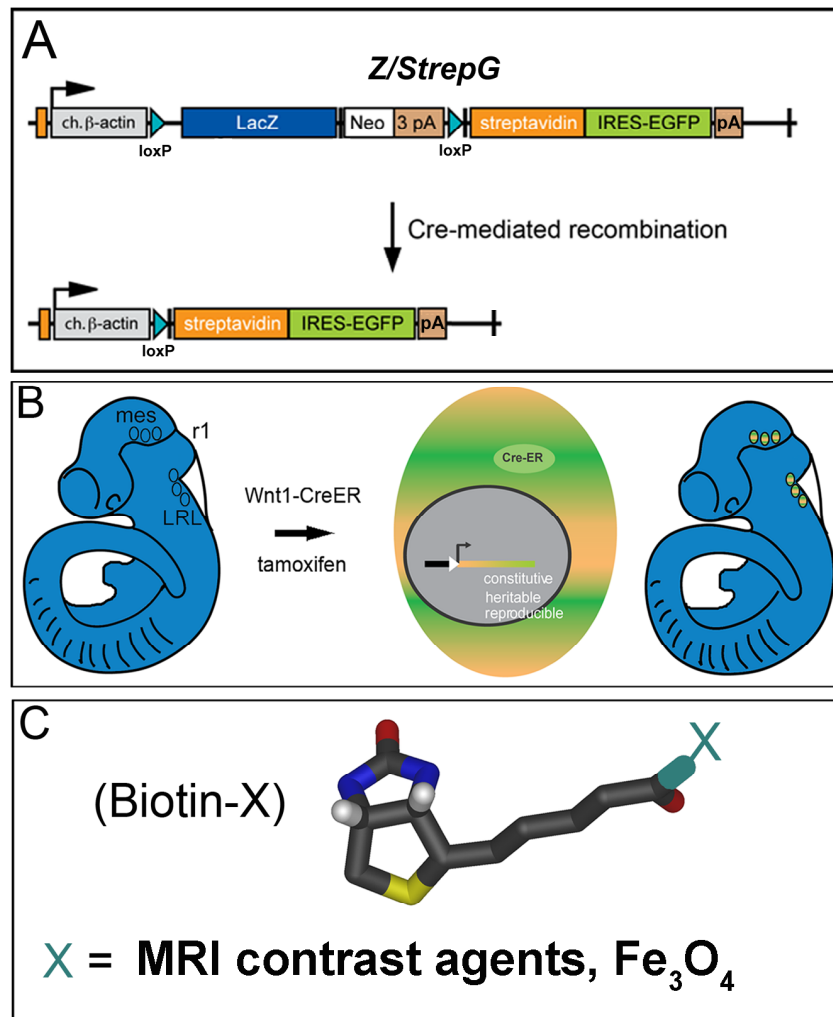


Figure 13. Z/StrepG concept design. (A) Prior to recombination, *Z/StrepG* constitutively expresses only *LacZ* in all cell types from the chick β -actin promoter. Following recombination, *LacZ* is excised and both streptavidin and GFP are expressed. (B) In this example, *Z/StrepG* is coupled with *Wnt1-CreER* for GIFM. Prior to tamoxifen administration, *LacZ* (blue) is expressed throughout the midgestation embryo. Post-tamoxifen, *Wnt1-CreER* expressing cells in the midbrain (mes) and hindbrain (LRL) recombine *Z/StrepG* and express streptavidin (orange) and GFP (green). Biotinylated contrast agents such as iron oxide (C) may then be delivered *in utero* or post-natal to assess three-dimensional *Wnt1* lineage contribution by MRI.

Chapter 2

Temporal expression of *Wnt1* in auditory progenitors defines the distribution and molecular profile of neurons in the cochlear nucleus and inferior colliculus.

Abstract

A central tenet of auditory system organization is the preservation of tonotopy at each anatomical processing level which recapitulates the frequency topography originating within the cochlea. The neuronal and axonal basis for tonotopy is established within the hindbrain and midbrain before the onset of hearing. The developmental mechanisms governing the distribution of neurons underlying tonotopic organization in the brain are relatively unknown. Genetic approaches in mice have demonstrated that *Wnt1*-expressing progenitors of the rhombencephalon and dorsal mesencephalon contribute to the cochlear nucleus and inferior colliculus, respectively. However, how the timing of *Wnt1* expression is linked to the spatial distribution and terminal fate of these progenitors within tonotopically organized structures has not been determined. Through genetic inducible fate mapping we show that the temporal control of gene expression in auditory primordia relates to how functional subdivisions of auditory centers are established. In particular, the timing of *Wnt1* expression in auditory progenitors predicts the extent and distribution of *Wnt1*-derived neurons across the tonotopic map within each structure. Moreover, *de novo* *Wnt1* expression in progenitors of the cochlear nucleus correlates with the establishment of biochemically distinct but functionally similar neurons. Conversely, our results provide evidence for a model of progressive lineage restriction to explain how progenitors contribute to the auditory midbrain following neurulation. These findings provide a deeper understanding of how functionally interconnected but spatially segregated auditory centers are generated during development.

Introduction

The central auditory system is comprised of several anatomically distinct brain regions that are essential for normal sound processing. The functional coordination between auditory centers requires the proper distribution of specific cell types during development. In addition, innervation at each anatomical level, from the brainstem to subcortical and cortical areas, is vital for processing auditory information (reviewed by Winer and Schreiner, 2005). The cochlear nucleus of the hindbrain is the initial site of convergence for auditory information in the brain, receiving topographically organized primary input from the cochlea of the inner ear (Osen, 1970). The precise mapping of afferents within the cochlear nucleus establishes the foundational tonotopic representation of sound within the brain (reviewed by Rubel and Fritzsch, 2002). Neurons within the cochlear nucleus project to and innervate the inferior colliculus (Cant and Benson, 2003; Malmierca et al., 2005), an obligatory midbrain processing center that integrates ascending auditory information critical to the function of higher order auditory centers (Aitkin and Phillips, 1984). Afferents of the cochlear nucleus terminate within the central nucleus of the inferior colliculus (ICC) in a laminar distribution that contributes to the tonotopic organization of the auditory midbrain (Cant and Benson, 2003; Malmierca et al., 2005; Malmierca et al., 2008; Loftus et al., 2010). Concurrent with the projection path of neurons within the cochlear nucleus is the differential expression of the calcium-binding proteins parvalbumin (PV), calretinin (CALR), and calbindin (CALB) (Fredrich et al., 2009).

A relatively unresolved issue is how the molecularly and functionally distinct types of neurons in the auditory hindbrain and midbrain are established during development. Interestingly, the innervation and neuronal organization responsible for tonotopy in the hindbrain and midbrain are largely established before the onset of hearing (Friauf and Kandler, 1990; Kandler and Friauf, 1993; Gurung and Fritzsche, 2004), suggesting that the functional organization of auditory centers may be related to cell fate specification in progenitor pools that contribute to the hindbrain and midbrain. These progenitor pools can be defined by distinct gene expression patterns (Wilkinson et al., 1987; Wilkinson et al., 1988; Murphy et al., 1989). One gene that has been instructive to understand the development of the auditory system is *Wnt1* (Zervas et al., 2004; Farago et al., 2006; Nichols and Bruce, 2006). *Wnt1* encodes a secreted glycoprotein that plays diverse roles in development, including proliferation and cell fate decisions (Lee et al., 2004; Ciani and Salinas, 2005), and is expressed in the hindbrain and midbrain primordia (rhombencephalon and mesencephalon, respectively) (Wilkinson et al., 1987; Zervas et al., 2004; Farago et al., 2006). Genetic fate mapping studies demonstrate that *Wnt1*-expressing progenitors in the lower rhombic lip (LRL) of rhombomeres 2-5 and the mesencephalon differentially contribute to the cochlear nucleus and inferior colliculus, respectively (Zervas et al., 2004; Farago et al., 2006). However an important gap in the field of auditory system development is determining the relationship between the timing of early gene expression in progenitors and the terminal fate and spatial address of neurons within the tonotopic representation of mature auditory centers.

To begin to bridge this gap we used genetic inducible fate mapping (GIFM: reviewed by Joyner and Zervas, 2006) to delineate how neuronal progenitors contribute to specific cell types and spatial locations in the cochlear nucleus and inferior colliculus based on the temporal control of *Wnt1* expression. We show that timing of *Wnt1* expression in auditory progenitors predicts the extent, distribution, and molecular profile of mature neurons. In addition, we used mutant analysis to provide evidence of a role for *Wnt1* in patterning the auditory structures. This study provides the framework to further investigate the common mechanisms by which *Wnt1* acts in establishing the cochlear nucleus and inferior colliculus.

Materials and Methods

Animals and Genotyping

Wnt1-Venus transgenic mice (generated by C. Bromleigh) were gratefully obtained from A. Joyner. This transgenic line was generated by subcloning a cassette encoding yellow fluorescent protein (*Venus*, green fluorescent protein variant) into a multi-cloning site between the translated and untranslated region of exon 1, which places *Venus* under the control of *Wnt1* regulatory elements. This same configuration was successfully used to control *CreER^T* expression in *Wnt1-CreER^T* transgenic mice (Zervas et al., 2004). The fidelity of this allele has recently been verified by *in situ* hybridization (Brown et al., 2011). *mGFP* (*Tau^{lox-}STOP-loxmGFP-IRES-NLS-LacZ-pA*) mice (Hippenmeyer et al., 2005) were generously provided by S. Arber and have recently been used to follow the *Wnt1* lineage

(Brown et al., 2009; Ellisor et al., 2009; Ellisor and Zervas, 2010). Mice were housed and handled in accordance with Brown University Institutional Animal Care and Use Committee guidelines. Genotyping was done as previously described (Ellisor et al., 2009; Brown et al., 2009). Operationally, *Wnt1-Venus* embryos were detected by whole-mount fluorescence using filters for GFP, while in tissue sections *Wnt1-Venus* is detected by anti-GFP antibodies (described below).

Embryonic Tissue Preparation

Wnt1-Venus transgenic mice were bred with Swiss Webster wildtype females (Taconic) to obtain litters at three different embryonic stages (E8.5, E10.5, E12.5). Embryos were dissected in PBS over ice and *Wnt1-Venus* embryos were identified by GFP fluorescence and confirmed by genotyping for GFP (Ellisor et al., 2009). Embryos were fixed in 4% PFA overnight at 4°C, cryoprotected, and embedded in OCT. Embryos (n=3 across two litters for each embryonic stage) were sectioned sagittally (12 µm) with a Leica cryostat and stored at 20°C. Sections were immunolabeled as described below.

Genetic Inducible Fate Mapping (GIFM)

GIFM experiments were conducted by crossing *Wnt1-CreERT^T;mGFP* males with Swiss Webster wildtype females (Taconic) as previously described (Brown et al., 2009; Ellisor et al., 2009; Ellisor and Zervas, 2010; Brown et al., 2011). The morning (0900) of the day a vaginal plug was detected was

designated as 0.5 days post-coitus. Tamoxifen was administered at a dose of 4 mg to timed pregnant females by oral gavage at 0900 (Brown et al., 2009; Ellisor et al., 2009). Mice were genotyped by obtaining a tail biopsy and performing PCR analysis on DNA from tail lysates. At 6 weeks of age *Wnt1-CreER^T; mGFP* fate mapped mice were deeply anesthetized with Nembutal (100 mg/kg) and intracardially perfused with 4% paraformaldehyde (PFA). Craniotomies were performed to extract the fate mapped brains, which were stored in PFA at 4°C until sectioning. Brains were embedded in 3% agarose in PBS and sectioned coronally (40 µm) with a Leica vibratome (Brown et al., 2009). Three fate mapped brains across two litters were processed for each marking and analysis stage.

Immunocytochemistry

Sections were immunolabeled as previously described (Ellisor et al., 2009). Briefly, sagittal sections from *Wnt1-Venus* embryos at E8.5, E10.5 and E12.5 were analyzed using an anti-GFP antibody (1:600, Molecular Probes, Cat # A-6455). Adult coronal sections (from fate mapping experiments) were immunolabeled with an anti-β-galactosidase (β-gal) antibody (1:500, Biogenesis, Cat # 4600-1409 and 1:500, Abcam, Cat # ab9361-250) to identify *Wnt1*-derived cells (Ellisor et al., 2009). In addition, anti-Calretinin (1:5000, Chemicon; Billerica, MA; Cat # AB1550), anti-Calbindin (1:1000, Swant, Cat # CB3a) and anti-Parvalbumin (1:1000, Sigma, Cat # P3088-.2ML) antibodies were used as biomarkers. The secondary antibodies, Alexa 488 (Invitrogen; Cat # A-21206 donkey anti-rabbit IgG; Cat # A-21202, donkey anti-mouse IgG; Cat # A-11055

donkey anti-goat IgG), Dylight 549 (Jackson ImmunoResearch Laboratories; Cat # 703-505-155, donkey anti-chicken) were prepared at 1:500.

Histology

Silver staining was performed as previously described (Gallyas, 1979). Briefly, lateral sagittal sections (40 μ m thick) from *Wnt1*^{+/+} and *Wnt1*^{sw/sw} mice (processed in parallel) were mounted on glass slides and incubated in pyridine:acetic anhydride (2:1) for 30 minutes. Tissues were rinsed 3x in ddH₂O for 5 minutes, followed by 45 minute incubation in ammoniacal silver nitrate. Tissues were rinsed 3x in 0.5% acetic acid for 10 minutes, incubated in developer solution (Gallyas, 1979) for 5 minutes, and terminated by incubation in 1% acetic acid for 10 minutes. Sections were then dehydrated through ethanol incubations, followed by xylene. Slides were coverslipped with Permount mounting media.

Microscopy and Cell Counting

Whole-mount images were collected with a Leica MZ16F stereoscopic epifluorescent microscope using PictureFrame3 software. Tissue section images were collected with a Leica DM600B epifluorescent microscope using Velocity 5.2 imaging software (Improvision). Low magnification images were captured with 2.5x and 5x objectives and high magnification images were obtained using a motorized stage with 10x and 20x objectives. Magnifications are indicated by scale bars. All images were pseudo colored live as part of the acquisition

palettes. Imaging data sets were exported to Adobe Photoshop CS3 where montages of representative data were generated. We counted neurons in the central nucleus of the inferior colliculus by acquiring 10x images spanning the entire central nucleus that were stitched together in Adobe Photoshop CS3. Profiles in the red channel corresponded to the nuclear β -gal labeling of fate mapped neurons in accordance with the genetics of our fate mapping alleles (Ellisor et al., 2009; Brown et al., 2009; Ellisor and Zervas, 2010). Images were imported into ImageJ and the data was converted to binary images and counted using the Analyze Particles function. Magnocellular neurons of the cochlear nucleus were manually counted to avoid the densely labeled granule cell layers that were prohibitive to the automated method described above. Manual counting was done by analyzing images in Photoshop CS3 with the Count Tool function. For each time point analyzed 3 representative sections from 3 separate animals (9 sections total per time point) were counted for each structure. The data are represented as the average number (+/- the standard deviation) of *Wnt1*-derived neurons per section. Statistical analysis using Student's *t*-test was performed to determine the significance of change in contribution between time points of analysis.

Results

Wnt1 expression in auditory primordia

We first assessed the distribution of *Wnt1*-expressing progenitors in *Wnt1-Venus* embryos to better understand where the initial populations of midbrain and

hindbrain progenitors resided at key developmental stages (Figure 1). We evaluated GFP-labeled domains by both whole-mount fluorescence and GFP immunocytochemistry at E8.5, E10.5 and E12.5. Specifically, GFP labeling in *Wnt1-Venus* transgenic mice allowed us to determine the state of *Wnt1* expression across development. At E8.5, *Wnt1* was diffusely expressed throughout the mesencephalon (mes) and faintly observed in posterior rhombomeres (rhomb) (Figure 1A, solid and open arrowheads, respectively), consistent with previous reports (Wilkinson et al., 1987; Zervas et al., 2004). We analyzed mid-sagittal sections to provide a more detailed view of *Wnt1* expression at E8.5 (Figure 1B-D). *Wnt1* expression spanned the dorsal-ventral (D-V) extent of the mesencephalic neuroepithelium (Figure 1C, bracketed area). However *Wnt1* was heterogeneously expressed and therefore progenitors which did not express *Wnt1* were readily observed within the expression domain. *Wnt1* expression in the rhomb was restricted to the dorsal tier of the neuroepithelium (Figure 1D, bracketed area). At E10.5, whole mount fluorescence revealed that *Wnt1* expression became confined to the dorsal and ventral midline and a ring of expression at the posterior limit of the dorsal mes (Figure 1E, solid arrowheads), in agreement with previous reports (Wilkinson et al., 1987; Gavin et al., 1990; Ellisor et al., 2009). In comparison to the E8.5 domain, *Wnt1* expression had expanded in the LRL (Figure 1E, open arrowheads) and extended caudally into the posterior hindbrain and spinal cord. Analysis of the E10.5 embryo *Wnt1* expression domains in a midline sagittal section showed the posterior mes expression domain (Figure 1F,G), as well as the broadened expression

throughout the LRL (Figure 1F,H). *Wnt1*-expressing progenitors in the dorsal mes at E10.5 appeared clonal within the neuroepithelium and were interspersed amongst non-expressing progenitors (Figure 1G). Similarly, progenitors that did not express *Wnt1* were found within the LRL at E10.5 (Figure 1H). The general whole-mount pattern of expression observed at E10.5 persisted until E12.5 (figure 1I-L), though the lateral aspects of the mes posterior ring showed diminished expression (Figure 1I, solid arrowheads), while the LRL expression domain continued to broaden (Figure 1I, open arrowheads). Sagittal section analysis confirmed that the mes expression domain was tightly restricted (Figure 1J,K) while the LRL had a broader domain of *Wnt1* expression (Figure 1J,L). The appearance of a broadened expression domain was likely due to the presence of early projections (Figure 1L, arrows) and migrating cells emanating from the LRL.

The Wnt1 lineage contributes to the cochlear nucleus in distinct temporal windows

We next addressed how the dynamically changing *Wnt1*-expressing progenitors contributed to auditory structures using GIFM. Specifically, we determined how the timing of *Wnt1* expression in progenitors was related to the organization of the cochlear nucleus using *Wnt1-CreERT^T;mGFP* transgenic mice. We administered tamoxifen to pregnant *Wnt1-CreERT^T;mGFP* mice between E8.5-E14.5 and analyzed the mature cochlear nucleus at 6 weeks of age. The *mGFP* reporter is driven by the *Tau* locus (Hippenmeyer et al., 2005), which allowed us to strictly assess the neuronal *Wnt1* lineage contribution (Ellisor et al.,

2009). Neuronal descendants of progenitors that expressed *Wnt1* at the time of tamoxifen administration expressed nuclear β -galactosidase (β -gal) and were identified through immunocytochemistry (Figures 2-6).

The cochlear nucleus is composed of three distinct divisions with overlapping function and organization: the anteroventral cochlear nucleus (AVCN), posteroventral cochlear nucleus (PVCN), and dorsal cochlear nucleus (DCN) (Osen, KK, 1988). The *Wnt1* lineage contributed to neurons within each of the cochlear nucleus subdivisions over a broad window of development (marking from E8.5-E12.5, Figure 2). The distribution of *Wnt1*-derived neurons differed amongst the individual nuclei and was dependent on when the progenitors expressed *Wnt1*. AVCN progenitors in the LRL, which expressed *Wnt1* at E8.5 and E10.5 gave rise to magnocellular neurons distributed in the dorsal aspect of the nucleus (Figure 2B,C). In contrast, *Wnt1*-expressing progenitors at E12.5 contributed to neurons more evenly throughout the D-V axis (Figure 2D). Granule cells within the microneuronal shell (MNS) of the AVCN were derived in greater abundance from progenitors which expressed *Wnt1* at E10.5 and E12.5 (Figure 2B-D, compare the MNS denoted in A). Similarly, early expression of *Wnt1* in PVCN progenitors at E8.5 resulted in a dorsal bias of contribution (Figure 2E). However, PVCN progenitors which expressed *Wnt1* at E10.5 or E12.5 contributed to neurons that were distributed throughout the PVCN core and MNS (Figure 2F,G). In contrast to the AVCN and PVCN, there was no discernible D-V bias in the distribution of *Wnt1*-derived DCN magnocellular neurons at any marking stage (Figure 2E-G). In addition, while AVCN/PVCN granule cells in the

MNS were primarily derived from later *Wnt1*-expressing progenitors, the MNS of the DCN was derived from progenitors expressing *Wnt1* at each marking stage (Figure 2E-G).

Through quantifying the contribution that *Wnt1*-expressing progenitors made to the magnocellular neurons within each nucleus, we demonstrated peaks of contribution from the *Wnt1* lineage to the cochlear nucleus (Figure 2H-J). Magnocellular neurons of the AVCN were predominantly derived from progenitors expressing *Wnt1* at E10.5, as the number of *Wnt1*-derived neurons increased fourfold compared to the contribution that resulted from marking *Wnt1*-expressing progenitors at E8.5 (increase from 38.9 ± 12.0 to 169.4 ± 47.2 *Wnt1*-derived neurons/section) (Figure 2H). Despite the extensive expression of *Wnt1* observed in the developing hindbrain at E12.5 (Figure 1L), the contribution of the *Wnt1* lineage to AVCN neurons declined (60.8 ± 34.4 *Wnt1*-derived neurons/section) (Figure 2H). By E14.5, progenitors expressing *Wnt1* no longer contributed to the cochlear nucleus (data not shown). The neuronal contribution of *Wnt1*-expressing progenitors to the PVCN resembled that of the AVCN; a sharp peak of contribution to the magnocellular core resulted from marking *Wnt1*-expressing progenitors at E10.5 (Figure 2E-G,J). A striking tenfold increase was observed with marking *Wnt1*-expressing progenitors at E10.5 versus at E8.5 (increase from 18.3 ± 14.4 to 188.4 ± 85.0 *Wnt1*-derived neurons/section) (Figure 2E,F,J). Similarly, despite broad expression of *Wnt1* in the developing hindbrain at E12.5, progenitors expressing *Wnt1* contributed to PVCN neurons in decreased numbers at this marking stage (65.1 ± 46.4 *Wnt1*-derived neurons/

section)(Figure 2G,J). In contrast to the AVCN and PVCN, very few neurons within the DCN magnocellular core were derived from *Wnt1*-expressing progenitors at E8.5 (8.0 ± 4.1 *Wnt1*-derived neurons/section) (Figure 2E,I). Unlike the sharp peak of contribution observed in the AVCN/PVCN when marked at E10.5, the contribution to the DCN occurred over an extended period (from E10.5-E12.5) (Figure 2E,I). The numbers of DCN neurons derived from E10.5 and E12.5 *Wnt1*-expressing progenitors were not significantly different (75.9 ± 19.9 and 70.2 ± 27.8 *Wnt1*-derived neurons/section, respectively) (Figure 2F,G,I). Thus prolonged expression of *Wnt1* in the developing hindbrain may be important for DCN neuronal specification.

In summary, progenitors of the cochlear nucleus which expressed *Wnt1* early (E8.5) gave rise to neurons distributed in the dorsal AVCN/PVCN or MNS of the DCN. Marking *Wnt1*-expressing progenitors at E10.5 resulted in a peak of contribution to the AVCN/PVCN and more abundant contribution throughout the DCN. There was a prolonged period of contribution to the DCN that resulted from progenitors which expressed *Wnt1* from E10.5-E12.5. However by E12.5 *Wnt1*-expressing progenitors gave rise to fewer AVCN/PVCN magnocellular neurons, but continued to contribute to the MNS of each nucleus.

Wnt1-expressing progenitors contribute to biochemically distinct neurons in the cochlear nucleus

Calcium-binding proteins, such as PV, CALB, and CALR, are expressed exclusively by neurons (Bredderman and Wasserman, 1974; Heizmann, 1984;

Rogers, 1987; Celio, 1990). Moreover, the expression of PV, CALB, and CALR correlates with the functional distribution of neurons in cochlear nucleus subdivisions of rat (Fredrich et al., 2009). Thus, we used the calcium-binding proteins as molecular markers to determine whether the timing of *Wnt1* expression in progenitors was related to the establishment of biochemically distinct neurons of the cochlear nucleus (Figures 3-5). For each subdivision of the cochlear nucleus we performed double immunocytochemistry to identify whether neurons derived from the *Wnt1* lineage (β -gal positive) co-localized with calcium-binding proteins (PV, CALB, or CALR positive).

The *Wnt1* lineage in the magnocellular core of the AVCN were PV positive, regardless of the stage of marking (Figure 3A-C, insets). Strikingly, *Wnt1*-derived neurons did not express CALB or CALR in the AVCN (Figure 3D-F, G-I). These findings indicate that *Wnt1*-expressing progenitors contributed specifically to a molecularly distinct subpopulation (PV+) in the AVCN. In contrast, PV-expressing DCN neurons were not derived from progenitors expressing *Wnt1* at any time point examined (Figure 4A-C). However, DCN progenitors expressing *Wnt1* from E10.5-E12.5 gave rise to neurons that expressed CALB (Figure 4E,F) or CALR (Figure 4I). CALB-expressing neurons were derived from progenitors expressing *Wnt1* that were marked earlier (E10.5 lineage, Figure 4E), while CALR-expressing neurons were derived from progenitors expressing *Wnt1* later (E12.5 lineage, Figure 4I). The biochemical make up of *Wnt1*-derived neurons in the PVCN shared similarities to the *Wnt1* lineage in the AVCN and DCN (Figure 5). Progenitors expressing *Wnt1* from E8.5-E12.5 gave rise to both PV+ and CALB+

neurons (Figure 5A-C, D-F). As was observed in the AVCN, *Wnt1*-expressing progenitors did not contribute to CALR-expressing neurons (Figure 5G-I).

The auditory midbrain is established through progressive lineage restriction after E9.5

We next determined how *Wnt1*-expressing progenitors in the mes (Figure 1) contributed to the auditory midbrain through GIFM as described for the cochlear nucleus. Progenitors expressing *Wnt1* at E8.5 (Figure 1A, solid arrowheads) gave rise to neurons sparsely distributed throughout the inferior colliculus (Figure 6B,E) along the rostral-caudal axis (Supplementary Figure S1). We validated the ICC by PV immunolabeling (Coleman et al., 1992; Vater and Braun, 1994; Lohmann and Friauf, 1996; Sharma et al., 2009), which demonstrated that *Wnt1*-expressing progenitors contributed to the ICC and PV-expressing neurons within it (Figure 6A-C, insets). A quantitative analysis revealed that *Wnt1*-expressing progenitors at E8.5 gave rise to an average of 919.3 ± 307.5 ICC neurons/section (Figure 6E). Marking *Wnt1*-expressing progenitors following neural tube closure at E9.5 resulted in a fourfold increase in the number of *Wnt1*-derived ICC neurons as compared to marking a day earlier (3923.8 ± 722.7 ICC neurons/section)(Figure 6E). The large increase in contribution suggested *de novo* expression of *Wnt1* as the mes undergoes morphometric changes necessary for neural tube closure. The evidence for *de novo* expression of *Wnt1* in auditory midbrain progenitors was further supported by the spatial distribution of *Wnt1*-derived neurons; the E9.5 *Wnt1* lineage was

largely confined to the ICC (Figure 6C, dashed line), whereas E8.5 marking resulted in contribution throughout the inferior colliculus (compare Figure 6B vs C). Interestingly, despite the preserved *Wnt1* expression domain, there was a rapid and large decline in contribution (181.7 ± 132.8 *Wnt1*-derived ICC neurons/section) to the ICC from progenitors expressing *Wnt1* marked at E10.5 (Figure 6D,E). The distribution of the E10.5 *Wnt1* lineage was confined to the dorsomedial aspects of the ICC (Figure 6D). However, neurons of the posterior external cortex of the inferior colliculus (ECIC) were derived from *Wnt1*-expressing progenitors from E8.5-E11.5 (Supplementary Figure S1).

These data provided further evidence of a temporal role for *Wnt1* in specifying the distribution of neurons within an auditory structure. As the location of neurons within tonotopically organized tissues correlates with their auditory function, the timing of *Wnt1* expression in auditory progenitors may be important in establishing waves of functional cohorts across the tonotopic map.

Wnt1 is required for proper establishment of midbrain and hindbrain auditory centers

While GIFM allowed us to resolve how progenitors expressing *Wnt1* contributed to the auditory midbrain and hindbrain, these experiments did not address the functional requirement for *Wnt1* in the development of these structures. We utilized mice homozygous for the *swaying* allele (*Wnt1*^{sw/sw}) to further investigate the role of *Wnt1* in the development of the inferior colliculus and cochlear nucleus. The *swaying* allele is a naturally occurring single base pair

deletion in the coding sequence of *Wnt1* believed to result in a truncated protein product (Thomas et al., 1991).

Wnt1^{SW/SW} mice had phenotypes ranging from mild to severe with the most obvious defects occurring in the midbrain and cerebellum (Figure 7). Although abnormalities in midbrain/cerebellum development have been previously described in *Wnt1^{SW/SW}* mice (Lane, 1967; Bronson and Higgins, 1967; Bally-Cuif et al., 1995), a detailed analysis of affected auditory structures has not been reported. We determined how *Wnt1* perturbation altered the inferior colliculus by comparing *Wnt1^{+/+}* and *Wnt1^{SW/SW}* tissues immunolabeled for PV and CALB (Figure 7B-D). PV is a broad marker of the ICC, while CALB is largely confined to the ECIC (Coleman et al., 1992; Vater and Braun, 1994; Lohmann and Friauf, 1996; Sharma et al., 2009). In the wild type auditory midbrain there was clear delineation between the ICC and ECIC, as the PV and CALB positive domains were mutually exclusive (Figure 7B). Although much of the staining was diffuse due to PV and CALB positive afferents, the cell bodies of PV and CALB-expressing neurons were readily identified in *Wnt1^{+/+}* mice (Figure 7B, arrows and arrowheads, respectively). In *Wnt1^{SW/SW}* brains which exhibited a mild phenotype, the medial aspects of the presumptive ECIC (Figure 7C, ECIC*) were expanded, and the commissural connections between the colliculi were not easily distinguished (compare bracketed areas in Figure 7B vs C). However, both PV and CALB-expressing neurons were abundantly identified (Figure 7C, arrows and arrowheads, respectively). In addition to the morphological and cellular differences, ICC afferents in mildly affected *Wnt1^{SW/SW}* mice were disorganized as

they passed through the brachium en route to higher order auditory centers (Supplementary Figure S2B vs D). *Wnt1^{sw/sw}* mice that were categorized with the most severe phenotype exhibited loss of bilateral symmetry, presence of ectopic cell types, and aberrant A-P patterning in the midbrain (Figure 7D). The presumptive ICC were displaced in the D-V axis, and contained few PV-expressing cell bodies (Figure 7D, ICC*, right side in this example). Though CALB neurons were easily identified (Figure 7C, arrowheads), there was no clear delineation of the ECIC. Presumptive superior colliculus tissue (SC*), based on morphology and laminated expression of CALB, was found in plane with the presumptive ICC (compare Figure 7A vs C). In addition, ectopic cerebellar cell types as determined by morphology and co-expression of PV/CALB were found in clusters throughout the midbrain (Figure 7C, open arrowheads). These results confirmed that *Wnt1* is required for normal specification of the auditory midbrain and its subdivisions, and for the proper organization of commissural connections and afferents of the inferior colliculus.

The anterior aspects of the cochlear nucleus were also affected in *Wnt1^{sw/sw}* mice (Figure 8A-F). The AVCN exhibited a reproducibly broadened morphology and abundance of PV-expressing neurons (Figure 8B versus C). In addition, though commonly reported in rat (De Camilli et al., 1984; Hurd and Feldman, 1994; Koszeghy et al., 2009), we observed the invariant presence of Purkinje-like cells (PLC) in the most anterior aspects of mutant DCN (Figure 8D-F, arrow). Interestingly, the gross morphology and marker expression of PVCN and posterior DCN were largely unaffected (Figure 8G-K).

Discussion

The cochlear nucleus and inferior colliculus are interconnected and share aspects of neuronal, axonal, and fibrodendritic organization that are necessary for their role in normal hearing. We tested the hypothesis that progenitors that give rise to neurons in these functionally connected domains share developmental mechanisms. The pattern of early *Wnt1* expression in auditory primordia dynamically changes, but becomes largely fixed following neurulation (E9.0) through E12.5. We used GIFM to test two competing models of how *Wnt1* expression in auditory progenitors relates to the establishment of these structures. By determining the terminal fate and distribution of *Wnt1*-expressing progenitors, we have discerned whether these auditory structures are derived entirely from an early progenitor pool (progressive lineage restriction model) or whether waves of *Wnt1* expression (*de novo* expression model) are related to the generation of unique cell types over time. Interestingly, both mechanisms are employed in the development of *Wnt1*-derived auditory structures.

Wnt1 in the auditory hindbrain

Our results show that *Wnt1*-expressing progenitors that contribute to the cochlear nucleus exhibit waves of *de novo Wnt1* expression. This model is best exemplified by the contribution observed in the DCN (as summarized in Figure 9). If the DCN was derived entirely from progenitors expressing *Wnt1* early, we would have observed similar contribution to both granule cells and magnocellular neurons with early marking of progenitors at E8.5. However we found that the

microneuronal shell is derived from *Wnt1*-expressing progenitors throughout the span of *Wnt1* expression while the large projecting neurons of the magnocellular core are largely generated after E10.5. In addition, the competency of progenitors to give rise to each of the biochemically distinct cell types does not diminish as development proceeds. Instead, progenitors marked late generate CALR positive neurons of the DCN indicating that *de novo* expression of *Wnt1* in progenitors must occur sometime within the LRL after E10.5. Quantifying the contribution *Wnt1*-expressing progenitors made to the cochlear nucleus subdivisions over time confirmed that *de novo* peaks of contribution occurred that were independent of early *Wnt1* expression. Taken together, these results show that despite the broad expression of *Wnt1* throughout the LRL germinal zone progenitors exist within the neuroepithelium that do not express *Wnt1* early but turn it on at later developmental stages. Thus the timing and induction of *de novo* *Wnt1* expression may be instructive for the specification of individual cell types and the circuits they form. In further support of this idea is our assessment of molecular marker expression in *Wnt1*-derived neurons of the AVCN and PVCN. Through antero- and retrograde dye tracing, Fredrich et al. found the expression of calcium-binding proteins correlated with the organization of projections from neurons of the cochlear nucleus in rat. They determined that all AVCN/PVCN neurons that send projections to the contralateral ICC express PV, while all ipsilateral projecting neurons express CALR. Conversely, in the DCN half of the neurons that provide contralateral innervation of the ICC express CALB, while neurons providing ipsilateral projections do not express any of the calcium-

binding proteins. Interestingly, we found that *Wnt1*-expressing progenitors contribute primarily to PV but not CALR-expressing neurons of the AVCN/PVCN, or to CALB-expressing neurons of the DCN. Thus, the (contralateral) axonal organization responsible for connecting the cochlear nucleus and inferior colliculus may be related to developmental programs initiated through the temporal regulation of *Wnt1*.

We also showed that *Wnt1* is required for the normal development of the cochlear nucleus. A perturbation in *Wnt1* results in a truncated and broadened morphology of the AVCN and the invariant presence of PLCs in the anterior DCN. The presence of these cells, often referred to as displaced Purkinje cells, have been found throughout the brainstem of rat, including the cochlear nucleus (Hurd and Feldman, 1994; Koszeghy et al., 2009). However PLC positioning is unpredictable, and whether they participate in auditory processing remains controversial. As the location of PLCs within the DCN of *Wnt1^{sw/sw}* mice was invariant, the expression of *Wnt1* may normally act to prevent their presence within this region. Interestingly, the PVCN and posterior DCN were unaffected in *Wnt1^{sw/sw}* mice. The mild anterior phenotype and preservation of posterior nuclei likely resulted from *Wnt* redundancy through the expression of *Wnt3a* (Ikeya et al., 1997).

Wnt1 in the auditory midbrain

As was observed in the cochlear nucleus, the inferior colliculus was not entirely derived from the earliest *Wnt1*-expressing progenitor pool in the mes

(summarized in Figure 9). Early *Wnt1* expression in the mes dynamically changes concomitant with the closure of the anterior neural fold. While whole-mount analysis revealed a broad *Wnt1* expression domain prior to neural tube closure at E8.5, examination of *Wnt1*-expressing progenitors in tissue section uncovered non-expressing progenitors intermingled within the expression domain. Marking *Wnt1*-expressing progenitors at this early time point resulted in sparse contribution through the entire extent of the inferior colliculus, likely reflective of the broad expression domain of *Wnt1*. However, the peak contribution to ICC neurons arose from *Wnt1*-expressing progenitors at E9.5. This result implies *de novo* *Wnt1* expression occurs in the dorsal mes as primary neurulation proceeds and the pattern of *Wnt1* expression is refined. Additionally, the contribution from *Wnt1*-expressing progenitors rapidly decreased, and *Wnt1*-derived neurons were confined to dorsomedial aspects of the ICC but continued to populate posterior ECIC with marking at E10.5 and beyond. A model of progressive lineage restriction may explain how progenitors contribute to the inferior colliculus following neural tube closure. Thus the auditory midbrain is established by a mixed model of contribution from the *Wnt1* lineage, where an early wave of *de novo* expression is replaced by a progenitor population which then becomes progressively restricted over time.

As was demonstrated for the cochlear nucleus, *Wnt1* is required for the development of the auditory midbrain. In both mildly and severely affected *Wnt1*^{SW/SW} mice, the central nucleus and external cortex of the inferior colliculus were present, but the auditory midbrain in severely affected mice were aberrantly

patterned. Severe mutants had ectopic anterior midbrain and cerebellar tissues surrounding the presumptive ICC. In one example half of the auditory midbrain was largely devoid of PV positive cell bodies, and commissural connections between each ICC could not be detected. In addition, the axonal output of the ICC was greatly disorganized. Misexpression of *Wnt1* perturbs the formation of the isthmus organizer required for patterning the adjacent midbrain and cerebellum (Adams et al., 2000; Matsunaga et al., 2002), yet how this perturbation may effect the development of the inferior colliculus and its subdivisions has not been determined. While it is likely that much of the patterning deficits we observe are secondary to the disruption of the isthmus organizer, *Wnt1* is required for the proper specification of the auditory structures and their connections. How this mutation affects the distribution of the *Wnt1* lineage in auditory centers, and consequently the processing of auditory information remains to be determined.

Tonotopy and the distribution of Wnt1-derived auditory neurons

The spatial address of auditory neurons and the organization of afferents dictate their functional identity, namely which isofrequency they relay within the tonotopic representation of sound. Our study provides evidence that the timing of *Wnt1* expression in cohorts of progenitors influences how neurons derived from this genetic lineage are ultimately distributed across the cochlear nucleus and inferior colliculus. Progenitors of the inferior colliculus expressing *Wnt1* at E9.5 are competent to give rise to all neurons across the tonotopic map, while later

Wnt1 expression delineates progenitors which are restricted towards a dorsal, low frequency response fate or posterior ECIC. In contrast, progenitors of the ventral cochlear nuclei which expressed *Wnt1* early gave rise to neurons which primarily populated the dorsal aspects of the AVCN and PVCN and are poised to respond to higher frequencies of sound (Luo et al., 2009). This dorsal bias was observed within the AVCN at each marking time point, and may reflect the underlying biology and temporal lineage contribution. Cumulative *Wnt1* fate mapping in which every cell with a history of *Wnt1* expression is heritably marked demonstrates that *Wnt1*-derived neurons contribute throughout the dorsoventral axis of each cochlear nucleus subdivision (Farago et al., 2006). The absence of substantial contribution from *Wnt1*-expressing progenitors to ventral domains observed in our approach may be explained by summing the ventral contribution from each marking stage. Alternatively, our results may provide evidence for a model in which the expression and activity of *Wnt1* controls the spatial distribution of auditory neurons. An earlier study identified a gradient of *Wnt1* expression, high to low across the D-V axis of the LRL (Landsberg et al., 2005). Given the mosaicism of marking innately due to the pharmacokinetics of tamoxifen administration (Joyner and Zervas, 2006) and the fact that progenitors expressing low levels of *Wnt1* would presumably have lower levels of *CreER* expression, the lack of substantial ventral labeling may be indicative of the importance of gene expression levels as well as timing. Thus, progenitors expressing and secreting the highest levels of WNT1 early would be immersed in the high end of the concentration gradient, and this position may be instructive

for depositing neurons in the dorsal tiers of the cochlear nucleus. Since much of the neuronal and axonal organization responsible for tonotopy is present before the onset of hearing (Friauf and Kandler, 1990; Kandler and Friauf, 1993), this model provides a mechanism in which auditory progenitors are imprinted for the neuronal organization underlying tonotopy at midgestation.

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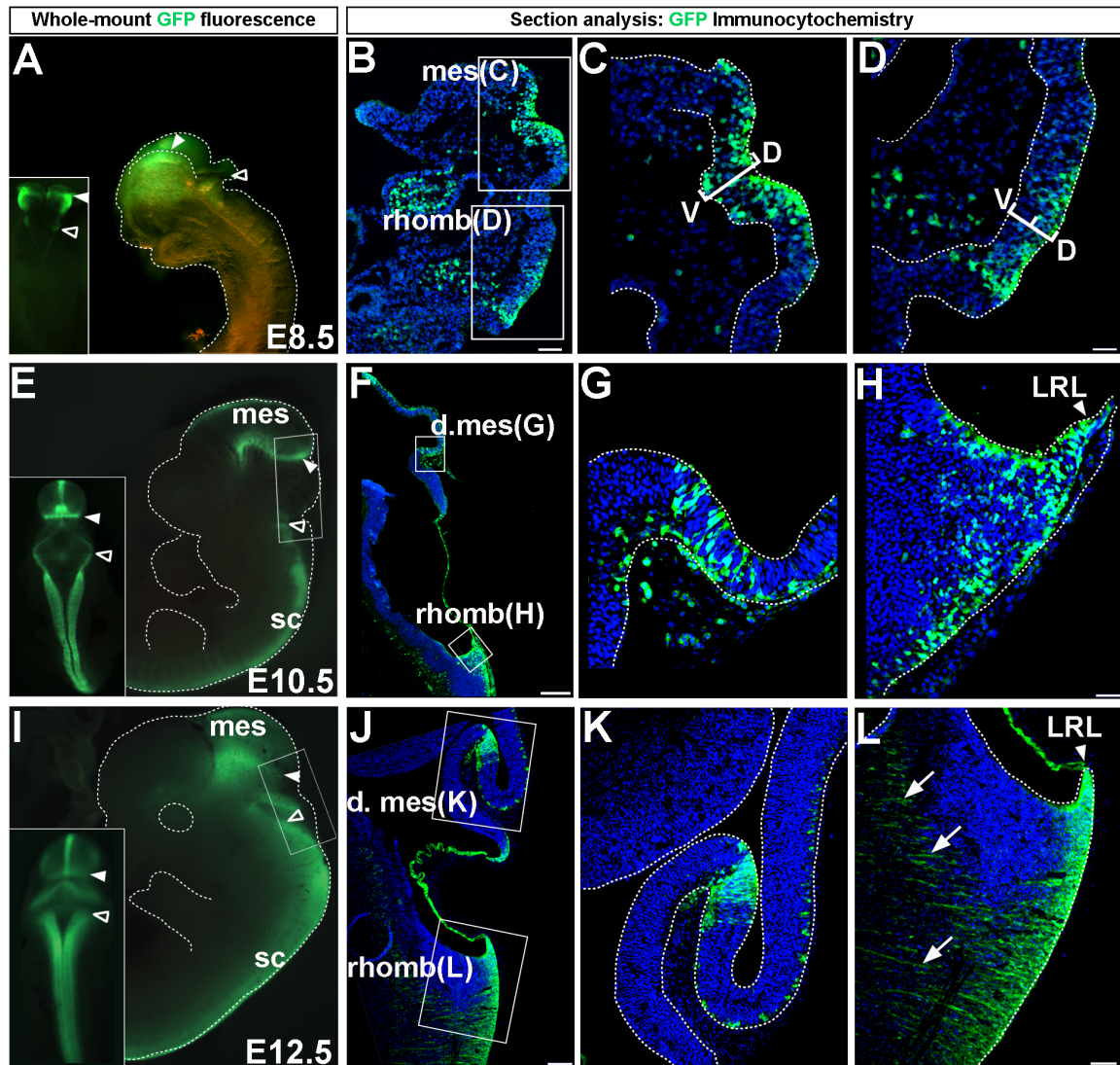


Figure 1. *Wnt1* expression in midbrain and hindbrain auditory primordia.

Figure 1. *Wnt1* expression in midbrain and hindbrain auditory primordia.

Wnt1 expression observed by GFP whole-mount fluorescence and GFP immunocytochemistry in *Wnt1-Venus* transgenic embryos. (A-D) Expression of *Wnt1* at E8.5. *Wnt1* was expressed diffusely throughout the mes and was faintly observed in the rhombencephalon (A, solid versus open arrowheads, respectively). Higher magnification in a sagittal section details *Wnt1* expression within the neuroepithelium (B-D). *Wnt1* was expressed through the dorsal-ventral extent of the mesencephalic neuroepithelium (C, bracketed area) while faint expression in the rhombencephalon was confined to the dorsal half of the neuroepithelium (D, bracketed area). (E-H) *Wnt1* expression at E10.5. *Wnt1* expression in presumptive auditory midbrain progenitors was confined to a ring pattern at the posterior limit of the dorsal mes (E, solid arrowheads) while auditory hindbrain progenitors expressed *Wnt1* through the A-P extent of the the LRL (E, open arrowheads). A mid-sagittal section revealed the spatial segregation of *Wnt1* expression in Mb and Hb auditory primordia (F). High magnification showed GFP positive cells remaining within the neuroepithelium (G,H). (I-L) *Wnt1* expression at E12.5. The pattern of *Wnt1* expression observed at E10.5 persisted through E12.5, though diminished within the posterior ring in dorsal mes and broadened within the LRL (I, solid versus open arrowheads, respectively). A mid-sagittal section confirmed the continued pattern of *Wnt1* expression observed at E10.5 (J versus F). High magnification showed a restricted *Wnt1* expression domain as compared to the broader profile at E10.5 (K versus G). The LRL showed broadened *Wnt1* expression in the neuroepithelium at E12.5 (L versus H) and GFP perdurance allowed the early tracking of progenitors as they migrate out of LRL and begin to form projections (L, arrows). Insets in (A,E,I) display whole-mount dorsal view. Abbreviations: mesencephalon (mes), dorsal mesencephalon (d. mes), rhombencephalon (rhomb), spinal cord (sc), lower rhombic lip (LRL), dorsal-ventral (D-V) Scale bars: (B) 63 μ m; (C,D) 32 μ m; (F) 260 μ m; (G,H) 32 μ m; (J) 130 μ m; (K,L) 63 μ m.

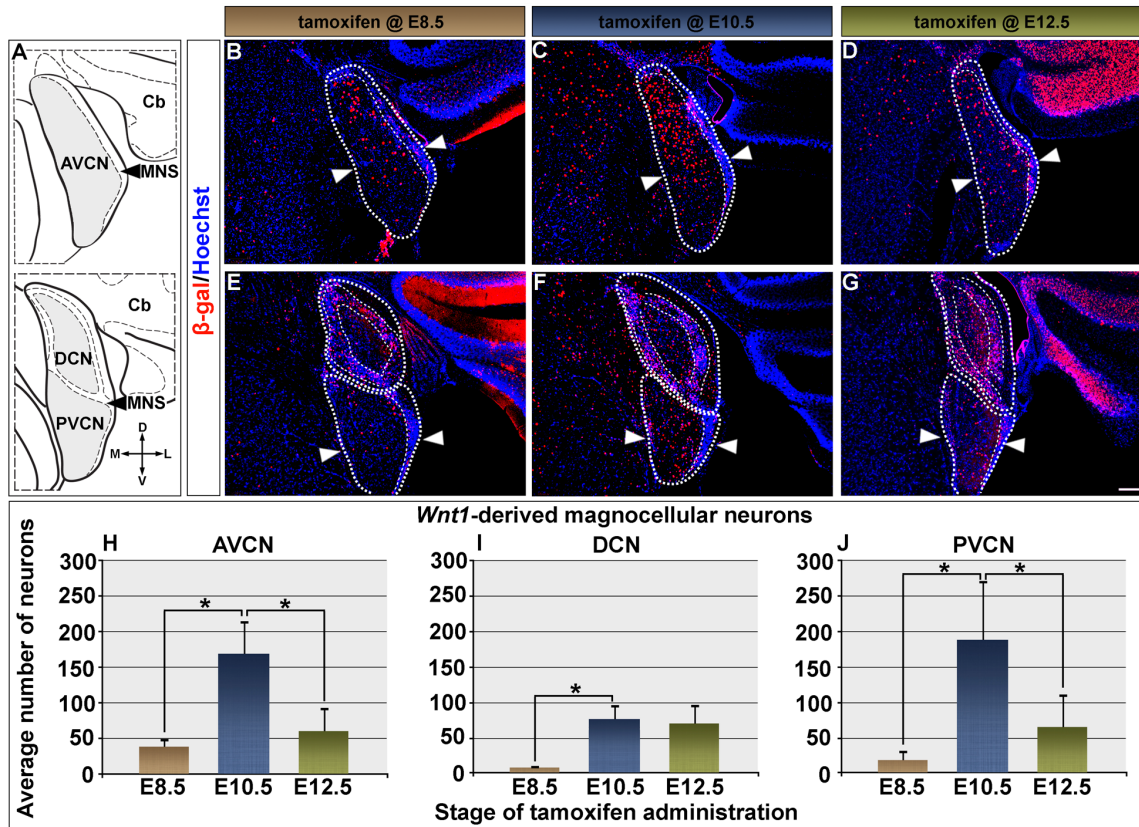


Figure 2. The timing of *Wnt1* expression in progenitors predicts the extent and distribution of neurons within the cochlear nucleus.

Figure 2. The timing of *Wnt1* expression in progenitors predicts the extent and distribution of neurons within the cochlear nucleus.

(A) Schematic representation of AVCN, DCN, and PVCN. Shaded grey area delineates the magnocellular core (MGC) of each nucleus counted in (H-J). Fine dashed lines represent the microneuronal shell, denoted by MNS, and the laterally located cerebellum is denoted as Cb. *Wnt1*-derived neurons were identified by nuclear β -gal immunocytochemistry in red (B-G). (B-D,H) The differential contribution of *Wnt1*-expressing progenitors to the AVCN. Early *Wnt1* expression at E8.5 in progenitors conferred a limited contribution (38.9 ± 12.0 *Wnt1*-derived neurons/section) to mature neurons predominantly found in the dorsal aspects (above area demarcated by arrowheads) of the AVCN (B, H). Progenitors expressing *Wnt1* at E10.5 provided peak contributions (169.4 ± 47.2 *Wnt1*-derived neurons/section) to the AVCN while a dorsal bias persisted (C,H). By E12.5 *Wnt1*-expressing progenitors ultimately contributed to the AVCN in decreased numbers (60.8 ± 34.4 *Wnt1*-derived neurons/section) throughout the D-V axis (D, H). (E-G, I) Magnocellular neurons of the DCN were derived from progenitors with late *Wnt1* expression. DCN progenitors which expressed *Wnt1* at E8.5 largely contributed to the MNS, with few *Wnt1*-derived MGC neurons (8.0 ± 4.1 *Wnt1*-derived neurons/section)(E, I). *Wnt1*-expressing progenitors at E10.5 gave rise to neurons throughout the D-V axis of the MGC and continued to populate the MNS (F, I). A prolonged peak of contribution from *Wnt1*-expressing progenitors to DCN MGC neurons existed between E10.5 (75.9 ± 19.9 *Wnt1*-derived neurons/section) and E12.5 (70.2 ± 27.8 *Wnt1*-derived neurons/section)(G, I). (E-G,J) *Wnt1* derived neurons populated the PVCN with a similar profile as the AVCN. E8.5 *Wnt1*-expressing progenitors contributed sparsely to the dorsal aspects of the PVCN (E,J). Peak contribution of *Wnt1*-expressing progenitors to the PVCN arose from E10.5 expression (F,J), as later E12.5 *Wnt1*-expressing progenitors contributed in fewer numbers throughout the D-V axis (G,J). Asterisks denote average numbers of *Wnt1*-derived neurons which were significantly different ($p < 0.005$). Scale bar: 130 μ m (B-G).

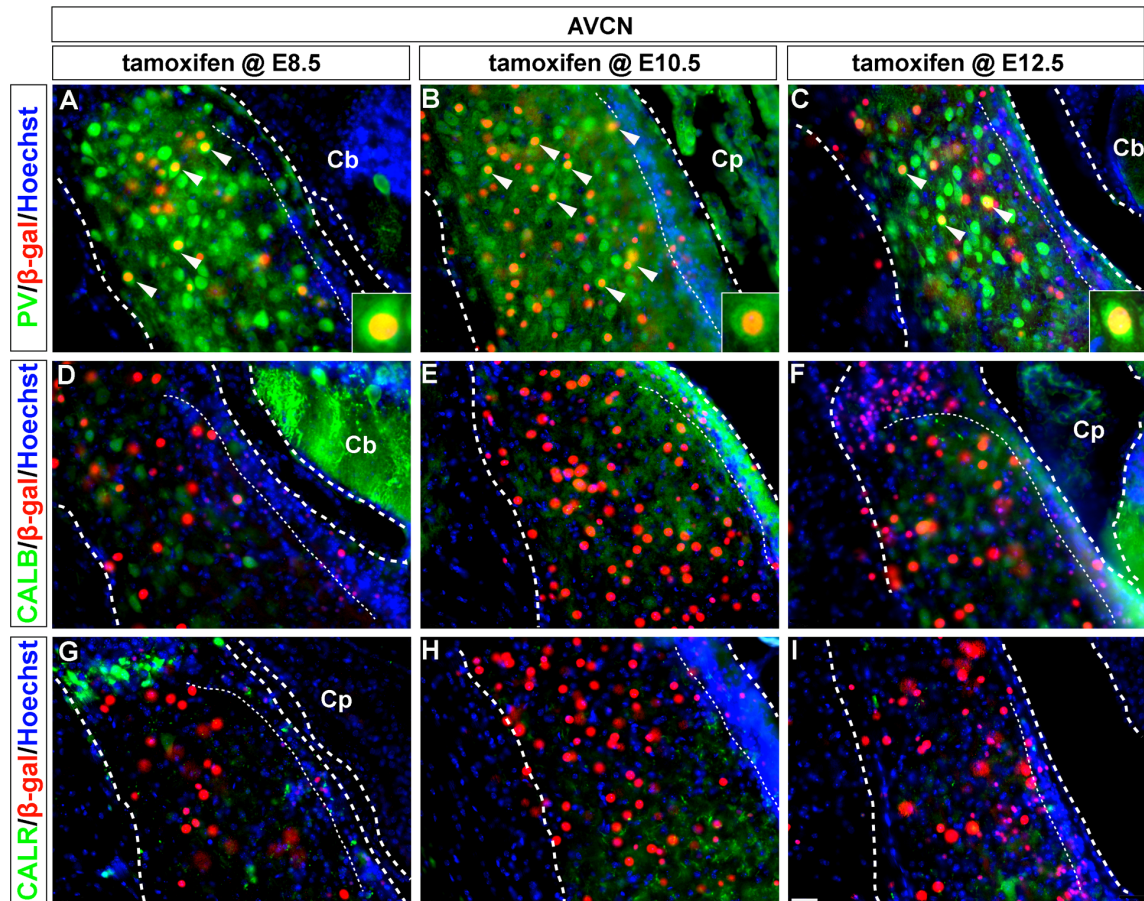


Figure 3. PV-expressing AVCN neurons are derived from *Wnt1*-expressing progenitors.

Wnt1-derived neurons were identified by nuclear β -gal immunocytochemistry in red, and biochemical markers were delineated in green (A-I). *Wnt1* expression between E8.5-E12.5 in progenitors defined biochemically distinct neurons of the AVCN which expressed PV (A-C, arrowheads, insets). CALB (D-F) or CALR (G-I) positive neurons in the AVCN were not derived from progenitors which expressed *Wnt1* between E8.5-E12.5. Broad dashed lines indicate the AVCN magnocellular core, while fine dashed lines depict the granule cell layer. Abbreviations: cerebellum (Cb), choroid plexus (Cp) Scale bar: 32 μ m (A-I).

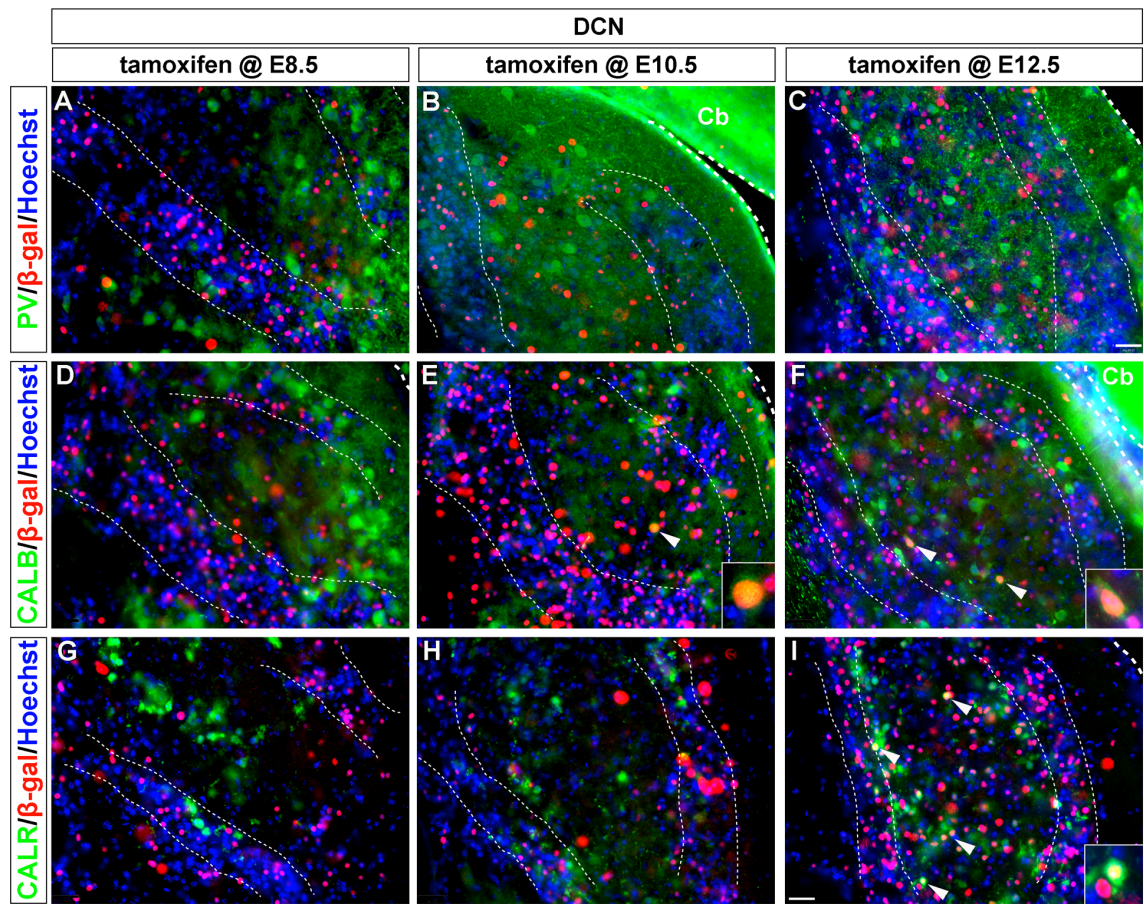


Figure 4. CALB and CALR-expressing DCN neurons are sequentially derived from *Wnt1*-expressing progenitors.

Wnt1-derived neurons were identified by nuclear β -gal immunocytochemistry in red, and biochemical markers were delineated in green (A-I). PV-expressing DCN neurons were not derived from progenitors which expressed *Wnt1* between E8.5-E12.5 (A-C). Progenitors which expressed *Wnt1* between E10.5-E12.5 gave rise to CALB positive neurons (E, F, arrowheads, insets) while CALR positive DCN neurons were derived from later E12.5 *Wnt1*-expressing progenitors (I, arrowheads, inset). Broad dashed lines indicate the separation of DCN from cerebellum (Cb), while fine dashed lines depict the granule cell layers. Scale bar: 32 μ m (A-I).

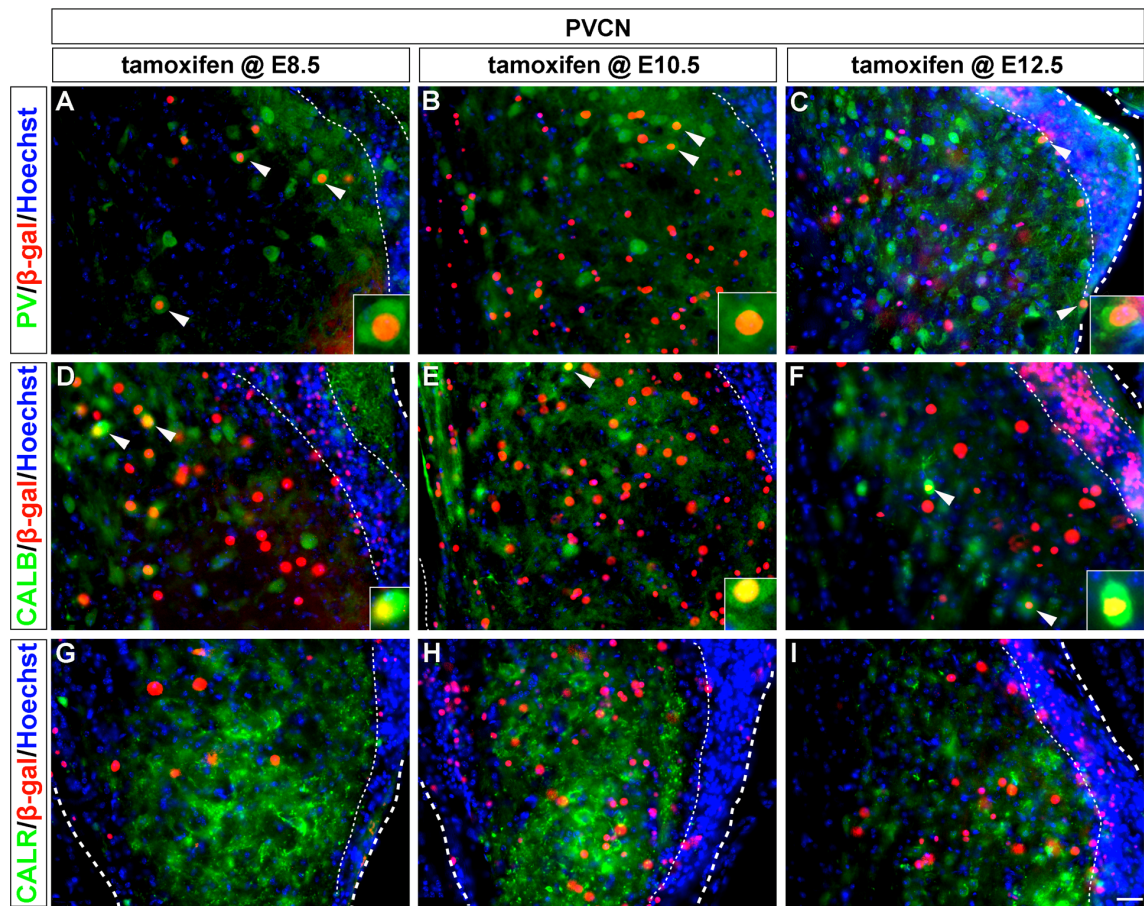


Figure 5. PV- and CALB-expressing PVCN neurons are derived from *Wnt1*-expressing progenitors.

Wnt1-derived neurons were identified by nuclear β -gal immunocytochemistry in red, and biochemical markers were delineated in green (A-I). PV positive (A-C, arrowheads, insets) and CALB positive (D-F, arrowheads, insets) neurons of the PVCN were descendant of progenitors which expressed *Wnt1* between E8.5 and E12.5. CALR positive neurons were not derived from progenitors which expressed *Wnt1* from E8.5-E12.5. Broad dashed lines indicate the lateral or medial edge of the PVCN, while fine dashed lines depict the granule cell layer. Scale bar: 32 μ m (A-I).

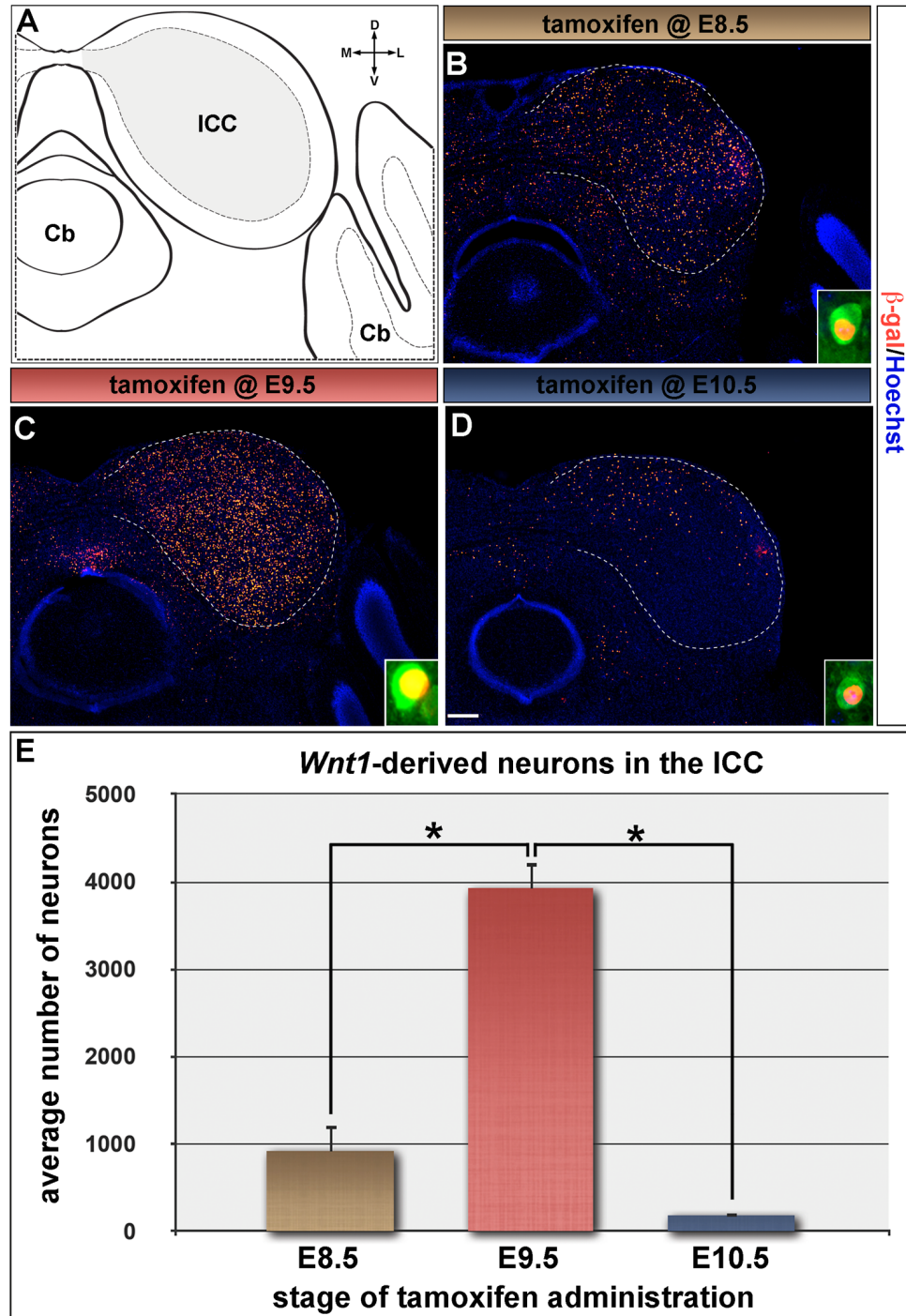


Figure 6. The inferior colliculus is established by progenitors expressing *Wnt1* from E8.5-E10.5.

Figure 6. The inferior colliculus is established by progenitors expressing *Wnt1* from E8.5-E10.5.

(A) Schematic representation of the auditory midbrain (hemisagittal view is shown). ICC is shaded in grey to delineate the area counted in (E), and represented by dashed line in (B-D). *Wnt1*-derived neurons were identified by nuclear β -gal immunocytochemistry in red (B-D). *Wnt1*-expressing progenitors at E8.5 gave rise to sparse neurons (919.3 $\pm$ 307.5 *Wnt1*-derived ICC neurons/section) throughout the inferior colliculus (B,E). The ICC is largely established by neuronal descendants of E9.5 *Wnt1* expression (3923.8 $\pm$ 722.7 *Wnt1*-derived ICC neurons/section)(C,E). *Wnt1*-expressing progenitors at E10.5 contributed only sparsely (181.7 $\pm$ 132.8 *Wnt1*-derived ICC neurons/section) to neurons which populated the dorsomedial ICC (D,E). Insets in (B-D) depict representative PV positive neurons of the ICC derived from *Wnt1*-expressing progenitors. Asterisks denote average numbers of *Wnt1*-derived neurons which were significantly different ($p < 0.001$). Scale bar: 260 μ m (B-D).

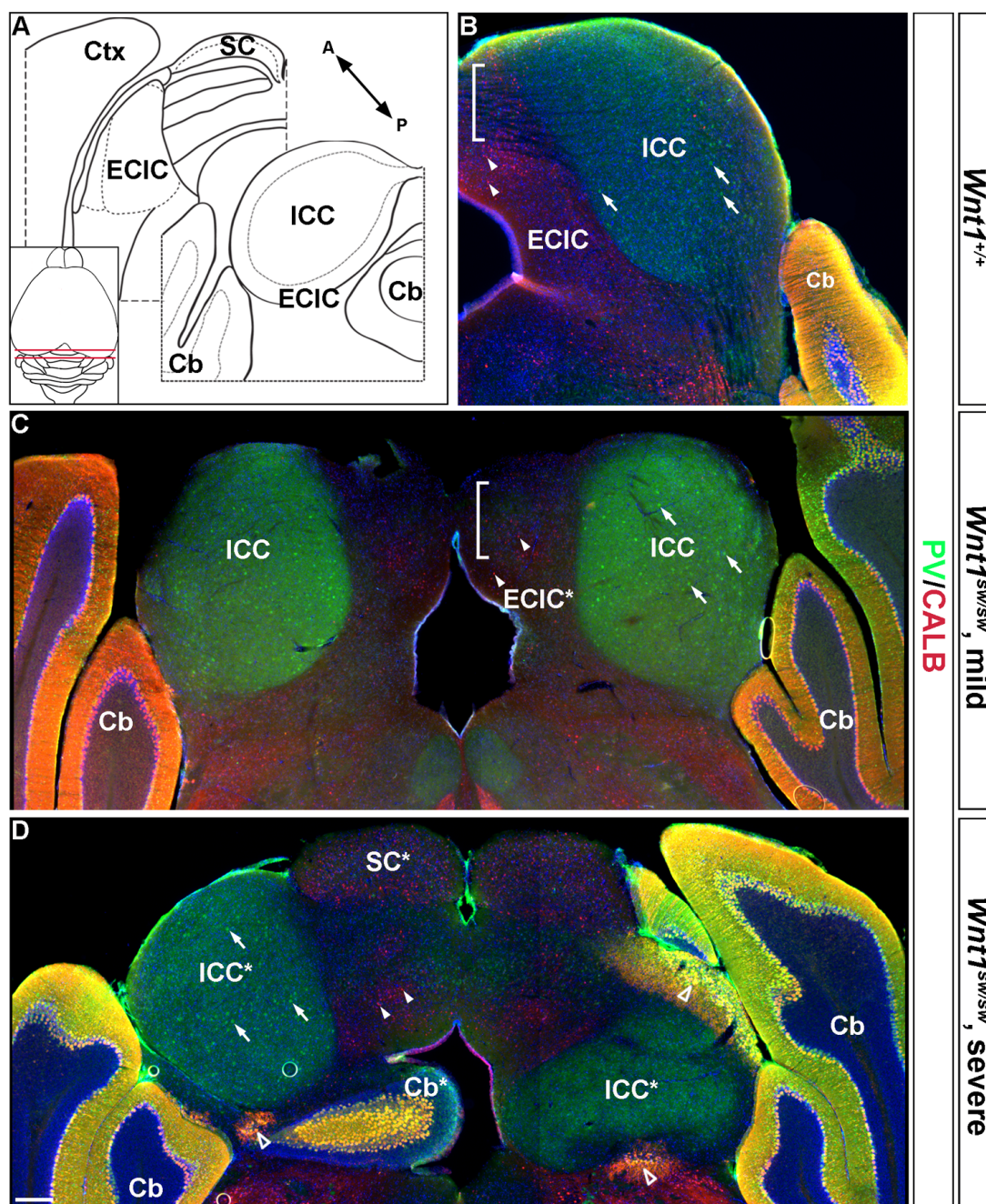


Figure 7. Patterning deficits of the auditory midbrain in *Wnt1^{sw/sw}* mice.

Figure 7. Patterning deficits of the auditory midbrain in *Wnt1^{sw/sw}* mice.

(A) Schematic representations of two wild type hemicoronal planes of section through the dorsal midbrain. Inset: dorsal view of a whole mount brain schematic depicts the anterior and posterior planes of section. The anterior section reveals the superior colliculus (SC) dorsally, and external cortex of the inferior colliculus (ECIC) laterally. Cortex (Ctx) surrounds the Mb laterally in this plane. The posterior plane details the ICC, and the cerebellum (Cb) surrounding laterally and ventromedially. (B) Hemicoronal wild type section representative of the posterior plane in (A) details normal staining for PV (green) and CALB (red). PV broadly stained the ICC while CALB was largely confined to ECIC. Examples of PV and CALB positive neurons are noted with arrows and arrowheads, respectively. Commissural connections between the colliculi are identified by the bracketed area. (C, B) Phenotypes varied from mild (C) to severe (D) in *Wnt1^{sw/sw}* mice. (C) Mice with mild phenotypes exhibited minor loss of bilateral symmetry, though general morphology of each ICC was maintained. In addition, the ECIC was malformed, denoted by ECIC*, but PV and CALB neurons were still specified. Commissural connections in the bracketed area were absent. (D) Mice with severe phenotypes displayed a loss of bilateral symmetry between each ICC (as determined by PV expression and denoted as ICC*), ectopic Cb tissues and cell types within the midbrain, and presence of presumed SC tissues normally positioned anteriorly. The left ICC* in (D) was established with largely normal morphology and contained PV-expressing neurons, while the right ICC* was perturbed and contained few PV neurons. Ectopic or malformed Cb tissue was present within the Mb (Cb*), and presumed ectopic Cb cell types which expressed both PV and CALB were abundant throughout the Mb (open arrowheads). Presumed ectopic anterior tissues of the SC (SC*) were observed in plane with the ICC in (D) as evident by the dorsal laminated morphology and expression of CALB. Scale bar: 260 μ m (B-D).

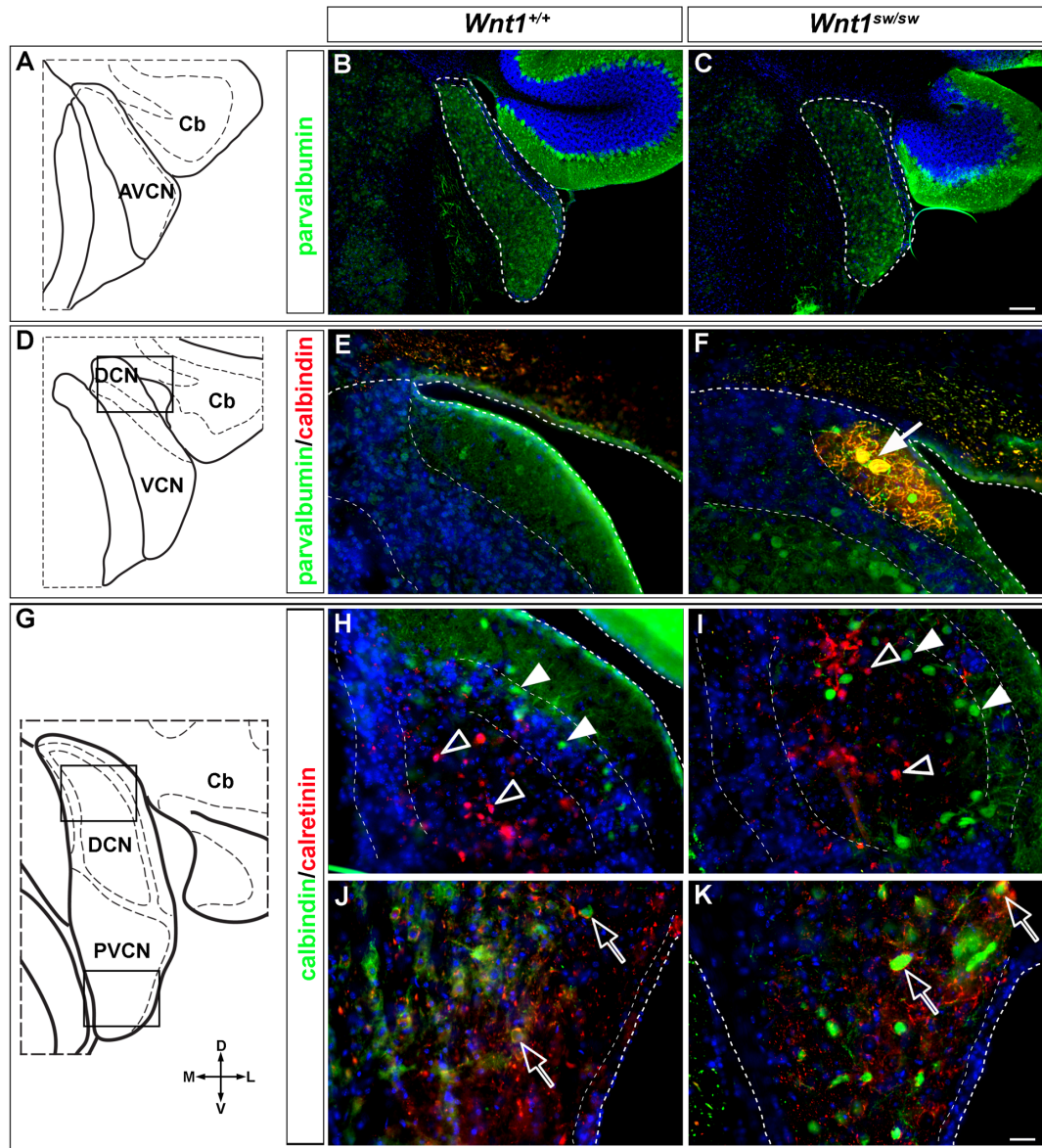


Figure 8. Altered morphology and ectopic cell types in anterior aspects of *Wnt1^{SW/SW}* cochlear nucleus.

Figure 8. Altered morphology and ectopic cell types in anterior aspects of *Wnt1^{sw/sw}* cochlear nucleus.

(A) Schematic representation of the normal AVCN morphology observed in (B). (C) A stereotypical broadened morphology was observed in the AVCN of *Wnt1^{sw/sw}* mice. PV-expressing neurons of the AVCN remained prevalent (B,C). (D) Schematic view of the sectioning plane and boxed area analyzed in (E) and (F), depicting the transition between AVCN and PVCN (denoted VCN) and the most anterior aspects of the DCN. While the general morphology of mutant posterior cochlear nuclei remained intact, an ectopic cell type characteristic of Purkinje-like cells (PLCs) (based on morphology and co-expression of PV/CALB) was commonly observed (E versus F). (G) Schematic view delineating the boxed areas of DCN and PVCN analyzed in (H-K). The general morphology was largely normal in mutant DCN (H versus I). CALB and CALR neurons were unaffected (solid and open arrowheads, respectively). Morphology and cell type specification was also normal in mutant PVCN (J versus K). Open arrows highlight CALB neurons surrounded by CALR positive processes. Scale bars: 130 μm (B,C); 32 μm (E,F,H,I,J,K).

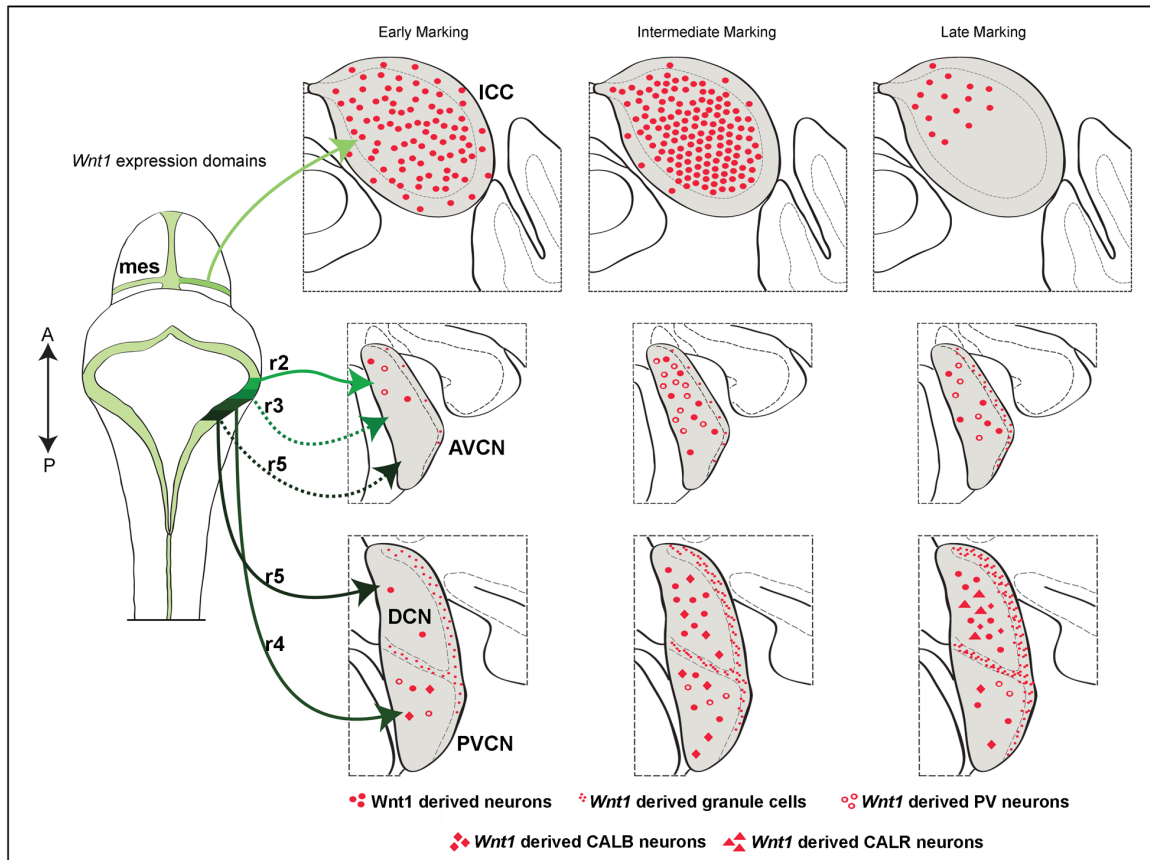
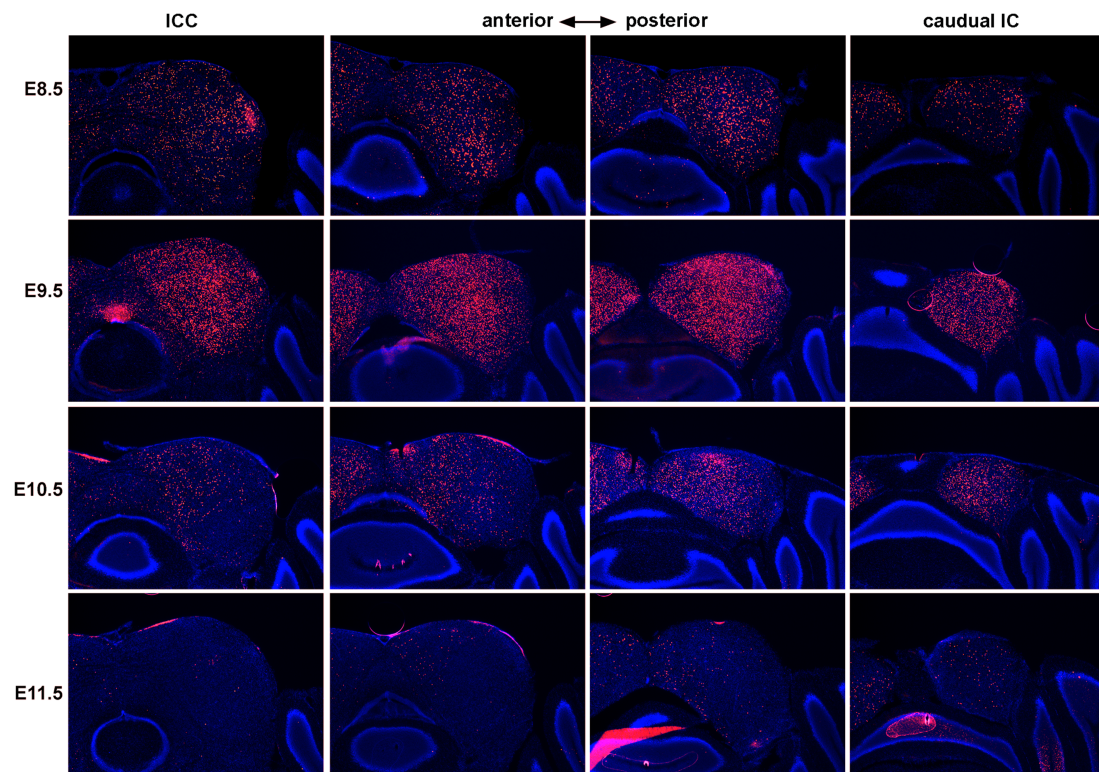


Figure 9. Schematic summary of the temporal allocation of *Wnt1*-expressing progenitors to mature midbrain and hindbrain auditory domains.

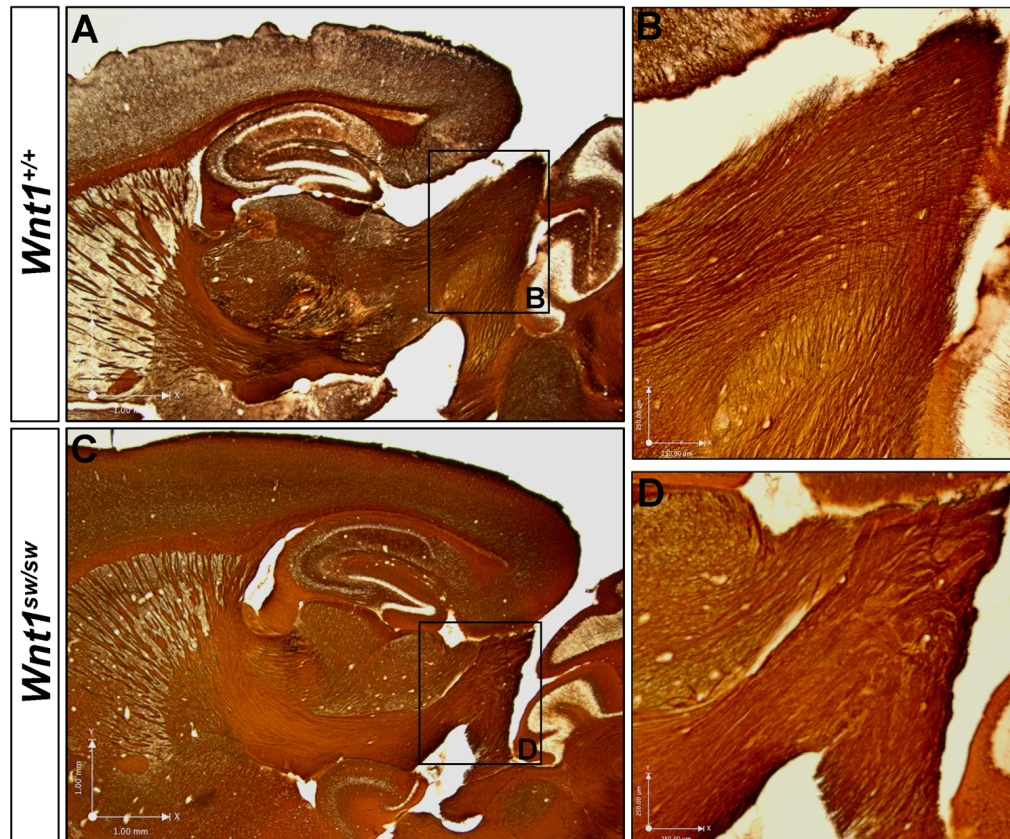
Figure 9. Schematic summary of the temporal allocation of *Wnt1*-expressing progenitors to mature midbrain and hindbrain auditory domains.

A dorsal view of the mouse embryo at midgestation detailing *Wnt1* expression domains in green, and the contribution the Mb and Hb primordia make to the inferior colliculus and cochlear nucleus (specific rhombomeric contribution mapped from Farago et al., 2006). The ICC was derived from *Wnt1*-expressing progenitors in the dorsal mes. Early, intermediate, and late marking stages are relative to specific structures as indicated below. Early (E8.5) *Wnt1*-expressing progenitors contributed diffusely throughout the inferior colliculus, while intermediate (E9.5) marking resulted in substantial contribution within the ICC. Late (E10.5) marking conferred sparse contribution to dorsomedial aspects of the ICC. The AVCN is derived from *Wnt1*-expressing progenitors in r2, r3, and r5. Marking early (E8.5) *Wnt1*-expressing progenitors resulted in sparse contribution to PV positive neurons in the dorsal tier of the AVCN. Intermediate marking (E10.5) revealed greater contribution to PV neurons, while late (E12.5) resulted in a drop off of *Wnt1*-derived neurons in the AVCN. The DCN is largely derived from *Wnt1*-expressing progenitors in r5. Early marking (E8.5) of *Wnt1*-expressing progenitors revealed a primary contribution to granule cells of the microneuronal shell. Magnocellular neurons of the DCN were derived from progenitors expressing *Wnt1* at an intermediate stage (E10.5), including CALB-expressing neurons. CALR-expressing neurons were derived from late (E12.5) *Wnt1* expression. The PVCN is derived largely from *Wnt1*-expressing progenitors of r4. The distribution of neurons derived from *Wnt1*-expressing progenitors resembles that of the AVCN, though both PV and CALB-expressing neurons were derived from each stage examined.



Supplementary Figure 1. Anterior-Posterior contribution of the *Wnt1* lineage from E8.5-E11.5.

Early (E8.5, E9.5) *Wnt1*-expressing progenitors contributed to neurons throughout the D-V, A-P extent of the auditory midbrain. Late (E10.5, E11.5) *Wnt1*-expressing progenitors primarily populated the posterior auditory midbrain.



Supplementary Figure 2. *Wnt1* is required for normal axonal organization in the brachium of the inferior colliculus.

Myelin-based silver staining allowed the visualization of axons leaving the inferior colliculus en route to higher order auditory centers. (A,B) Wild type axons exiting the auditory midbrain formed organized, parallel fibers. (C,D) *Wnt1*^{sw/sw} mice displayed clumped, disoriented axonal bundles.

Chapter 3

***Z/StrepG*: a novel transgenic reporter for *in vivo* lineage assessment**

Introduction

Genetic fate mapping approaches in mouse have revolutionized our understanding of how early cell populations contribute to tissues throughout mammalian development. The advent of inducible site-specific recombinases (Feil et al., 1996) have provided the means to resolve how progenitors expressing specific genes at critical time points of development establish the multitude of cell types within an organ. This methodology, termed genetic inducible fate mapping (GIFM), utilizes two genetically modified alleles to indelibly mark a progenitor population and all the cells descendant from it (reviewed by Dymecki, 2000; Joyner and Zervas, 2006). One allele encodes a drug-responsive Cre recombinase (CreER), while the other allele encodes a conditional reporter gene that remains quiescent in the absence of Cre activity. Typically, *CreER* is driven by genetic elements unique to a progenitor population of interest, such that whenever the selected gene is expressed in development, *CreER* will be coincidentally expressed (Brown et al., 2009). Upon its translation, CreER remains sequestered in the cytoplasm through its interaction with heat shock protein 90 (Hsp 90), which binds the modified estrogen receptor (ER) ligand binding domain of CreER (Feil et al., 1996). Low levels of the drug tamoxifen can be introduced *in utero* at developmental time points of interest, where it interacts with the ER domain of CreER, displacing Hsp 90 and releasing it from cytoplasmic sequestration. Therefore, tamoxifen administration allows CreER to translocate to the nucleus. Through site-specific recombination, CreER then catalyzes the removal of DNA flanked by 34 base-pair *loxP* sequences

oriented in the same direction (Sauer, 1998). In conditional reporter alleles, the flanked sequence typically encodes a stop cassette which precedes the coding regions of the reporter gene, rendering it quiescent. Once recombination has occurred, the stop cassette is removed and the reporter is constitutively and heritably expressed within the progenitors and all cells they give rise to across development.

Many reporter alleles currently used in fate-mapping studies typically express enzymatic or fluorescent gene products. For example, well established reporter alleles include the *R26R* reporter (Soriano, 1999), which conditionally expresses beta-galactosidase (β -gal), or *Z/EG* (Novak et al., 2000) a conditional eGFP reporter. More recently, reporters have been generated that allow for the specific analysis of neuronal lineages (*Tau^{mGFP}*; Hippenmeyer et al., 2005; Brown et al., 2009), or the contribution that arises from progenitors within the intersection of gene expression domains (Awatramani et al., 2003; Farago et al., 2006). However, the assessment of lineage contributions in fate mapping studies employing these reporters has often required static, postmortem analysis. A reporter which could provide three-dimensional imaging of genetic lineage contribution *in vivo* could be an advancement in cell lineage tracing. Specifically, a live-imaging reporter could allow the assessment of how progenitors within a developing embryo longitudinally contribute to tissues over time, or how the course of disease may affect particular cell types within an organ. A significant extension of this idea, is that isolated progenitors expressing a live-imaging reporter could be followed in cell transplantation studies. To this end, we

received a small pilot grant (Institute for Brain Science, Brown University) to design and engineer our novel reporter, termed *Z/StrepG*. This allele was used to establish a conditional transgenic reporter line that expresses either *LacZ* (when unrecombined) or a bicistronic message of *Streptavidin-IRES-eGFP* after Cre-mediated recombination. Streptavidin is a bacterial protein from *Streptomyces avidinii* which binds the small molecule biotin with an affinity of K_d 10^{-15} M, ranking amongst the strongest of non-covalent interactions observed in molecular biology (Argarana et al., 1986). Streptavidin forms a homotetrameric complex which binds 4 molecules of biotin, and because of this binding and their unusually high affinity, streptavidin-biotin interactions have been used in a wide array of biochemical assays and purification procedures (Bayer and Wilchek, 1980; Wilchek and Bayer, 1988; Diamandis and Christopoulos, 1991). We designed our construct to exploit streptavidin as a conditional reporter in GIFM studies, enabling marked cells to be preferentially enriched for (bio)chemicals tagged with biotin. Specifically, we were interested in providing genetically-defined lineages the capacity to accumulate biotinylated moieties to function as contrast agents that could be detected by magnetic resonance imaging (MRI). Following Cre-mediated activation of the reporter, we hypothesized that delivery of biotinylated-contrast agents either *in utero* or to the adult animal would allow for the direct imaging of genetically-defined lineages *in vivo* by MRI. We demonstrated that *pZ/StrepG* undergoes recombination both *in vitro* and *in vivo* resulting in the expression of streptavidin and GFP proteins. Yet we were unable to demonstrate the specific retention of biotinylated compounds in cells

expressing our construct *in vitro*. Additionally, the transgenic line of mice we generated was designed to express our reporter ubiquitously in all cell types, however the reporter displayed uneven and biased expression within glial cells of the brain.

Although our immediate goals were not met, the important concept of transiently accumulating MRI-contrast agents in defined cell populations in the context of development or disease merits re-examination of our methods and the investigation of other design strategies. Our study serves to provide the rationale for future designs, and to provide analysis and review of the challenges encountered through our transgenic approach.

Materials and Methods

Animals and Genotyping

Z/StrepG mice were generated as described below. *Z/EG* reporter mice (Novak et al., 2000) were purchased from Jackson Laboratories. *En1^{Cre}* mice (Kimmel et al., 2000) were kindly provided by A. Joyner, NYU. Mice were housed and handled in accordance with Brown University Institutional Animal Care and Use Committee guidelines. Mice were genotyped by PCR for the presence of Cre and GFP as previously described (Ellisor et al., 2009; Brown et al., 2009).

Subcloning of pZ/StrepG

The backbone vector for *pZ/StrepG* (*pCALL2*, Figure 1A) originally described by C. Lobe (Novak et al., 2000), was kindly provided by James Li, UCONN Health Center. The cDNA encoding *streptavidin* was synthetically generated by GenScript (Piscataway, NJ) based on the sequence for a recombinant form, *Stv-13* (Sano et al., 1995). *Streptavidin* was engineered to contain unique 5' (BglII) and 3' (XhoI) restriction sites used to subclone within the multi-cloning site (MCS) of *pCALL2* (see Figure 1). Prior to the introduction of *Stv-13*, cDNA coding for *IRES-eGFP* was excised from *pIRES2-EGFP* vector (CloneTech, Mountain View, CA) and subcloned within the MCS using XhoI (5') and NotI (3'). In this configuration, *LacZ* (Figure 1B, blue cassette) is strongly expressed by the ubiquitous chick β -actin promoter enhanced by the cytomegalovirus enhancer (CMV). Three polyadenylation (3xPolyA) sites downstream of *LacZ* prevent further transcription beyond the *LacZ*/neomycin (stop) cassette. Upon Cre-mediated recombination between *loxP* sites (Figure 1, blue triangles) *LacZ*/Neo-3xPolyA is excised, bringing *streptavidin-IRES-eGFP* (Figure 1, orange/green cassette) under the control of the chick β -actin promoter (Figure 1C).

Explant preparation, culture, and transfection

Explants were micro-dissected and prepared as previously described (Brown et al., 2009). Briefly, the mesencephalon/rhombomere 1 (midbrain/cerebellum primordia) of E11.5 *En1^{Cre}* mouse embryos were micro-dissected and

cut along the dorsal midline to create an “open book” preparation. This embryonic explant tissue was used as a source of Cre expressing cells to test the recombination and expression of *pZ/StrepG*. The explants were cultured in complete media: DMEM+Glutamax/10% FBS/1% Pen-Strep prior to and after Lipofectamine 2000 mediated transfection (all Gibco/Invitrogen reagents, Carlsbad, CA). Lipid-based transfection of the explants was done as previously described (Bauer et al., 2000). Briefly, explants were incubated in serum/antibiotic free media for 1.5 hours prior to transfection. The ratio of DNA:Lipofectamine that was optimized for the transfection experiments was 1:5 (1 µg DNA: 5 µl Lipofectamine). After 5 hours the transfection media was replaced with complete media. Expression was monitored over 3-4 days by whole mount GFP fluorescence. Explants were subsequently washed with PBS, fixed in 4% paraformaldehyde, and immunolabeled for GFP and TH as described below.

Embryonic Tissue Preparation

Mouse embryos were dissected in PBS over ice and *En1^{Cre};Z/StrepG* embryos were identified by GFP fluorescence and confirmed by genotyping for Cre and GFP (Ellisor et al., 2009). Chick embryos (from *in ovo* electroporation experiments described below) were assessed by GFP whole-mount fluorescence or X-gal staining (described below). Embryonic mouse and chick tissues were prepared for immunocytochemistry experiments using methods previously described (Brown et al., 2009). Briefly, both mouse and chick embryos were

fixed in 4% PFA overnight at 4°C, cryoprotected, and embedded in OCT. Embryos (n=3) were sectioned sagittally (12 μ m) with a Leica cryostat and stored at 20°C. Sections were immunolabeled as described below.

Brain tissue preparation

Mice were genotyped by obtaining a tail biopsy and performing PCR analysis on DNA from tail lysates. At 6 weeks of age *En1^{Cre};Z/StrepG* mice were deeply anesthetized with Nembutal (5 mg) and intracardially perfused with 4% paraformaldehyde (PFA). Craniotomies were performed to extract the fate mapped brains, which were stored in PFA at 4°C until sectioning. Brains were embedded in 3% agarose in PBS and sectioned horizontally (40 μ m) with a Leica vibratome (Brown et al., 2009). At least three brains across two litters were processed *Z/StrepG* expression analysis.

In ovo chick electroporation

Hamburger-Hamilton (HH) stage 9-10 chick embryos were transfected by electroporation as previously described (Muramatsu et al., 1997; Timmer et al., 2001). Briefly, fertilized eggs (Charles River Laboratories, Wilimington, MA) were cultured on their sides at 38.5°C for 33-38 hours. The top side was marked for orientation. A small hole was made in the pointed end of the egg to allow a needle and syringe to remove 3 ml of albumen. The embryo was exposed through a small window cut in the marked side of the egg. Native (unrecombined, *LacZ*-expressing) or recombined (*streptavidin/GFP*-expressing)

pZ/StrepG (1 µg/µl with 0.5% Trypan Blue) was delivered to the lumen of the neural tube spanning the entire mesencephalon and metencephalon with a glass micropipette. 1-2 drops of PBS were added to the embryo prior to placing electrodes on either side of the neural tube, making contact with the vitelline membrane. Following a charge and pulse sequence (3x 50 ms at 20V), the egg was sealed with tape. Expression of the vectors was assessed 18-24 hours post-transfection through GFP whole mount fluorescence (to detect expression of the recombined construct), X-gal histochemistry (to detect expression of the native unrecombined construct), and tissue section immunocytochemistry described below.

Cell Culture

HEK293 cells were grown in complete media: DMEM/10% FBS/1% Pen-Strep (Gibco/Invitrogen) at 37°C, 5% CO₂. Cells were grown to 80% confluency prior to Lipofectamine 2000 (Invitrogen) mediated transfection in 3 cm plates containing a 24x40 mm glass coverslip to which the cells adhered. Complete media was replaced with 1.6 ml of DMEM, and cells were incubated for 1 hour. 600 ng of DNA was mixed with 100 µl DMEM, while 20 µl of Lipofectamine 2000 was separately mixed with 100 µl of DMEM. These mixtures sat at room temperature for 5 minutes, and were then combined and incubated for 20-55 minutes at room temperature. The DNA-lipofectamine mixture was added to the 3 cm plates, and incubated for 5 hours at 37°C, 5% CO₂. The transfection media was then removed and replaced with 3 ml of complete media. Expression was

assessed 18-24 hours post-transfection. Cultures were then either rinsed with PBS and fixed for 2 minutes with 4% paraformaldehyde (PFA) in PBS, or were cultured in the presence of biotin conjugates. For the fixed cultures, Atto 550-Biotin (Sigma Aldrich, St. Louis, MO) or Biotin-IgG (biotinylated goat IgG isotype control, GeneTex, Irvine, CA) was added to cells at 10 ng/ml, 100 ng/ml, or 1 µg/ml in PBS/0.2% Triton X-100 (PBT) at 4°C overnight. Living cells in culture were incubated with biotin conjugates at the same doses described above by adding them directly to the culture media for 4 hours at 37°C, 5% CO₂. Media was replaced with normal complete media and incubated for 2 hours at 37°C, 5% CO₂. Cells were rinsed with PBS and briefly fixed with PFA. Transfected cells were identified through GFP immunocytochemistry described below. Atto 550-Biotin fluoresces in the red spectrum and was directly detected through fluorescence microscopy. Biotin-IgG was detected in parallel with GFP through immunocytochemistry. The coverslips were mounted on glass slides prior to microscopy.

Generating ES cell lines, chimeric mice and founder lines

129/Ola ES cells (BayGenomics) were maintained in DMEM(high glucose)/ 15% FBS/ 1 mM sodium pyruvate/ 1 mM nonessential amino acids/ 2 mM L-glutamine/ 10⁻⁶ M β-mercaptoethanol/ 10 ng/ml leukemia inhibitory factor (LIF). ES cells were grown on a feeder layer of mouse embryonic fibroblasts to obtain 1-2x10⁵ cells for electroporation. Linearized (ScaI digested) *pZ/StrepG* (1 µg/µl) was electroporated (250 V, 500 µF) into ES cells and clones that were

positive for *Z/StrepG* integration were selected for with 150 µg/ml G418. Positive clones were further screened by PCR analysis to confirm the integrity of the 5' and 3' ends and the GFP domain between. Clones with fully integrated *Z/StrepG* were then screened by Southern analysis for single-copy integration. DNA was digested with EcoRV, separated by gel electrophoresis, and transferred to Zeta-Probe nylon membrane (Bio-Rad). The membrane was hybridized with a ³²P-labeled 600 bp fragment encompassing GFP. Three clones harboring putative single copies of *Z/StrepG* were used to generate chimeric mice. ES cells were injected into the blastocoel cavity of 3.5 day old B6(Cg)-Tyr^{c-2J}/J embryos (Jackson Laboratories). The host embryos were albino, and the 129/Ola ES cells carry the agouti gene allowing for coat color assessment of chimerism. Two of the clones produced viable chimeras, and F1 progeny were screened for germ line transmission as indicated by coat color and PCR screening for the presence of *LacZ* and *GFP* in DNA obtained from tail biopsies. Each chimera produced progeny positive for *Z/StrepG*. X-gal histochemistry (described below) on ear biopsies demonstrated weak *LacZ* expression in progeny of one of the lines. The other line showed strong *LacZ* expression comparable to the *Z/EG* positive control, and this line was established as the founder line for *Z/StrepG*.

Immunocytochemistry (ICC)

Sections were immunolabeled as previously described (Ellisor et al., 2009). Briefly, sections from *En1^{Cre}*, *En1^{Cre};Z/EG*, and *En1^{Cre};Z/StrepG* embryos and explant tissues at E11.5 were analyzed using an anti-GFP antibody (1:600,

Molecular Probes). Horizontal adult sections from *En1^{Cre};Z/StrepG* animals were immunolabeled with anti-GFP and anti- β -galactosidase (β -gal) antibodies (1:500, Biogenesis) to identify *Z/StrepG*-expressing cells. In addition, anti-MAP2 (1:1000, Sigma Aldrich), anti-GFAP (1:500, Millipore), anti-CD11b (1:500, AbD Serotec), and anti-ABCA2 (1:500, kindly provided by S. Shioda, Showa University School of Medicine, Tokyo, Japan) antibodies were used as biomarkers. Sections from electroporated chick embryos were analyzed with anti-GFP, anti- β -gal, and anti-streptavidin (Vector Laboratories, 1:100). Cells from cell culture experiments were also analyzed with anti-GFP, anti- β -gal, and anti-streptavidin. The secondary antibodies, Alexa 488 (Invitrogen; donkey anti-rabbit IgG; donkey anti-mouse IgG; donkey anti-rat IgG; donkey anti-goat IgG), Alexa 555 (Invitrogen; donkey anti-rabbit IgG; donkey anti-mouse IgG; donkey anti-rat IgG; donkey anti-goat IgG) Dylight 549 (Jackson ImmunoResearch Laboratories, donkey anti-chicken) were prepared at 1:500.

X-gal histochemistry

X-gal staining was done as previously described (Zervas et al., 2004; Ellisor et al., 2009). Briefly, transfected chick embryos, *Z/EG* and *Z/StrepG* ear biopsies, and sagittal *En1^{Cre}* and *En1^{Cre};Z/StrepG* adult sections were fixed for 5 minutes in 4%PFA. Tissues were then washed 2x 10 minutes in X-gal wash buffer (2 mM MgCl₂, 0.1% Ipegal CA-30, 0.05% deoxycholate, in PBS). Tissues were then incubated in X-gal reaction buffer (50 ml composed of: 106 mg

potassium ferrocyanide, 82 mg potassium ferricyanide, 2 ml X-gal substrate stock solution at 25 mg/ml, and 48 ml X-gal wash buffer) for 1-4 hours at 37°C.

Microscopy

Whole-mount images were collected with a Leica MZ16F stereoscopic epifluorescent microscope using PictureFrame3 software. Tissue section images were collected with a Leica DM6000B epifluorescent microscope using Velocity 5.2 imaging software (Improvision). High magnification images were obtained using a motorized stage with 10x and 20x objectives. True magnifications are indicated by scale bars. All images were pseudo colored live as part of the acquisition palettes. Imaging data sets were exported to Adobe Photoshop CS3 where montages of representative data were generated.

Results

Construct Design

Streptavidin cDNA containing unique 5' (BglII) and 3' (XhoI) restriction sites was synthetically made using the sequence published for a recombinant form of core streptavidin, *Stv-13* (Sano et al., 1995). *Stv-13* was selected over full-length streptavidin because it was shown to be more stable and more accessible to large biomolecules conjugated to biotin, while still forming the homotetrameric core complex (Sano et al., 1995). Notably, *Stv-13* was qualitatively shown to have enhanced affinity for biotin. The vector *pCALL2* was used as the backbone construct (Figure 1A). This vector was successfully used

in the generation of the conditional *Z/EG* reporter line of mice (Novak et al., 2000). Because of the ubiquitous strong promoter and conditional expression of reporters before and after recombination, this construct allowed for flexibility in screening for transgene function and expression at each stage of development (see methods and results below). Prior to subcloning *Stv-13*, *pCALL2* was modified through the addition of *IRES-eGFP* sequence into the multi-cloning site (XhoI 5', NotI 3') downstream of the *loxP*-flanked *beta-geo* stop cassette (Figure 1A). Subsequently, *Stv-13* was introduced 5' to the IRES element, yielding *pZ/StrepG* as detailed in Figure 1. Prior to Cre mediated recombination, this vector should produce only β -galactosidase (β -gal expressed from *LacZ*), and should be constitutively expressed in all cell types under the control of the ubiquitous chick β -actin promoter (Figure 1B). Following recombination between the *loxP* sites, the *beta-geo* stop cassette would be excised, and *streptavidin-IRES-eGFP* brought under the control of the chick β -actin promoter (Figure 1C). Because of the internal ribosome entry site (IRES), bicistronic message of both reporters is constitutively expressed, yielding cells that should express both streptavidin (*Stv-13*) and GFP proteins.

In vitro testing of pZ/StrepG in explant culture

To test the ability of the construct to recombine and express the reporters, we used *En1^{Cre}* mice (Kimmel et al., 2000) which express high levels of Cre recombinase throughout the developing mesencephalon (mes) and rhombomere 1 (r1), the midbrain and cerebellum primordia, respectively (Li et al., 2002;

Zervas et al., 2004). The mes/r1 region of E11.5 *En1^{Cre}* embryos was microdissected (Brown et al., 2009) (Appendix 1) yielding a “butterfly” shaped piece of tissue in which the ‘wing’ edges contain dorsal tissue, and the midline containing the most ventral tissue. An example of this dissection is represented in Figure 2, where *En1^{Cre};Z/EG* tissue is shown to highlight the GFP positive area indicating the activity of *En1^{Cre}* expression (Figure 2A,B). The *Z/EG* reporter is configured similarly to *Z/StrepG*, except in place of *streptavidin-IRES-eGFP*, is *GFP* alone.

En1^{Cre} explants were cultured for 24 hours prior to lipofectamine mediated transfection of *pZ/StrepG*. The transfected explants were monitored over 4 days to assess transfection and for recombination through GFP fluorescence. Though sparse fluorescence was observed in living cells using a fluorescent stereoscope (not shown), GFP immunocytochemistry and high magnification fluorescence microscopy revealed clearly identified GFP positive cells (Figure 2C). A subset of the ventral mes (midline area of explant) is where dopamine neurons are generated (Zervas et al., 2004), so we assessed the health of the explant tissue over time and whether we had also transfected dopamine neuron progenitors that had differentiated by immunolabeling for tyrosine hydroxylase (TH, dopamine biosynthetic enzyme)(Figure 2D). Dopamine neurons were easily identified, and remained healthy for up to 7 days in culture. These results confirmed that our cultures developed properly and established that our culture conditions were conducive to dopamine neuron differentiation and growth. However, marked (transfected, GFP positive) neurons were not co-localized with

TH (Figure 2D: inset), indicating that we primarily transfected non-dopamine neuron progenitors. These experiments demonstrated that *pZ/StrepG* was able to be ‘activated’ (recombined) in neuronal progenitors, and that neurons remained healthy over the time course of expressing the reporters. Though not directly shown, streptavidin and GFP expression did not appear to be detrimental to cell growth, neuronal differentiation, or biotin metabolism *in vitro*.

Testing of pZ/StrepG expression through in ovo chick electroporation

Though explant culture provided the means to test expression in embryonic mouse tissue, lipid-based transfection provided only limited transfection which precluded labeling of high numbers of cells throughout the tissue preparation.

We hypothesized that we could observe robust expression of our reporter using the developing chick embryo. *In ovo* chick electroporation allows for the precise delivery of large amounts of DNA to the developing neuroepithelium (Muramatsu et al., 1997). Chick embryos develop within the egg following electroporation, providing an *in vivo* expression system to test the efficacy of *pZ/StrepG* and to determine whether high levels of expression was detrimental to developing tissues. This last point is of particular importance, as high levels of streptavidin expression could create a “biotin sink” in which essential endogenous biotin is sequestered from enzymes which require it as a co-factor (Knowles, 1989; Jitrapakdee and Wallace, 2003).

Several hundred nanograms of the native and recombined forms of *pZ/StrepG* were independently delivered to the lumen of the neural tube of Hamburger-Hamilton (HH) stage 9-10 chick embryos. The DNA filled the lumen spanning the mesencephalon/metencephalon boundary (midbrain/cerebellum) prior to electroporation. Pulses of current were applied laterally across the embryo, delivering the construct to one half of the neural tube (see methods). The eggs were sealed following electroporation allowing the embryo to continue developing normally. Expression was assessed 24 hours later (at HH 17) after the neural tube had closed. Electroporation of *pZ/StrepG* led to high levels of expression of the unrecombined *LacZ*- (β -gal) expressing construct, or the activated recombined *streptavidin/GFP*-expressing construct as determined by whole-mount analysis (Figure 3A-C). Electroporation specificity of the activated construct was readily apparent by comparing the internal control (non-electroporated) side of the embryo in a dorsal view (Figure 3C). There were no gross morphological perturbations resulting from unilateral expression of any of the *pZ/StrepG* gene products. Double immunolabeling for β -gal and GFP in tissue sections of embryos electroporated with unrecombined *pZ/StrepG* confirmed β -gal expression and showed an absence of GFP expression, validating that the stop cassette effectively prevented transcription downstream of *LacZ* (Figure 3D). Immunolabeling for GFP and streptavidin following electroporation of the recombined construct showed that the expression of both proteins was largely coincident, although not every transfected cell was positive for both protein products (Figure 3E,F).

These results definitively confirmed the expression capacity of *pZ/StrepG* as a conditional genetic allele. All three gene products were effectively expressed to no obvious detriment of the cells or development of the embryo.

Testing for intracellular streptavidin-biotin interaction

While *in ovo* chick electroporation provided an ideal system to test for construct expression *in vivo*, it did not provide an amenable platform for testing the delivery and binding of biotin conjugates (not shown). Thus, we turned to mammalian cell culture utilizing HEK293 cells. This experimental approach allowed for robust transfection and flexibility of culture conditions for the testing of biotin binding and retention. HEK293 cells were grown in 3 cm plates that contained a glass coverslip to which the cells adhered. Cells were transfected with activated *pZ/StrepG* using lipofectamine, and expression was assessed 18-24 hours post-transfection (Figure 4). Biotin conjugates were then added to the cultures in two separate paradigms: (1) delivered directly to the culture media to allow living cells to uptake biotin; (2) delivered to cells which were briefly fixed for 2 minutes with 4% paraformaldehyde and permeabilized with detergent. The first approach (1) allowed us to assess whether living cells could transport biotin conjugates across the cell membrane and be bound by cytoplasmic streptavidin. In the case that HEK293 cells in culture were unable to incorporate the biotin conjugates, approach (2) allowed us to bypass transport by permeabilizing the membrane and still test for streptavidin-biotin interactions. Because the cells adhered to the glass coverslips, we were able to utilize immunocytochemistry

and high magnification fluorescence microscopy after mounting the coverslips on glass slides to visualize cells which expressed *pZ/StrepG* gene products and determine whether they contained biotin conjugates (Figure 4).

Within 24 hours post-transfection the construct was robustly expressed, and validated that HEK293 cells transfected with the recombined form of *pZ/StrepG* expressed both GFP and streptavidin (Figure 4A). We then added biotin directly conjugated to the red fluorophore Atto 550 (Atto 550-Biotin) to living cultures or plates containing cells which were briefly fixed. Atto 550-Biotin was added at concentrations from 10 ng/ml to 1 µg/ml, in agreement with levels found in common cell culture media (all data in Figure 4 represent conditions using a concentration of 100 ng/ml). Following an incubation of 4 hours, media containing Atto 550-Biotin was removed from the living cultures and replaced with normal media for 2 hours. The cultures were then washed several times with phosphate buffered saline (PBS), briefly fixed, and immunolabeled for GFP. Because cells may not readily take in biotin conjugated to Atto 550, we permeabilized cells that were briefly fixed and incubated them with Atto 550-Biotin in PBS containing 0.2% Triton X-100 (PBT). These cells were immunolabeled for GFP in parallel (Figure 4B,C). However, whether we supplemented Atto 550-Biotin to culture media, or added it to fixed/permeabilized cells, we were unable to detect the specific retention of biotin in transfected cells compared to non-transfected cells. Both approaches yielded background fluorescence that obscured an appreciable increase of fluorescence in cells expressing streptavidin/GFP (Figure 4B,C: representative data from fixed cell

paradigm). Transfected cells displayed red fluorescence indicating the presence of Atto 550-Biotin at levels indistinguishable from neighboring cells (Figure 4B,C arrows vs. arrowheads). We varied the incubation period and the number and duration of detergent washes which did not substantially alter background to signal (not shown). For example, decreasing the incubation period (from 4 to 2 hours in culture) and increasing the amount of time post-biotin removal (from 2 to 4 hours in culture) did not reduce background fluorescence. Similarly, doubling the amount of time cultures were washed with PBT in either paradigm had no effect. As biotin was expected to be incorporated in all cells through general metabolic processes, but hypothesized to be in greater abundance in cells overexpressing streptavidin, the use of a biotin conjugate directly linked to a fluorophore may have created conditions with unmanageable background.

We then used a different biotin conjugate which was not directly conjugated to a fluorophore and could be detected through our general immunocytochemical approach (Figure 4D,E). We substituted Atto 550-biotin with biotin-IgG (biotin conjugated to a non-specific goat IgG isotype) to allow us to detect biotin using fluorescent secondary antibodies that recognized goat immunoglobulins. Biotin-IgG was added to living or fixed cells as described above, and was indirectly detected along with GFP using our immunocytochemistry protocol. A secondary antibody conjugated to a red fluorophore was used to detect the presence of biotin-IgG. However, whether we added biotin-IgG to living or fixed cells, the non-transfected cells were positive for biotin-IgG at indistinguishable levels as compared to transfected cells (Figure 4D:

representative data from fixed cell paradigm). To control for the baseline of background fluorescence, we applied a threshold value for the red channel (Figure 4E) that was calculated from the total red fluorescence of the negative control (Figure 4F). This control consisted of cultures that did not receive biotin-IgG, but the secondary antibody used to detect it was applied in parallel to the experimental conditions (as exemplified in Figure 4F). We adjusted the fluorescence levels of the red channel in the negative control so that background red fluorescence approached zero. This “threshold” value for background fluorescence was then applied to data from the experimental conditions (Figure 4D versus E). Prior to threshold adjustment, transfected cells were detected as both GFP and biotin-IgG positive (Figure 4D, insets 1 and 2). However, applying the threshold to the same data resulted in no appreciable detection of biotin-IgG within the same transfected cells (Figure 4E, insets 1* and 2*).

Potential problems remained in this last approach, including background levels of fluorescence, the ability of living cells to uptake biotin conjugated to an immunoglobulin, and whether streptavidin in fixed cells retained its capacity to bind biotin. Through careful examination of controls, no definitive conclusion could be made as to whether biotin was specifically accumulated and retained in streptavidin-expressing cells.

Screening ES cell lines for Z/StrepG integration, and founder lines for germ line transmission

The *in vitro* and *in ovo* experiments described in the previous sections demonstrated the Cre-responsiveness and expression capacity of our allele. Although the conjugates we used to test for biotin-streptavidin interactions gave inconclusive results, we had begun, in parallel experiments, to generate transgenic mice harboring our reporter.

Transgenic mice are traditionally generated through pronuclear injection of a linearized genetic construct in fertilized eggs (Nagy, 2003). However this approach often results in the random integration of many copies of the transgene in tandem, and could create unpredictable Cre-based recombination events leading to transgene or chromosomal loss (Lewandoski and Martin, 1997). To circumvent these issues, we employed electroporation techniques more commonly used in the generation of gene-targeted lines which provide greater chance of single-copy transgene integration (Baer et al., 2000). We generated embryonic stem (ES) cell lines harboring *Z/StrepG* that integrated randomly in the genome by the electroporation of *pZ/StrepG*. ES cell lines were selected for using the neomycin positive selection cassette contained within the construct (Figure 5A) by screening for G418 resistance. We also used primers designed to amplify the 5' and 3' ends of the transgene and a primer set recognizing GFP to check for full integration and integrity of the reporter genes (Figure 5A,B). All ES cell lines were positive for GFP (n = 48), while only ~30% of the lines contained integrated *Z/StrepG* with properly intact ends. Eight positive clones were further

screened by Southern blot analysis, three of which were putative single copy integrants based on the signal of the Southern probe (P^{32} radiolabeled *GFP*) hybridizing to a single band (example in Figure 5B').

Chimeric mice were generated by Brown University Transgenic Facility by injecting ES cell lines into blastocyst stage embryos which were subsequently implanted into pseudo-pregnant females (Nagy, 2003). Three ES cell lines were injected, and two produced viable chimeras with discernible chimeric coat color (example in Figure 5C). These two chimeras were then bred with B6(Cg)-Tyr^{c-2J}/J mice (Jackson Laboratories) to produce F1 progeny to screen for germ line transmission of *Z/StrepG*. PCR genotyping for the presence of both *GFP* and *LacZ* in F1 progeny determined that both chimeras provided germ line transmission of *Z/StrepG* (example in Figure 5D). Because of the ubiquitous β -actin promoter and the configuration of the unrecombined transgene (Figure 5A), β -gal should have been expressed in all tissues of F1 progeny positive for *Z/StrepG*. Thus, we also phenotyped ear biopsies of F1 mice by X-gal to screen for the expression of *LacZ* from the *Z/StrepG* allele (Figure 5E). This analysis reconfirmed that progeny from each chimera contained *Z/StrepG*; however the ear biopsies provided evidence that *Z/StrepG* was not ubiquitously expressed in progeny from one of the clones which contained very few cells stained for β -gal activity (Figure 5E, *Z/StrepG* founder 1). The second line demonstrated comparable expression of *LacZ* to the positive *Z/EG* control and was further characterized for transgene expression and function.

Characterizing Z/StrepG founder mice

We first tested the transgene function and expression *in vivo* by breeding *Z/StrepG* mice with *En1^{Cre}* mice. As stated earlier, *En1^{Cre}* expression mediates Cre based recombination throughout the developing midbrain and cerebellum primordia (mes and r1, respectively). Thus, embryos positive for both alleles should express GFP and streptavidin throughout the mes and r1 and β -gal in the non-*En1* expressing domains. We expected to readily observe expression of the recombined form of the transgene by GFP whole-mount fluorescence in midgestation *En1^{Cre};Z/StrepG* embryos because the backbone construct we used to generate *Z/StrepG* was also used for the *Z/EG* reporter allele (Novak et al., 2000). We predicted that GFP levels would be comparable to the GFP whole-mount fluorescence of *En1^{Cre};Z/EG* embryos (see Figure 2A for comparison). However, faint GFP fluorescence was observed in both E11.5 and E14.5 *En1^{Cre};Z/StrepG* embryos as compared to wild type controls (Figure 6A-D). Wild type, *Z/StrepG*, and *En1^{Cre};Z/StrepG* embryos were indistinguishable morphologically, confirming that *Z/StrepG* underwent proper recombination in the presence of Cre expression and that transgene expression was not detrimental to the developing embryo. However, GFP whole mount fluorescence was substantially fainter than the *Z/EG* reporter (compare Figures 2A vs 6C at E11.5). We then evaluated tissue sections from E14.5 *En1^{Cre};Z/StrepG* embryos through immunocytochemistry, which revealed that not all cells within the current or derived *En1^{Cre}* expression domain were expressing GFP (Figure 6E). This likely contributed to the faint levels seen by whole-mount fluorescence.

We also assessed the transgene expression and function in the adult brain. The brains of *Z/StrepG* and *En1^{Cre};Z/StrepG* mice were analyzed at six weeks of age for global transgene expression as well as cell type specific expression (Figures 7 and 8). X-gal staining of sagittal sections obtained from adult *Z/StrepG* and *En1^{Cre};Z/StrepG* mice revealed transgene expression throughout the brain, however *LacZ* expression was diffuse and not ubiquitous in every region of the brain (Figure 7A). Areas within cortex, midbrain, and brainstem in particular showed areas void of transgene expression (Figure 7A-C). In the absence of *En1^{Cre}* expression during development, the transgene does not recombine and β -gal is expressed in the midbrain and cerebellum (Figure 7B). As was confirmed during development, the expression of *En1^{Cre}* in the developing midbrain and cerebellum recombines the reporter, thereby removing the *LacZ* expression cassette and completely eliminating the expression of β -gal in the mature midbrain and cerebellum (Figure 7D, E, compare B vs E). In both *Z/StrepG* and *En1^{Cre};Z/StrepG* tissues, the cortex provided internal controls for comparison across each animal as this area is not derived from an *En1*-expressing domain (Figure 7C,F).

To further investigate the nature of *Z/StrepG* expression we performed immunocytochemistry against β -gal, GFP, streptavidin, and cell specific markers. GFP and β -gal ICC revealed many of the transgene expressing cells to have an elaborate, bushy morphology (Figure 8). Examples of this morphology could be found throughout the brain, including the midbrain (Figure 8A,B,D,E,G,H), cortex (Figure 8C,F,K), and cerebellum (Figure 8I). The morphology of these cells

appeared glial, which we tested with markers specific to microglia (CD11b, Figure 8A-C), oligodendrocytes (ABCA2, Figure 8D-F) and astrocytes (GFAP, Figure 8G-I) to determine whether the expression of *Z/StrepG* was biased towards glial lineages.

Microglia express *cluster of differentiation molecule 11b* (CD11b, also known as Integrin alpha M) (Rosen and Gordon, 1990; Hickman et al., 2008). CD11b immunostaining revealed that *Z/StrepG* was not expressed by microglia, neither the native or recombined form of the allele (Figure 8A,B vs C). As microglia are not derived from the neural ectoderm (Chan et al., 2007), we were not expecting them to be positive for GFP expression (not descendant of *En1^{Cre}* expressing progenitors). However, all cells should have the capacity to express the unrecombined form, yet no microglia were found to express β -gal (Figure 8J).

Another main class of glia are oligodendrocytes, which express *ATP-binding cassette transporter 2* (ABCA2 (Zhou et al., 2001). Surprisingly, though they constitute the majority of glia, ABCA2-expressing cells were not positive for any *Z/StrepG* gene product (Figure 8D-F).

Interestingly, only one of the other main class of glia were shown to express *Z/StrepG*. *Glial fibrillary acidic protein* (GFAP) is expressed primarily by astrocytes (Fuchs and Weber, 1994). This marker indicated that a small number of *Z/StrepG*-expressing cells were positive for GFAP (Figure 8G-I), and were most abundantly found throughout the white matter of the cerebellum (Figure 8I).

Transgene expression was not limited to glia alone (Figure 8J,M,N). Neurons of the cerebellum expressed the recombined transgene, as both

Purkinje cells and granule cells were positive for GFP (Figure 8J, solid and open arrowheads, respectively). As the vast majority of *Z/StrepG*-expressing cells throughout the rest of the brain were not positive for any of the glial markers tested, we used a neuronal marker to assess whether the bushy, glial-looking cell types were instead neurons. *Microtubule associated protein 2* (MAP2) is expressed in the cell bodies and dendrites of mature neurons (Figure 8K-N). Interestingly, the bushy cell types expressing *Z/StrepG* did not co-express MAP2, indicating that these cell types were not neuronal (Figure 8K,L). It is possible that this cell type is in fact glial, but were not readily identified using the subset of glial markers in this study. However, *Z/StrepG*-expressing neurons in the cortex which lacked the bushy morphology were identified as neurons based on MAP2 immunolabeling (Figure 8M,N).

These results show that the expression of *Z/StrepG* *in vivo* occurred in discrete populations of cells within the brain. The lack of substantial neuronal expression hinders the use of this transgenic animal in fate mapping studies in which neurons are to be assessed in an unbiased fashion. While the strongest expression of *Z/StrepG* occurred in unidentified (presumably glial) cell types, we did not detect the streptavidin protein product through immunocytochemistry (not shown). This may have been due to an inability of the antibody to recognize streptavidin expressed in mouse nervous tissue, a lack of streptavidin translation from the bicistronic message, or rapid turnover of the protein product.

Discussion

Fate mapping in three dimensions

Determining the three-dimensional lineage contribution of genetically defined progenitor populations using current fate mapping reporter alleles requires labor intensive stereological assessment of serially sectioned tissues. In addition, the continued *in vivo* longitudinal assessment of the population over time is not possible. We sought to generate a transgenic reporter capable of providing the biomolecular means to accumulate MRI-contrast agents in genetic lineages thereby allowing three-dimensional mapping *in vivo*. Our reporter was designed to provide a molecular switch, whereby every cell during development and in the mature animal would express the native, inert form of the streptavidin reporter, but upon Cre expression in a subset of cells the reporter would be recombined. This recombination event, which acts as a switch to turn on streptavidin expression, was to provide a molecular scaffold for the accumulation of biotinylated MRI-contrast agents. If this switch was made during development in a genetically defined population of interest, all cells descendant from this population would express streptavidin. By administering exogenous biotinylated MRI-contrast agents later in development or in the mature animal, we hypothesized that the streptavidin-expressing population would create a sink for the contrast agent allowing for the direct detection of this population *in vivo* by MRI. This accumulation of the contrast agent would be transient, and would allow for the repeated assessment of the population through the re-administration of the biotinylated contrast agent. This would provide longitudinal tracking of a

developmentally dynamic population and how it comes to reside within the adult structure, or the ability to track how a population of cells changes during the course of disease or aging.

Iron oxides as contrast agents for MRI, Pros and Cons

Our experimental design was to deliver biotinylated iron oxide (biotin-Fe₃O₄) as a contrast agent in our system. Implicit to our design was the ability to provide transient accumulation of biotin-Fe₃O₄. Iron oxides exhibit supraparamagnetic properties (Renshaw et al., 1986), and transgenic studies aimed at increasing iron concentrations within cells through the manipulation of iron homeostasis genes have shown the accumulation of iron to be detectable by MRI (Weissleder et al., 2000; Cohen et al., 2005; Deans et al., 2006; Cohen et al., 2007). By overexpressing the transferrin receptor (Weissleder et al., 2000), ferritin (Cohen et al., 2005), or both (Deans et al., 2006) cells were able to continually accumulate endogenous or supplemented iron and were detected by MRI. However the continued elevation of iron in cells was shown to promote the formation of reactive oxygen species (ROS) which can lead to cytotoxic effects (Grabill et al., 2003; Deans et al., 2006). Our approach would allow streptavidin to be expressed (and presumably turned over) throughout the lifetime of cells which recombined our reporter. Thus, biotin-Fe₃O₄ could be exogenously administered prior to imaging and would decrease the amount of time iron is accumulated in the cells, thereby decreasing the likelihood of ROS production and cell damage/death. This is critical to the continued assessment of a

population over time, especially when probing the state of a tissue during the course of disease.

Two reporter genes are better than one

Another advantage of our design was the incorporation of GFP expression mediated by the bicistronic message (Figure 1). As we demonstrated, *streptavidin-IRES-eGFP* mediated the concurrent expression of both protein products (Figure 3E,F, Figure 4A). Thus, MRI studies aimed at tracking the contribution of progenitors to tissues, or the loss of certain cells during disease/aging, could be verified at the cellular level at the conclusion of the experiment. The expression of GFP from our construct allowed for detailed cellular morphology, whether expressed *in vitro* (Figure 4) or *in vivo* (Figure 8).

The expression of GFP also confers flexibility for other applications involving the specific isolation of stem or progenitor cells through fluorescence-activated cell sorting (FACS). For example, genetically defined stem cells used in studies of cell replacement therapies could be obtained from embryonic tissue following expression of our reporter and isolation through FACS. These cells could then be transplanted in to a diseased host to reestablish compromised tissue. The engraftment and maintenance of the transplanted population could be monitored over time through the administration of biotin-Fe₃O₄ and detection by MRI. Thus, the degree of engraftment could be measured against the relative improvement of the disease state. As noted, detailed postmortem analysis of engraftment could be assessed through GFP immunocytochemistry.

Problem areas and suggestions for future designs

Despite conceptual advantages to our design, once incorporated into the mouse genome our reporter was not ubiquitously expressed in all cell types. This was likely due to transgene silencing effects resulting from the random integration of the transgene in an area of chromatin which remains transcriptionally inactive in some cells (Neuhuber et al., 1994; Robertson et al., 1995; Garrick et al., 1998). Because our construct was modeled after other transgenic reporters displaying ubiquitous expression (Lobe et al., 1999; Novak et al., 2000), simply re-deriving the line would likely produce mice with widespread expression.

Additional concerns at the molecular level were encountered through testing the function our construct. Specifically, we were unable to demonstrate the accumulation of biotinylated compounds in cells expressing our reporter *in vitro*. This may have been due to our experimental approaches, as non-specific background fluorescence obscured the detection of the biotin conjugates in transfected cells. It is also possible that stores of endogenous biotin, or free biotin in the culture media, preferentially occupied streptavidin binding sites and precluded the biotin conjugates from accumulating. Depriving the cultures of native biotin for finite periods prior to adding the biotin conjugates could circumvent this concern at least *in vitro*. Alternatively, cells may lack the ability to transport biotin in these conjugated forms. In this regard, the specific incorporation of biotin conjugates of interest (i.e. biotin-Fe₃O₄) would need to be

assessed before redesigning the reporter. If cell populations of interest are innately unable or inefficient at transporting biotin conjugates, then the expression of cytoplasmic streptavidin will not provide a sink for biotin accumulation. One solution to this putative problem that would not require a complete redesign of our approach would be the replacement of core streptavidin in our reporter with an extracellular, membrane-bound form of streptavidin (Gotoh and Matsumoto, 2007). The presence of the biotin binding domains on the surface of cells would preclude the requirement of cells to incorporate biotin-Fe₃O₄ intracellularly. This may also reduce the background signal from neighboring cells which do not express the reporter, as they would not be able to incorporate or bind biotin conjugates.

Of necessity in our design is the conditional nature of the reporter, through regulation of Cre/*loxP* recombination. However, other inducible systems for transgene expression exist, such as the use of tetracycline responsive elements (Gossen and Bujard, 1992). In this system, the tissue specific expression of the tetracycline transactivator (tTA) and presence of a tTA-responsive reporter gene can control the off and on state of the reporter through delivery of tetracycline. This approach was used to differentially express the ferritin gene in hepatocytes *in vivo*, allowing for the accumulation of endogenous iron and detection of the cells by MRI (Cohen et al., 2007). An advancement on this platform more suitable to the goals of our transgenic reporter, would be the incorporation of a *loxP*-flanked stop cassette inhibiting the expression of the tTa-responsive ferritin reporter. In this configuration, tetracycline would only induce expression of the

reporter in cells which have undergone Cre-mediated recombination. This reporter could then be combined with the multitude of cell or tissue specific Cre/CreER drivers, providing spatiotemporal lineage specificity with temporal induction of the reporter. Thus, genetically defined cell populations could sequester and accumulate endogenous iron at time points of choosing through the administration of tetracycline. This reporter would be poised to provide the transient accumulation of iron, thereby circumventing the increased risk of ROS production while maintaining the capacity for repeated assessment of cells or tissues of interest by MRI.

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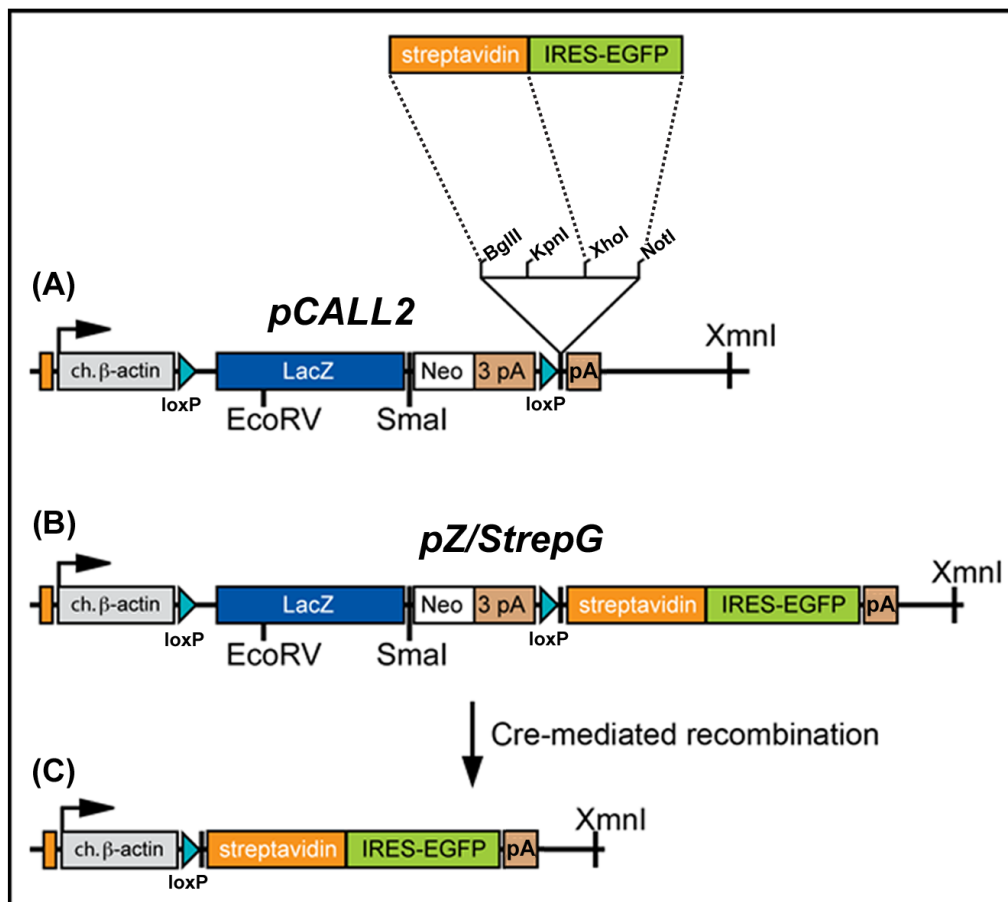


Figure 1. *pZ/StrepG* construct design.

cDNA coding for core *streptavidin* was introduced 5' to *IRES-eGFP*, which was subsequently subcloned into the multi-cloning site of *pCALL2* (A) utilizing BglII (5') and NotI (3') restriction sites. In this configuration (B), *LacZ* is constitutively expressed from the chick β -actin promoter and *streptavidin-IRES-eGFP* is not expressed because of the presence of the stop cassette encoded by a triple repeat poly-adenylation sequence (3x pA). Upon Cre mediated recombination between loxP sites (blue), *LacZ-Neo-3x pA* is excised, bringing *streptavidin-IRES-eGFP* under the control of the chick β -actin promoter inducing its permanent and constitutive expression (C).

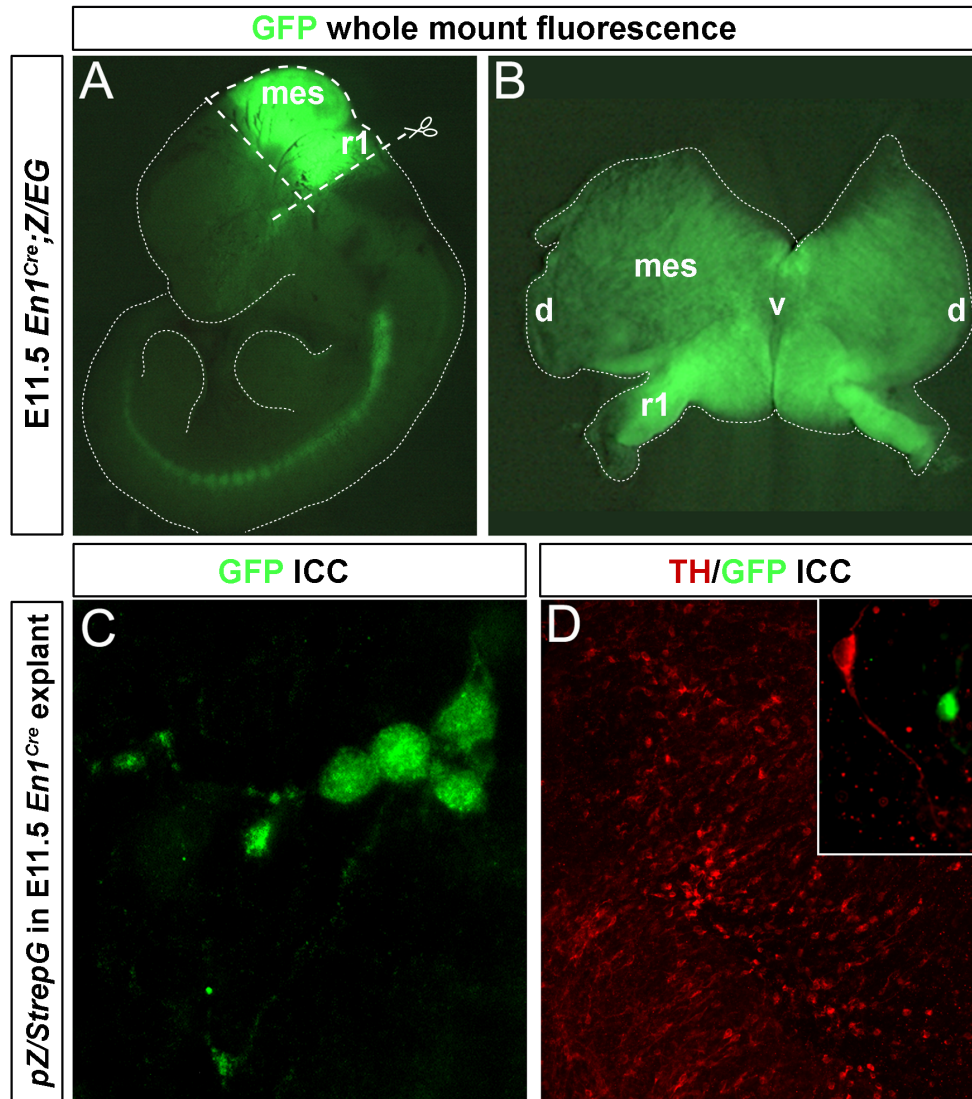


Figure 2. *In vitro* testing of *pZ/StrepG* in explant culture.

(A) Sagittal whole mount GFP fluorescence image of E11.5 *En1^{Cre};Z/EG* embryo, showing marking of entire mesencephalon (mes) and rhombomere1 (r1) as a result of Cre expression and recombination. Large dashed lines indicate the incision paths for microdissected tissue. (B) Microdissected mes/r1 region from embryo in (A) representative of the tissue transfected with *pZ/StrepG*, d: dorsal, v: ventral. (C) GFP positive neurons labeled by GFP immunocytochemistry (ICC) in a *pZ/StrepG* transfected E11.5 *En1^{Cre}* mes/r1 explant indicating successful recombination of *pZ/StrepG* following transfection within Cre expressing cells. (D) TH immunostaining (red) of *pZ/StrepG* transfected E11.5 *En1^{Cre}* mes/r1 explant, inset: high mag image of GFP positive neuron adjacent to TH labeled neuron.

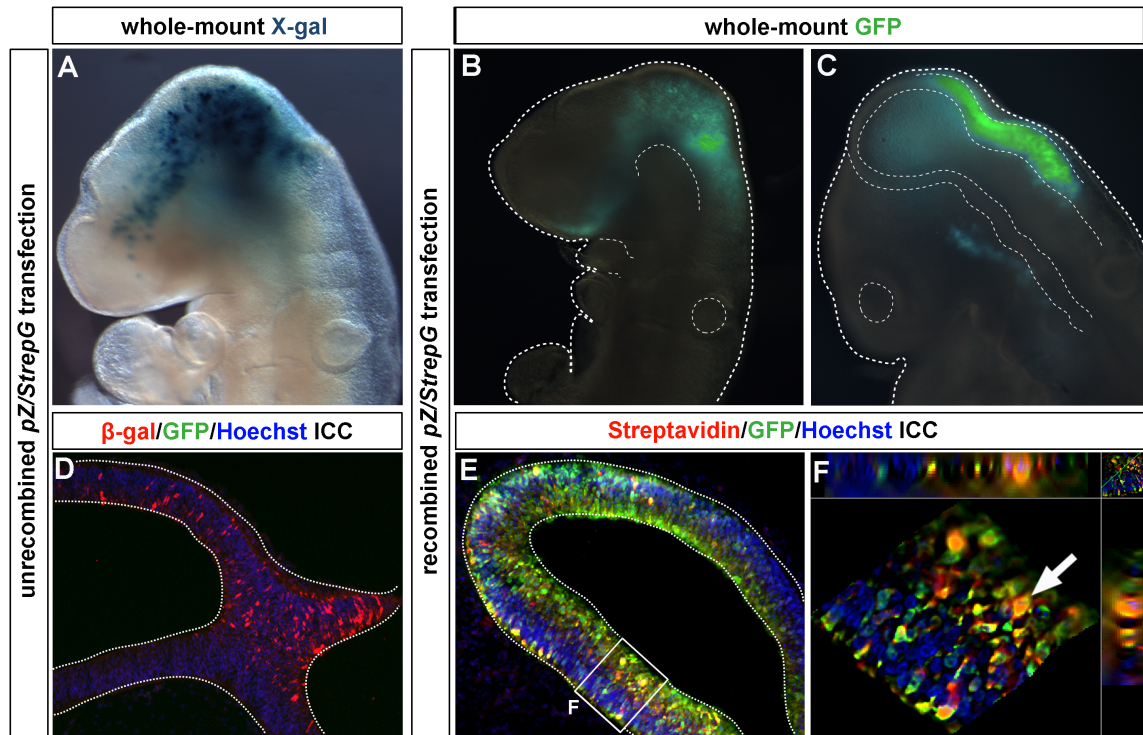


Figure 3. Testing of *pZ/StrepG* expression through *in ovo* chick electroporation.

(A) X-gal staining 24 hours post-transfection of the unrecombined form of *pZ/StrepG* demonstrated robust *LacZ* expression in a whole-mount sagittal HH stage 17 chick embryo. (B,C) Whole-mount analysis of GFP expression 24 hours post-transfection of the recombined form of *pZ/StrepG*. An off-dorsal view showed the unilateral transfection resulting from electroporation across one half of the neuroepithelium (C). Immunocytochemistry (ICC) of sagittal sections through the chick in (A) revealed cells expressing *LacZ* (β -gal) and not GFP, which indicated no leakiness of expression through the stop cassette in the unrecombined form of *pZ/StrepG* (D). ICC for streptavidin and GFP in sagittal sections of the chick transfected with the recombined form of *pZ/StrepG* in (B,C) revealed co-localization of each protein resulting from their combined expression (E,F). Higher magnification and Z-series analysis of an area in (E) provided greater detail of streptavidin/GFP expressing cells, arrow indicates the cell present in the X-Z and Y-Z planes (F).

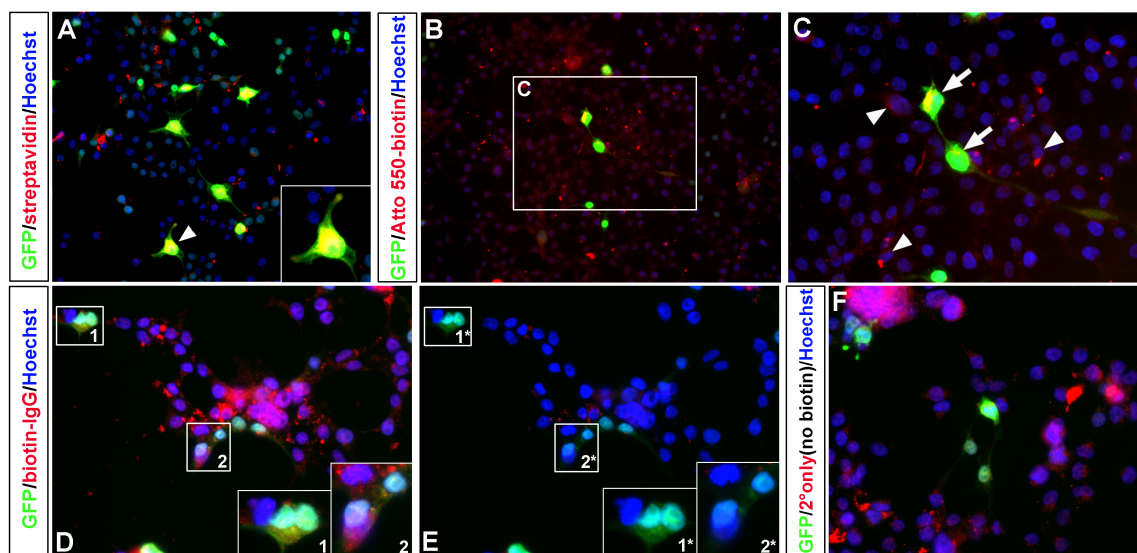


Figure 4. Testing for intracellular streptavidin-biotin interactions in transfected HEK293 cells.

(A) Both streptavidin and GFP were expressed from the recombined form of *pZ/StrepG* in transfected HEK293 cells (inset higher mag of cell denoted with arrowhead). (B,C) The use of biotin directly conjugated to a red fluorophore (Atto 550-biotin) revealed that transfected streptavidin/GFP expressing cells contained Atto 550-biotin (arrows in C), yet surrounding non-transfected cells were also positive for Atto 550-biotin (arrowheads in C). The use of biotin-IgG allowed for the detection of biotin in transfected cells through immunocytochemistry (D-F). Prior to thresholding the levels of red fluorescence calculated from the “no biotin-IgG” condition (F), transfected cells were positive for biotin-IgG (D, insets 1,2). However, applying threshold levels revealed little to no signal for presence of biotin-IgG (E, insets 1*,2*).

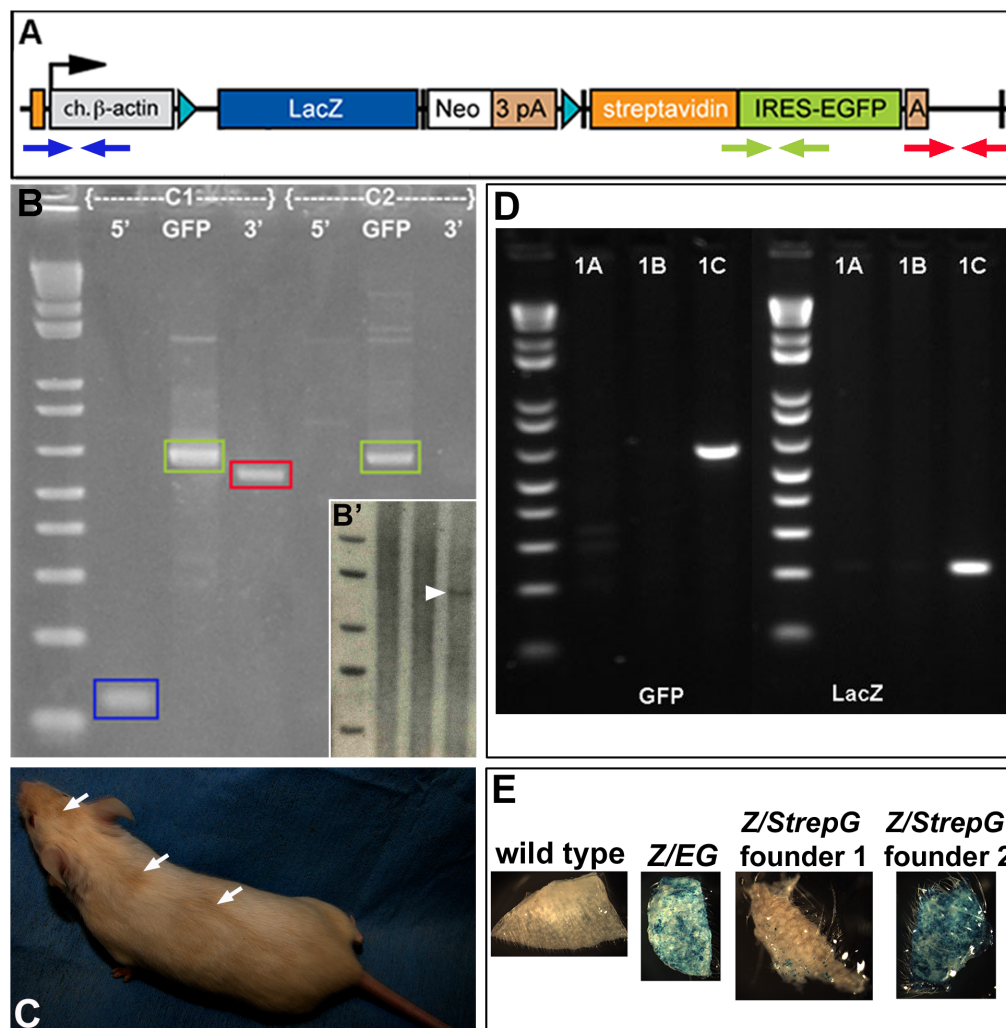


Figure 5. Screening for transgene integration and germ line transmission.

(A) Schematic representation of *Z/StrepG*, detailing the primer sites used to screen for transgene integration by PCR analysis in (B). PCR screening of ES cell lines generated through the electroporation of linearized *pZ/StrepG* revealed that only 30% (n=48) of cell lines contained the entire intact transgene (B). Inset (B') shows one example of an ES cell line which contained a single copy of *Z/StrepG* as determined through Southern blot analysis. Single copy integrant ES cell lines were used to generate chimeras identified through coat color chimerism (arrows), as exemplified in (C). Progeny of chimeras were screened for germ line transmission of *Z/StrepG* through PCR genotyping (D) as well as ear biopsy phenotyping (E). PCR genotyping confirmed germ line transmission from two chimeras through two independent PCR reactions (D). Two founder lines were positive for germ line transmission, however phenotyping analysis through X-gal staining of ear biopsies revealed one of the lines (*Z/StrepG* founder 1) severely under-expressed *Z/StrepG*, while the other founder line had comparable levels of β -gal activity as the *Z/EG* positive control (E).

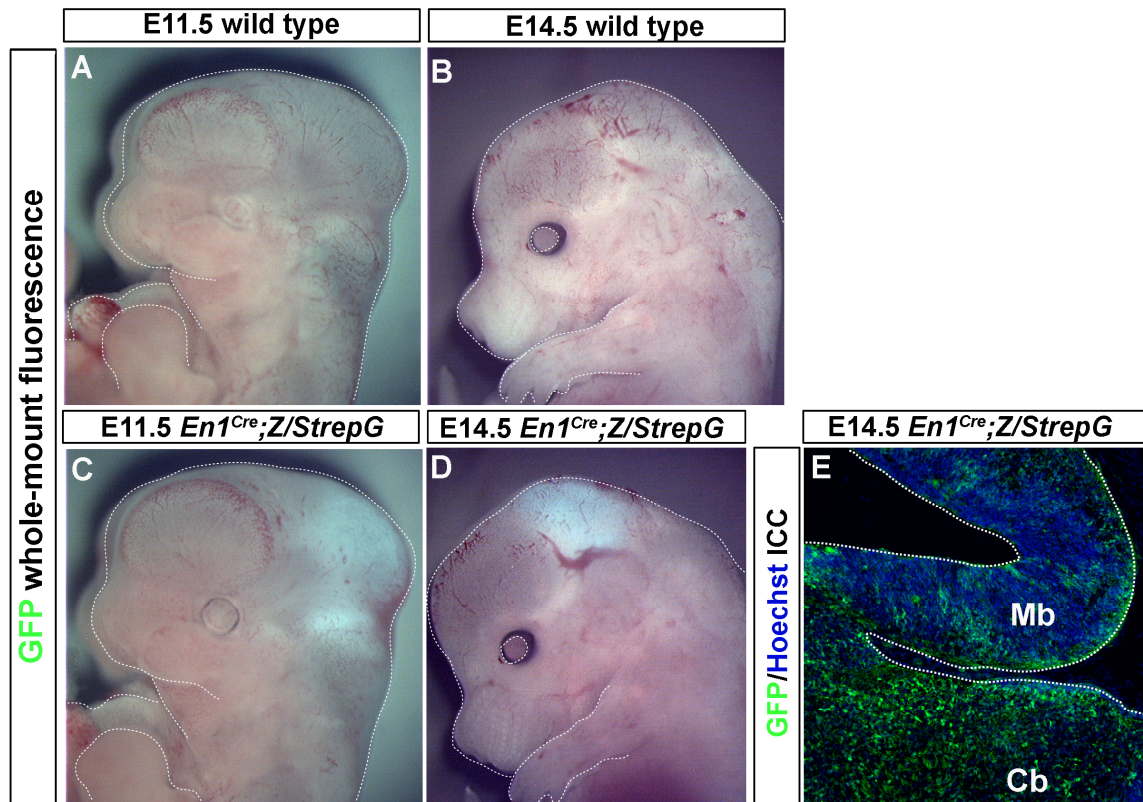


Figure 6. Analysis of *Z/StrepG* recombination and expression during development.

(A) E11.5 and (B) E14.5 wild type negative control embryos showed no GFP fluorescence. (C-E) *Z/StrepG* effectively underwent recombination *in vivo* and expressed GFP following Cre-mediated recombination resultant of *En1^{Cre}* expression. GFP expression was very faint, but clearly identified by whole mount fluorescence microscopy in E11.5 (C) and E14.5 (D) *En1^{Cre};Z/StrepG* embryos. Sagittal ICC tissue section analysis of *En1^{Cre};Z/StrepG* embryos at E14.5 showed that not all cells within the *En1^{Cre}* expression domain (Mb, Cb) expressed the recombined reporter (E).

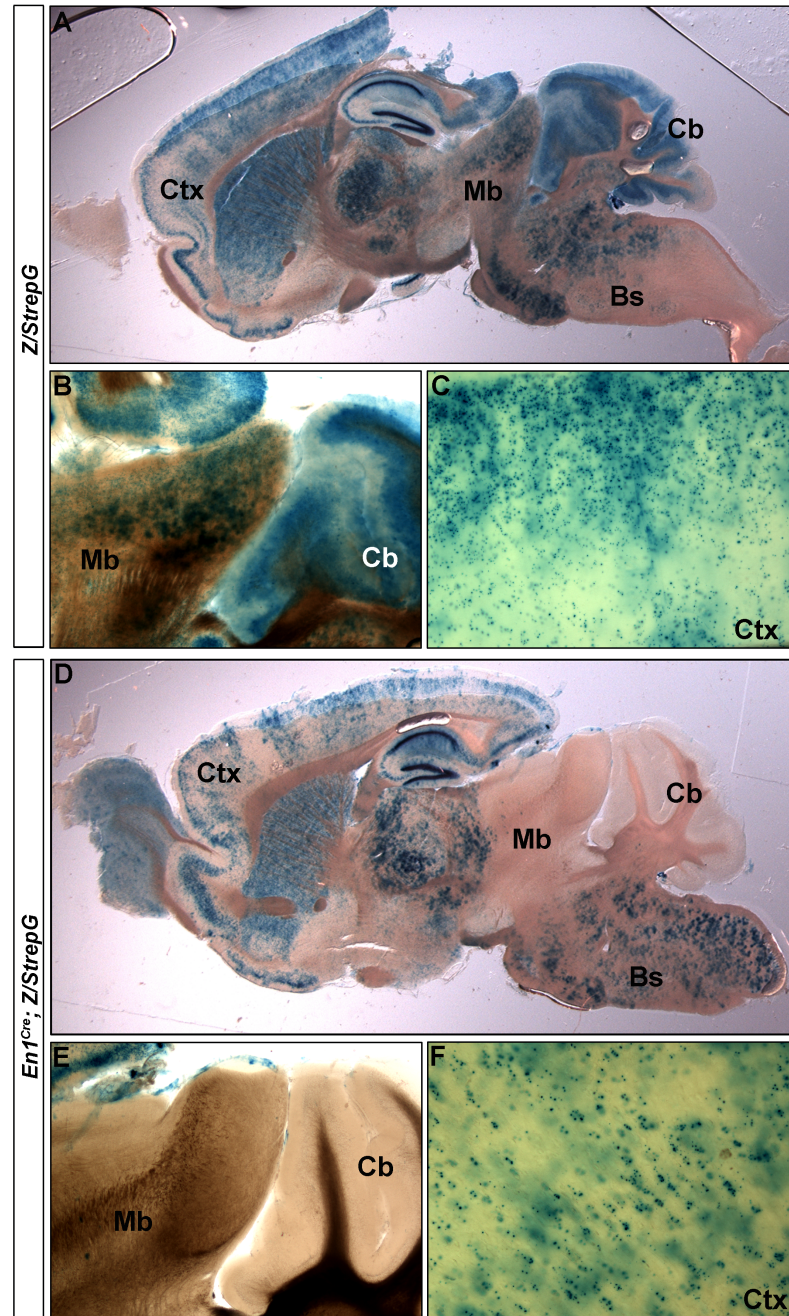


Figure 7. Analysis of *Z/StrepG* recombination and expression in the adult brain.

(A) X-gal staining of sagittal sections revealed that *Z/StrepG* was stochastically expressed throughout the adult brain, areas devoid of expression were found throughout the brain, including cortex (Ctx), midbrain (Mb), cerebellum (Cb), and brainstem (Bs). When crossed to *En1^{Cre}* mice, *Z/StrepG* was recombined in the developing Mb and Cb, thereby removing the *LacZ* expression cassette, and completely eliminating β -gal activity in the adult Mb and Cb (D, compare B and E). Areas not derived from *En1* expression continued to express *LacZ*, such as Ctx (C, F).

Cells which expressed *Z/StrepG* throughout the brain exhibited bushy, glial-like morphology (A-I,K,L). The use of glial markers confirmed that *Z/StrepG* was not expressed by microglia (CD11b negative)(A-C) or oligodendrocytes (ABCA2 negative)(D-F). A minority of bushy cell types were positive for the astrocyte marker GFAP (G-I), and were most abundant in the white matter of the cerebellum (I). Neurons of the cerebellum expressed *Z/StrepG* (D, solid arrowhead denotes Purkinje cell, open arrowhead denotes granule cells). The neuronal marker MAP2 identified *Z/StrepG*-expressing cells in the cortex to be neurons (M,N), while the more abundant, bushy morphology cells did not co-localize with MAP2 (K,L). Abbreviations denote where in the brain the images are taken: midbrain (Mb), cerebellum (Cb), cortex (Ctx).

Chapter 4:

Discussion

The primary focus of the research described in this thesis was to determine how timing of gene expression was coupled to cell fate decisions in the auditory system. To accomplish the research goals, I utilized sophisticated mouse genetic fate mapping approaches to investigate the development of two auditory centers established by segregated germinal zones defined by the expression of *Wnt1*. Additionally, the generation and characterization of a novel fate mapping reporter allele was presented. This allele was hypothesized to provide the molecular capacity for three-dimensional *in vivo* lineage assessment by MRI. Careful analysis determined that this allele and strategy requires further investigation to prove its effectiveness. However, we utilized a specialized reporter allele that allowed us to mark and track neural progenitors, which provided clarity in determining how the *Wnt1* lineage contributed to auditory neuron progenitors and to biochemically and functionally distinct cohorts of neurons in the auditory midbrain and hindbrain.

Two models of Wnt1 lineage contribution to the auditory system

In agreement with previous reports (Wilkinson et al., 1987; Gavin et al., 1990; Rodriguez et al., 2000; Zervas et al., 2004; Landsberg et al., 2005; Nichols and Bruce, 2006; Ellisor et al., 2009), we demonstrate that the progenitor domains which give rise to the cochlear nucleus and inferior colliculus are defined by the expression of *Wnt1*. Accordingly, the findings in this thesis show that progenitors in these domains substantially and differentially contribute to these structures dependent on when they express *Wnt1*. Given the roles for

canonical WNT signaling in neuronal fate decisions (Lee et al, 2004) we determined the modes of *Wnt1* lineage contribution to auditory neurons. One model of how the *Wnt1* lineage establishes these structures is progressive lineage restriction. In this model, the early expression of *Wnt1* defines progenitors that are competent to give rise to all the cell types distributed throughout the mature structure. As development proceeds, the competence of the progenitor population is restricted, and the terminal fates of cells derived from this population becomes more limited.

To definitively test this model, we used GIFM to indelibly mark *Wnt1*-expressing progenitors at distinct developmental stages, allowing us to assess the distribution and fate of cells they give rise to. By marking a fully and truly competent progenitor population early, the entire mature tissue would express the marker as well. The earliest expression of *Wnt1* is in the definitive midbrain and hindbrain primordia from E8-E8.5. Marking at this stage, therefore, should confer *Wnt1* lineage contribution throughout the cochlear nucleus and inferior colliculus, if in fact these progenitors are fully competent to give rise to every cell type of each structure. Our results suggest that within the mes, the early expression of *Wnt1* defines a fully competent progenitor pool. Accordingly, we observed contribution throughout the full extent of the inferior colliculus and its subdivisions when marking progenitors at E8.5, however the contribution was reproducibly sparse. This may be explained by the mosaic nature of GIFM, as not every cell expressing CreER will effectively mediate recombination of the reporter upon delivery of tamoxifen (Joyner and Zervas, 2006). Alternatively, the

sparse contribution may reflect the diffuse *Wnt1* expression domain at E8.5. If early *Wnt1* expression defines competent progenitors of the inferior colliculus, perhaps the full induction of *Wnt1* expression in the mes upon neural tube closure more accurately depicts the fully competent progenitor domain. In support of this idea is the E9.5 *Wnt1* fate map. At this stage, the anterior neural tube has closed and the expression of *Wnt1* in the dorsal mes is tightly refined to a dorsal midline stripe and ring at the poster limit. This pattern persists until at least E12.5, indicating an appropriate range of *Wnt1* expression for lineage assessment for restriction models. Marking progenitors at E9.5 demonstrated the peak of *Wnt1* lineage contribution to the inferior colliculus, with a four-fold increase of *Wnt1*-derived neurons in the central nucleus (ICC) as compared to marking at E8.5. Thus, *Wnt1*-expressing progenitors from E8.5-E9.5 are competent in establishing the full array of auditory midbrain neurons. In accordance with a progressive restriction model, *Wnt1*-expressing progenitors marked at later stages do not contribute equally to neurons throughout the inferior colliculus. Despite a robust *Wnt1* expression domain at E10.5, these progenitors sparsely contributed to neurons in the dorsal ICC and posterior external cortices. By E12.5, cells expressing *Wnt1* in the dorsal mes no longer contribute to neurons of the inferior colliculus. Taken together, these results indicate that following neural tube closure and full induction of *Wnt1* expression in the dorsal mes, the inferior colliculus is established by progressive *Wnt1* lineage restriction.

Interestingly, a model of progressive restriction does not explain *Wnt1* lineage contribution to the cochlear nucleus. Our E8.5 *Wnt1* fate map conclusively determined that *Wnt1*-expressing progenitors in the rhombencephalon at this early stage do not give rise to all the neuronal types of the cochlear nucleus. Notably, the contribution was largely confined to dorsal magnocellular neurons of the AVCN/PVCN, and to granule cells of the DCN. Moreover, as development progressed from E8.5-E12.5, we did not observe a restriction in the fates of *Wnt1*-derived neurons in the cochlear nucleus. In contrast, within the DCN we discovered that the competence of the *Wnt1* lineage increased from E10.5-E12.5. These results are explained by a model in which *Wnt1* expression is induced *de novo* in progenitors of the cochlear nucleus. Further, these findings suggest that progenitors which do not express *Wnt1* early, but are induced at later time points (correlating with the emergence of distinct cell types), must exist within the progenitor domain of the cochlear nucleus. This might have been unexpected because most studies describe the expression of *Wnt1* to be inclusive of the entire germinal zone (Rodriguez et al., 2000; Landsberg et al., 2005; Nichols and Bruce, 2006). These studies commonly employed *in situ* hybridization techniques and substrate-dependent histochemistry to investigate the expression of *Wnt1*, which provides for general tissue, but not always cellular, assessment of gene expression. Through a transgenic approach in which *Wnt1* regulatory elements were harnessed to express a fluorescent reporter allele, we gained detailed cellular resolution of *Wnt1*-expressing cells. This allowed us to clearly identify cells which do not

express *Wnt1* within the gene expression domain of the developing hindbrain. It is likely that these cells induce *Wnt1* expression *de novo*, and are related to the specification of unique neuronal types that are generated at successive time points.

Functional organization of the Wnt1 lineage in the auditory system

The distribution of neurons, their dendritic fields, and the axons which innervate them form the physical substrate for tonotopic organization of auditory centers. While extracellular cues, such as members of the ephrin receptor family likely guide the innervation of auditory afferents in the brainstem (Cramer, 2005; Huffman and Cramer, 2007), less is known of what coordinates the migration and final distribution of auditory neurons. Our *Wnt1* fate mapping demonstrated a relationship between the timing of *Wnt1* expression in progenitors, and the distribution of auditory neurons within the tonotopic maps of the cochlear nucleus and inferior colliculus.

In the midbrain, progenitors which expressed *Wnt1* early (E8.5-E9.5) were distributed throughout the ICC, encompassing the entire presumptive tonotopic map. However, progenitors expressing *Wnt1* at E10.5 reproducibly populated the dorsal aspects of the ICC. These neurons would likely respond to frequencies in the low end of the tonotopic spectrum. Of future interest would be assessing the distributions of progenitors which express *Wnt1* during more discrete periods between E9.5 and E10.5. Currently, GIFM marks progenitors over thirty hours post-tamoxifen administration due to the pharmacokinetics of

the drug (Joyner and Zervas, 2006). Marking the *Wnt1* lineage during shorter time bins between E9.5 and E10.5 could reveal a ventral (high frequency) to dorsal (low frequency) mode of contribution. In this regard, the directed proliferation of the *Wnt1* lineage over time could mediate the distribution of ICC neurons across the future tonotopic map. Interestingly, this relationship was more clearly represented by the *Wnt1* fate maps in the cochlear nucleus.

In the AVCN and PVCN, progenitors expressing *Wnt1* early (AVCN: E8.5-E10.5, PVCN: E8.5) gave rise to neurons which were biased towards the dorsal tiers, while later expression (E12.5) conferred contribution throughout the dorsal-ventral axis of these structures. The large projecting neurons of the DCN were derived from progenitors which expressed *Wnt1* late, and exhibited no discernible bias in dorsal-ventral distribution. The tonotopic maps in cochlear nucleus subdivisions are inverted with respect to the map in the ICC, such that higher frequencies are represented dorsally, and lower frequencies ventrally. Perhaps the timing of *Wnt1* expression in auditory progenitors of the hindbrain is related to differential modes of migration which establish the dorsal (high frequency) and ventral (low frequency) tiers of the AVCN and PVCN. This is consistent with a role for canonical WNT signaling in promoting the delamination and cell motility of neural crest cells (Burstyn-Cohen et al., 2004).

The role(s) of Wnt1 in auditory system development

WNT signaling is essential to development, affecting mechanisms of cell proliferation, cell fate specification, cell and tissue polarity, cell movement, stem

cell maintenance, and axon guidance (Ciani and Salinas, 2005; van Amerongen and Nusse, 2009). Of these processes, *Wnt1* (or the *Drosophila* ortholog *wingless* (*wg*)) directly influences cell proliferation (Dickinson et al., 1994; Ikeya et al., 1997; Megason and McMahon, 2002; Giraldez and Cohen, 2003; Panhuysen et al., 2004), cell fate (Dogan and DiNardo, 1992; Saint-Jeannet et al., 1997; Dorsky et al., 1998; Muroyama et al., 2002; Lee et al., 2004), and cell movement (Burstyn-Cohen et al., 2004; de Melker et al., 2004). It is possible that *Wnt1* affects each of these processes in the development of the auditory system. However, because of the combinatorial affects of convergent *Wnt* pathways, the particular function of any *Wnt* is likely context dependent. Our differential *Wnt1* fate maps of the auditory system support this notion.

In the context of auditory midbrain development, we observed that the E9.5 *Wnt1* lineage contributes substantially to the entire inferior colliculus. Marking the *Wnt1*-expressing population just 24 hours later resulted in only minor contributions to specific areas. Thus, a relatively small number of cells in the dorsal mes at E9.5 are responsible for an incredible expansion necessary for the establishment of the mature structure. A likely scenario is that *Wnt1* promotes this large proliferation and contributes to the expansion of the *Wnt1* lineage. In strong support of this mechanism, the ectopic and continued expression of *Wnt1* in *En1*^{+/*Wnt1*} mice resulted in the overproliferation of dorsal midbrain progenitors (Panhuysen et al., 2004). Knocking *Wnt1* into the *En1* locus caused a rostral and caudal expansion of the *Wnt1* expression domain, which persisted until adulthood. These mice display a significantly larger inferior colliculus, that is 4.8

times the volume of wild type controls. Interestingly, the cytoarchitecture, though enlarged, was mostly normal. These results, combined with our E9.5 *Wnt1* fate map of the dorsal midbrain, define an early proliferative mechanism for *Wnt1* in the development of the inferior colliculus.

Interestingly, expression analysis and later stage GIFM confirmed that *Wnt1* continues to be expressed in the posterior dorsal mes well beyond E10.5 (through at least E14.5: Wilkinson et al., 1987), but that these cells do not substantially contribute to the neuronal composition of the auditory midbrain. Thus, there may be early cell-autonomous or autocrine, and late paracrine roles for *Wnt1* in midbrain development. *Wnt1* expression from E10.5 onward may be important for promoting the continued proliferation of the E9.5 *Wnt1* lineage. Alternatively, as the fate mapping allele we employed is only expressed in neuronal lineages (Hippenmeyer et al., 2005), later expression of *Wnt1* may promote the generation of glia. More interesting in relation to the functional connections of the auditory midbrain, is the possibility that late WNT1 mediates aspects of axonal guidance. This possibility is not undocumented for WNT1 (Lyuksyutova et al., 2003). While attempting to identify factors involved in anterior-posterior guidance of post-crossing commissural axons in the developing spinal cord, WNT1 was shown to promote axon elongation in tissue culture. In support of this and other roles for *Wnt1* is our analysis of *Wnt1^{sw/sw}* mutants.

The *Wnt1^{sw}* allele encodes a naturally occurring point deletion in *Wnt1*, causing a frame shift which introduces an early stop codon in exon 3, believed to result in a nonfunctional protein product missing the last 50% of the carboxy-

terminus (Thomas et al., 1991). We exploited this line of mice to investigate the mechanism whereby *Wnt1* functions in the auditory centers of the brain. *Wnt1^{sw/sw}* mice exhibit variability in phenotypes and are often viable (though not in Mendelian ratios). This is in contrast to mice homozygous for a null allele of *Wnt1*, which die perinatally (Thomas and Capecchi, 1990; McMahon and Bradley, 1990). Thus, it is more likely that the *Wnt1^{sw}* mutation results in a hypomorphic or gain of function allele. In either case, the protein can be regarded as a perturbed form of WNT1. As we demonstrate, the perturbation of *Wnt1* results in mild to severe disruptions of auditory midbrain morphology. In mild cases, the ICC and surrounding cortices are present and largely normal, though not perfectly symmetrical bilaterally. In severe cases, there is a complete loss of bilateral symmetry, and the presumptive subdivisions of the inferior colliculus were malformed and not easily distinguished. Interestingly, there were anterior and posterior ectopic cell types embedded within the auditory midbrain, and a qualitative loss of parvalbumin (PV) positive cell bodies in the presumptive ICC. Collectively, these results indicate that, in addition to the role of expanding the lineage through proliferation, *Wnt1* is required for the normal patterning and specification of cell fate in the inferior colliculus. However, whether *Wnt1* directly affects these processes remains to be determined. The normal expression of *Wnt1* in the dorsal mes is required for the maintenance of the IsO, the signaling center at the junction between the developing midbrain and hindbrain (Adams et al., 2000; Matsunaga et al., 2002). This organizer patterns the adjacent midbrain and cerebellum through the actions of *Fgf8* (Crossley et al., 1996; Liu et

al., 1999). Thus, some of the patterning deficits we observe in *Wnt1^{sw/sw}* mice are likely secondary to the disruption of the IsO. The variability in phenotypes may then be attributed to stochastic and differential levels of perturbed WNT1, which in turn disrupt the IsO to varying degrees.

One prominent deficit common to both mildly and severely affected *Wnt1^{sw/sw}* mice was the loss or displacement of commissural connections between the colliculi. Moreover, the axonal output of the inferior colliculus was greatly disorganized in mildly affected mice. Late *Wnt1* expression may therefore be instructive in establishing inter-collicular projections, and/or to promote the outgrowth of anterior projections en route to the medial geniculate body in the thalamus. This is consistent with how WNTs in general and WNT1 functions in axon outgrowth (Lyuksyutova et al., 2003). Lastly, the presence of a late WNT1 signal in the mes may serve as an attractant or remodeling cue for ascending input from developing hindbrain auditory centers. Projections from the cochlear nucleus reach the midbrain as early as E15 in rat (Kandler and Friauf, 1993), on par with the late *Wnt1* expression in the dorsal mes (E15 in rat roughly corresponds to E13 in mouse (Clancy et al., 2001)). This suggests an intriguing putative mechanism of molecular matching whereby auditory neurons of a temporally defined lineage are functionally connected by axonal projections.

While the inferior colliculus was largely established by the E8.5, E9.5 *Wnt1* lineages, the cochlear nucleus received contributions from *Wnt1*-expressing progenitors from E8.5-E12.5. This prolonged period of contribution may suggest other mechanisms for *Wnt1* in the context of hindbrain

development. As described above, the *Wnt1* lineage in the cochlear nucleus is not progressively restricted over time. We show that waves of *de novo Wnt1* expression are differentially correlated with the distributions and emergence of unique cell types. These results suggest that, in contrast to its role in the auditory midbrain, *Wnt1* may not play a primary role in promoting the proliferation of cochlear nucleus progenitors. Rather, this thesis demonstrates that the timing or induction of *Wnt1* expression may play a central role in specifying the fate of cochlear nucleus neurons. For example, in the AVCN, the *Wnt1* lineage gives rise to PV-expressing neurons, but not calbindin (CALB) or calretinin (CALR) - expressing neurons. In the PVCN, the *Wnt1* lineage contributes to PV and CALB-expressing neurons, but not CALR-expressing neurons. Perhaps most interestingly, the *Wnt1* lineage gives rise to CALB and CALR-expressing neurons, but not PV-expressing neurons in the DCN. Through GIFM, we demonstrated that progenitors which contribute to CALB-expressing DCN neurons expressed *Wnt1* prior to, and then concurrently with, progenitors which establish the CALR-expressing population. Thus, early versus late *Wnt1* expression in DCN progenitors may affect neuronal cell fate decisions. Additionally, granule cells of the DCN were derived from *Wnt1*-expressing progenitors over an extended period of development, while those of the AVCN and PVCN were more abundantly derived from later *Wnt1* lineages. These results support a temporal role for *Wnt1* in specifying distinct cell types within each of the cochlear nucleus subdivisions. An important unifying finding is that the *Wnt1* lineage contributed to a functional class of auditory hindbrain neurons.

This was surprising because I initially hypothesized that biochemically distinct (PV, CALB, CALR) neurons would be differentially derived based on the timing of *Wnt1* expression. While this was true for CALB and CALR neurons of the DCN, the biochemical signature in each case correlates with neurons that form contralateral excitatory circuits (Fredrich et al., 2009). Again, this could be a convergence of mechanisms where lineage allocation and neural circuits are coupled.

Of further importance is deciphering how the timing of *Wnt1* expression in hindbrain primordia is related to the formation of a presumed WNT1 gradient, and whether the graded activity of WNT1 is instructive for cell fate decisions in the cochlear nucleus. *Wnt1* mRNA is expressed in a gradient, high to low, from dorsal to ventral in the lower rhombic lip (LRL) (Landsberg et al., 2005). This may affect levels of WNT1 translation and secretion, thereby establishing a corresponding WNT1 gradient high to low, from dorsal to ventral. This is in agreement with the observed graded activity of WNT signaling in the developing spinal cord (Megason and McMahon, 2002). For example, it is well established that Wg acts both locally and at a distance to affect cell fate and tissue organization in the *Drosophila* embryo (Struhl and Basler, 1993; Couso et al., 1993; Zecca et al., 1996; Neumann and Cohen, 1997; Strigini and Cohen, 2000). Further, in the wing imaginal disc where a gradient of Wg is formed in the dorsal-ventral axis, the effects of secreted Wg are concentration dependent (Zecca et al., 1996). In this example, low levels of Wg induces expression of *vestigial*, which promotes wing-blade cell fate; high levels of Wg activates *neuralized*

expression, required of bristle cell fate (Zecca et al., 1996). In the mammalian hindbrain, the levels of WNT1 could affect cell fate in a similar fashion. In further support of this assertion are our observations that the *Wnt1* lineage primarily establishes excitatory cell types, such as the PV-expressing neurons of the AVCN/PVCN. These cells are presumed to be multipolar type I neurons which provide excitatory contralateral innervation of the inferior colliculus. Other genetic fate mapping studies support a role for *Wnt1* in specifying excitatory cell types of the cochlear nucleus.

Our findings provide a comprehensive assessment of timing, *Wnt1* gene expression and *Wnt1* lineage allocation as well as functional evidence for the role of *Wnt1* in the auditory system. An outstanding question is what are the downstream molecular targets of *Wnt1* and WNT1 signaling and how might they help decipher a pathway used in development. Through cumulative genetic fate mapping, Fujiyama et al. demonstrated that progenitors that express the bHLH transcription factors *Math1* and *Ptf1a*, give rise to the excitatory and inhibitory cell types of the cochlear nucleus, respectively. Interestingly, the *Math1* expression domain is contained within the dorsal tier of *Wnt1* expression in the LRL (Landsberg et al., 2005), while *Ptf1a* is believed to be expressed just ventral to or slightly overlapping with the weakest ventral domain of *Wnt1* expression (Yamada et al., 2007; Fujiyama et al., 2009). Differing levels of WNT1 may regulate the expression of these transcription factors, such that high levels promote *Math1*, while low levels promote *Ptf1a*, similar to the differential control of factors which specify fate in the *Drosophila* wing imaginal disc. Of importance

is determining whether these genes are regulated through canonical Wnt signaling. This has been demonstrated for neural crest lineages.

Neural crest cells are a transient progenitor population derived from the LRL which give rise to diverse tissue types during development including much of the peripheral nervous system (Le Douarin and Smith, 1988). Gene inactivation studies demonstrated a role for *Wnt1* and *Wnt3a* in promoting the expansion of neural crest stem cells (Ikeya et al., 1997). More recently however, *Wnt1* was shown to be sufficient for specifying sensory neuron fate in neural crest stem cells (Lee et al., 2004). Moreover, sustained β -catenin activity did not result in the expansion of neural crest stem cells, instead sensory neurons were specified at the expense of other neural crest derivatives. Thus, it is likely that differing levels of WNT1 can regulate cell fate in the developing hindbrain. Whether the graded expression of *Wnt1* mRNA in the LRL differentially affects the fate of cochlear nucleus neurons remains to be determined. Though Wnt signaling through β -catenin is both necessary and sufficient for specifying the fate of sensory neurons from neural crest (Lee et al., 2004), *Wnt1* is not essential (Ikeya et al., 1997). In either *Wnt1*, or *Wnt3a* homozygous null embryos, the neural crest lineages were largely unaffected (Ikeya et al., 1997). In compound mutants lacking both *Wnt1* and *Wnt3a* however, there were significant reductions of neurogenic lineages. Thus, *Wnt1* and *Wnt3a* are able to compensate for each other in the developing hindbrain. This likely explains the lack of a significant cochlear nucleus phenotype in *Wnt1^{sw/sw}* mice. The severity of the midbrain phenotype was no indicator of the hindbrain phenotype; the cochlear nucleus

exhibited very subtle but stereotyped changes in *Wnt1^{sw/sw}* mice. Interestingly, only the anterior half of the cochlear nucleus was affected. It is possible that more striking changes would be uncovered if we were to follow the perturbed *Wnt1^{sw}* lineage over time through GIFM, such as a change in distributions or fate of *Wnt1*-derived neurons. In addition, further understanding the individual roles of *Wnt1* and *Wnt3a* in the context of hindbrain development may contribute to our understanding of cell fate decisions for cochlear nucleus neurons.

Longitudinal in vivo tracking of genetically defined cell lineages

Chapter 3 described our transgenic strategy to couple GIFM and contrast-enhanced MRI to visualize cell lineage contribution in living animals. Though unsuccessful in our approach, the conceptual merits of our design should encourage and inform future studies which aim to provide longitudinal *in vivo* tracking of cell lineage. For the purpose of this discussion, the complications we encountered and suggestions for alternative strategies described in Chapter 3 will not be elaborated upon. Instead, the potential applications that prompted us to pursue the development of our design will be discussed.

In the context of developmental genetics studies, our reporter was designed to be coupled with any of the hundreds of cell or tissue specific Cre drivers that enable conditional recombination. Following recombination, the reporter was hypothesized to confer specific enrichment of exogenously delivered biochemically tagged MRI contrast agents, either *in utero* or in postnatal animals. This would have allowed for the direct imaging of populations

that express the recombined reporter by MRI. If for example, the Cre driver is expressed by an early progenitor population, how that population expands and changes morphometrically to form tissues during development could be evaluated *in utero* by repeated MRI assessment. Additionally, the full extent of cell lineage contribution could be evaluated in mature animals.

In relation to the study described in Chapter 2, this reporter could have provided the means to visualize how the E9.5 *Wnt1* lineage in the dorsal mes rapidly proliferates and expands to form the developing inferior colliculus. Of further interest, using the reporter to fate map the mutant lineage in *Wnt1^{sw/sw}* mice could reveal how the perturbation of *Wnt1* affects the development of the midbrain and cerebellum over time. Other studies have followed the migration of transplanted ES cells preloaded with contrast agent by MRI *in vivo* (Hoehn et al., 2002). Hypothetically, we could have evaluated the migratory streams of cochlear nucleus progenitors as they exit the LRL and move tangentially to form the mature subdivisions.

One confounding factor to these hypothetical studies is resolution capacity. The study which followed the migrations of transplanted cells did so by achieving voxel size resolution of $78\ \mu\text{m}^3$ using a 7T magnet (Hoehn et al., 2002). Using a 3T system we could hope to gain $120\ \mu\text{m}^3$ resolution, which may not be adequate for some of the studies described above. However, this resolution could be appropriate for assessing terminal lineage contribution in the adult, or for direct imaging of grafted tissues in cell transplant studies.

We incorporated the GFP cassette in our reporter to provide flexibility in screening for expression, to assess the accuracy of fate maps obtained by MRI, and to isolate genetically defined progenitors by FACS. This last application of GFP expression is prerequisite to studies of cell based therapies in rodent models of degenerative disease.

The progressive loss of dopaminergic neurons underpins the pathology and symptoms of Parkinson's Disease, characterized by severe motor abnormalities (Olanow and Tatton, 1999). Rodent models have come along way in determining the efficacy of cell based therapies that can ameliorate symptoms (Hall et al., 2007). Typically, heterogenous populations of cells obtained from embryonic tissue (Bjorklund et al., 1983) or ES cells (Kim et al., 2002; Thompson et al., 2005) are transplanted into lesioned animals. However, assessing relative improvements in the disease state with the degree of successful engraftment requires postmortem analysis. Additionally, correlating the success and maintenance of engraftment is technically challenging.

Chapter 2 demonstrated how the *Wnt1* lineage contributes to auditory structures, including the inferior colliculus in the midbrain. However, *Wnt1*-expressing progenitors in the mes also contribute to the entire dopaminergic system in the ventral midbrain (Zervas et al., 2004; Brown et al, 2011). Activating the expression of our reporter in *Wnt1*-expressing cells of the ventral mes, could have allowed for the specific isolation of dopaminergic progenitors by GFP based FACS. This population could then be transplanted into the brains of lesioned animals, and the degree of engraftment monitored over time by MRI. This

approach could assess whether genetically defined dopaminergic progenitors are more capable to replenish populations lost to disease as opposed to heterogeneous fetal or ES cell derived grafts. As this would be accomplished *in vivo*, the degree of engraftment could be measured against behavioral improvements over time.

The above example highlights one potential use of our transgenic strategy in studies of cell based therapies, which is pertinent to other research interests of our lab. It serves, however, to demonstrate the flexibility we intended to incorporate in our approach. Because of the inherent uncertainty that comes with generating any novel transgenic mouse line, we chose a design that would confer multiple applications critical to advancing genetic approaches to study development and disease.

Summary

We designed, generated, and tested a novel transgenic reporter capable of providing three-dimensional *in vivo* lineage assessment by MRI. Though our strategy requires further design, the conceptual merits highlight the power of the mouse as a genetic model organism for the study of development and disease. In addition, we used a reporter allele that allowed for the assessment of neuronal lineage defined by the timing of gene expression with a genetic inducible fate mapping approach in mice. Specifically, we sought to examine the role of *Wnt1* in establishing two critical auditory centers in the mammalian brain, the cochlear

nucleus and inferior colliculus. These centers are functionally connected, and are tonotopically organized. Because the primordia of these structure express *Wnt1* during development, we tested the hypothesis that the timing of *Wnt1* expression was related to mechanisms that govern the functional organization of auditory centers such as cell fate specification. We used genetic approaches in mouse to follow that fate of *Wnt1*-expressing cells, and uncovered differential mechanisms for each auditory structure. We demonstrated that the inferior colliculus develops through progressive *Wnt1* lineage restriction, and provide insight for *Wnt1* in mediating the proliferation of auditory midbrain progenitors. In contrast, the cochlear nucleus of the hindbrain is established by progenitors which exhibit *de novo* expression of *Wnt1* over a broad window of development. We correlate waves of *de novo* *Wnt1* expression with the emergence of unique neuronal types in cochlear nucleus subdivisions, and provide molecular evidence that these neurons establish contralateral circuits which connect the auditory hindbrain and midbrain. Through mutant analysis, we demonstrate the requirement of *Wnt1* in patterning the auditory midbrain, and for organizing intercollicular projections, as well as projections which connect the auditory midbrain and thalamus.

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