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Structural Transformations of Synaptic Partners During Growth and Competition of the Giant Nerve Terminal, the Calyx of Held, in the Medial Nucleus of the Trapezoid Body in the Developing Postnatal Mouse

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**Structural Transformations of Synaptic Partners During Growth and
Competition of the Giant Nerve Terminal, the Calyx of Held, in the Medial
Nucleus of the Trapezoid Body in the Developing Postnatal Mouse**

Dakota Richard Jackson

**Dissertation submitted
to the Rockefeller Neuroscience Institute at West Virginia University**

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in

Neuroscience

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Abstract

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Dakota Richard Jackson

Across many parts of the nervous system, initial neural circuit assembly involves stereotyped stages of early axonal proliferation within target territories, selective reinforcement of a subset of inputs and the removal of exuberant or unneeded connections via either activity or experience-dependent mechanisms, in a process referred to as synaptic competition. The mechanisms and associated structural transformations that underlie and define these stages of neurodevelopment have been examined in a variety of brain regions including the peripheral neuromuscular junction, the central climbing fiber innervation of cerebellar Purkinje cells and retinal ganglion cell innervation of the thalamic lateral geniculate nucleus. Each of these connections are considered representative examples of sites of synaptic competition. We and others have established the globular bushy cell (GBC) innervation of principal cells (PC) of the medial nucleus of the trapezoid body (MNTB) in the auditory brainstem via the large calyx of Held (CH) as an additional representative, or model, system for the study of terminal growth and competition.

We utilized dense reconstruction of pre and postsynaptic elements from serial block-face scanning electron microscopy (SBEM) image volumes collected during key developmental timepoints from both prehearing (postnatal days (P)2, 3, 4, 6, & 9) and adult (P30) mice. In this document, I report our most recent studies of MNTB ultrastructure, which revealed additional structural elements that define the progressive growth, competition and subsequent maturation of the CH and its innervation of PCs.

We found evidence that all developing CHs extend 3 types of collateral arbors including short filopodia ($<5.85\text{ }\mu\text{m}$), long filopodia ($>5.85\text{ }\mu\text{m}$), and growth cone tipped collaterals of sufficient length to innervate adjacent cells or cells within $60\text{ }\mu\text{m}$, but in some cases up to $100\text{ }\mu\text{m}$, away from the innervation territory of the CH. Moreover, as the proportion of polyinnervated PCs with competing CH inputs declines from 23% at P6 to 11% at P9, we found a significant reduction in the length of CH collaterals until few if any were found by maturity (P30).

Using the first live imaging data collected from a living brain slice with the lattice light sheet microscope, we revealed with high spatial and temporal resolution that CH collaterals exhibited some of the fastest motility recorded in developing neural systems (average growth cone rate of motility: $2.1\text{ }\mu\text{m per min}$). We also observed interactions among long filopodial collaterals that resulted in retraction and altered growth trajectories suggesting possible repulsive contact-mediated guidance of CH growth and innervation. These may be the predominant locations where competing CHs physically interact, since they typically expand over non-overlapping territories across the cell surface of the PC soma.

Postsynaptically, we provided the first systematic description of two types of invaginating spine (type 1 and 2) that reside specifically within recesses underneath and penetrating the developing CH. Similar to the emergence of CH collaterals, we found a strong association of type 1 spine emergence with the initial expansion of the terminal. Moreover, for the first time, somatic spine mats, composed of so-called type 2 spines that intertwine to assemble a macrostructure called the somatic spine mat, were described in the vertebrate CNS. From inspection of EM images, we suggest three modes of intercellular communication between the CH and its synaptic partner, the PC: chemical synaptic transmission, signaling via cell adhesion proteins, trans-endocytosis of spine mats into the CH.

We hypothesize that the unifying event to synchronize CH growth and emergence of collaterals and postsynaptic somatic processes is a transformation of spontaneous activity to a bursting pattern. Preliminary experiments reveal that interference with synaptic transmission using viral vector induced expression of tetanus toxin leads to morphological changes in the CH and slower growth of the postsynaptic cell body. Further experiments are proposed that combine electrophysiology and dynamic imaging with molecular and physiological perturbation to further explore these linkages.

Dedication

For my family: Having your unwavering support and encouragement is what has facilitated my entire education.

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Introduction and Literature References:

Early Neural Development and Circuit Formation

The nervous system is composed of a complex and highly organized assemblage of specialized cells called neurons, capable of rapidly transmitting electrochemical signals necessary for processing sensory stimuli, memory storage, and coordinating the planning and execution of actions in response to environmental challenges. In order for the nervous system to produce the large variety of requisite biologic computations, diverse subpopulations of neurons exhibit unique differences in structure and connectivity that arise following a complex mode of intrinsic and extrinsic regulation of gene transcription during development (Pearson and Doe, 2004). An important question that has yet to be answered satisfactorily, however, is how the hundreds (*C. elegans*)(White et al., 1986) to billions (roughly 86 billion neurons in the human brain (Azevedo et al., 2009)) of neurons autonomously assemble in order to provide a substrate for processing all the implicit functions of sensation, perception and intention (Azevedo et al., 2009).

The formation of a mature neural network requires a concerted sequence of events, including the production of new cells, differentiation and migration of neurons, and the generation of functional and specific synaptic connections between neurons. Mice offer advantages to understanding brain development in mammals, due to their small size and genetic and structural similarities to primates, including humans (Mural et al., 2002; Bryda, 2014; Snyder et al., 2018). Moreover, their shorter gestation period of 18-21 days requires many of the same developmental processes to occur in a more compressed timeframe relative to humans

(Murray et al., 2010). In mice, formation of the neural plate begins around embryonic day 7 (E7) and neurulation occurs between E8.5-10 (Kaufman, 1992). Thus, many of the ~100 million neurons in the mouse brain are largely formed during an approximate 11-day period of late embryonic development, although some neurogenesis continues into the early postnatal (P) period (De Felipe et al., 1997).

Axon Formation

Following neurogenesis, cells must begin to take on the polarized morphology indicative of a neuron. A key question is what determines how axons find appropriate sites to deliver their electrochemical signals? Moreover, what mechanisms determine if these cells will produce hundreds to thousands of synaptic contacts, which can reach a density of about one trillion synaptic connections per cubic centimeter of tissue in cortical tissue (Drachman, 2005)? These questions were initially proposed over a century ago by Santiago Ramón y Cajal (Ramón y Cajal, 1892) and partially answered by his fastidious work with embryonic histological sections. From his study of fixed tissues, Ramón y Cajal described observations of neural processes tipped with a complex structure called a growth cone, characterized by an expanded lamellipodium often studded with finger-like filopodia (Ramón y Cajal, 1890). Ramón y Cajal realized these structures were largely absent in the more developed cells of the mature brain and such an observation likely helped spur his hypothesis that growth cones were influential in the development of the brain (de Castro et al., 2007). In more recent years, a clearer picture of neural development has emerged largely from work with dissociated and acute tissue cultures using methods that allow cells *in vitro* to survive for periods sufficient to observe the sequential stages

of neuron development (Dotti et al., 1988; Rodríguez-Hernández et al., 2014; Jedrzejczak-Silicka, 2018).

Among the first descriptions of the process of structural neural differentiation came from the study of dissociated, post-mitotic hippocampal neurons (Dotti et al., 1988; Goslin and Banker, 1989). Here it was found that multiple short, appendages tipped with growth cones and interstitial filopodia bud from spherical neurons within a day of dissociation and culture. However, among these short appendages, key differences could be observed. First, a single neurite with a ‘smooth and uniform’ morphology was shown to elongate up to five times more quickly than others, at up to 60 $\mu\text{m}/\text{day}$ (12 $\mu\text{m}/\text{day}$ for dendrites) (Dotti et al., 1988). Moreover, once a single neurite achieved a length greater than 10 μm relative to others, it could be reliably identified as an axonal extension. This process was further distinguished from the ‘branched and tapered morphology of the more slowly elongating dendrites (Dotti et al., 1988; Goslin and Banker, 1989). In part, these differences in neurite motility are likely driven by differences in underlying cytoskeletal structure and dynamics.

The major structural elements fundamental to aiding in the development of neurites are comprised of actin and tubulin. In brief, actin filaments and microtubules are formed from ATP-bound actin monomers and GTP-bound α - β -tubulin heterodimers to form long polymer chains, respectively. The elongation of polymer chains occurs primarily in a polarized fashion through addition of subsequent subunits to the “barbed end” of actin filaments (Pollard and Cooper, 2009) or the “plus-end” of microtubules at the exposed β -subunit (Chretien et al., 1992). Actin-enriched areas of an axon, such as dynamic and planar lamellipodia that tip growing axons and are often studded with thin projections called filopodia, are driven to elongate through mechanical forces exerted by extending actin filaments (Blanchoin et al., 2014). In filopodia,

parallel associations of enriched actin filaments collectively orient their barbed ends driving elongation towards the cell membrane to extend the tip of the process. In lamellipodia, however, actin filaments are arranged in a web-like orientation driving filament elongation in various directions leading to a non-uniform expansion of the veiled lamellar structure. Microtubules, meanwhile, are enriched in the main process of the axons and dendrites although the arrangement differs significantly between them (Barnes and Polleux, 2009). Axons exclusively exhibit a uniform and polarized arrangement of microtubules such that active polymerization occurs unilaterally at the distal plus-end of axonal microtubule bundles (Athamneh et al., 2017). Meanwhile, dendrites exhibit a more disorganized arrangement of microtubules wherein the majority of microtubule polymerization occurs at intermediary points along the length of a maturing dendrite without significant bias towards the somatic or distal end of the dendrite (Baas et al., 1988).

Long-distance axonal pathfinding is thought to be mediated by a lamellipodial-like structure termed a growth cone (Lowery and Van Vactor, 2009). The process of growth cone extension recapitulates the initial steps in process formation. During the initial formation of neuronal processes, a small cellular protrusion forms along the edge of the cell membrane. This early process is invaded by elongating actin filaments that generate the required force (up to 3 pN) to extend the cell membrane to form thin filopodial structures (Torre et al., 2007). In order to stabilize these filopodia, microtubules must invade previously actin-enriched areas. A similar process is responsible for branch formation in neurites as the initial actin-mediated extension of short filopodia is followed by subsequent invasion of microtubule bundles that lengthen and stabilize collateral branches (Gallo, 2011). Moreover, interaction of actin filaments and microtubules is perhaps best illustrated in the cytoskeletal arrangement and dynamics within

neuronal growth cones. Growth cones are composed of two primary domains: a peripheral domain (P-domain) that encompasses radial filopodia and a veiled lamellipodium each composed primarily of dynamic actin filaments, and a central domain (C-domain) with a cytoskeleton predominated by microtubules that extend from the axon to the edge of the central domain near the base of the growth cone lamellipodium (Figure 1)(Geraldo and Gordon-Weeks, 2009; Lowery and Van Vactor, 2009). In order for a growth cone to advance forward, microtubules must first invade territory previously occupied by actin in the P-domain (Sabry et al., 1991). Then, microtubules in the C-domain of the growth cones are bundled and tubulin is preferentially recruited to the plus-end of the polymer at the base of the growth cone which advances the position of the central domain of the growth cone and allows the distal axon segment to extend (Tanaka et al., 1995). Importantly, the recruitment of new tubulin molecules necessary for sustained microtubule elongation in axon growth requires transport of new constituent cytoskeletal molecules from elsewhere in the neuron along the length of the axon via molecular motors. This conclusion was initially based on seminal studies of axonal transport that utilized radioisotopic pulse labeling techniques. These experiments provided evidence for at least two modes of transport, a fast (100-400 mm/day) and slow (1-10 mm/day; (Brown, 2003; Roy et al., 2005)) component, were required to fully explain the observed kinetics of axonal cargos (Grafstein and Forman, 1980). Non-membrane bound cytoskeletal elements such as tubulin are transported rapidly over short distances at average rates of $\sim 1 \mu\text{m/s}$ ($\sim 86 \text{ mm/day}$) with average peak velocities of $\sim 2 \mu\text{m/s}$ ($\sim 173 \text{ mm/day}$) (Wang and Brown, 2002). Thus, the dynamics of actin and microtubule polymerization/depolymerization are rapid and must be tightly regulated through a myriad of interacting proteins that coordinate cytoskeletal interactions and control

neurite morphology during rapid neurite extension and motility (Dent et al., 2011; Dogterom and Koenderink, 2019).

Axon Pathfinding

Soon after his initial description of the growth cone (Ramón y Cajal, 1890), Ramón y Cajal referred to then current cell biologic evidence from other systems including microbiology as he theorized the growth cone could provide a mode of chemotactic guidance. Cajal suggested growth cones utilize secretions from other cells in the nervous system in a fashion that mirrored the contemporary findings of Pfeffer who was carefully describing and providing evidence for chemotactic behaviors in cells from bacteria to leukocytes and even fern spermatozoids (Pfeffer, 1884, 1888; Ramón y Cajal, 1892). Amazingly, Ramón y Cajal made these predictions without directly observing a living growth cone, a feat that would not be achieved until neuronal tissue culturing became possible in 1910 (Harrison, 1910). This forward thinking “neurotropic hypothesis” Ramón y Cajal proposed failed, however, to incorporate the idea that repellant signals too importantly aid in accurate axonal pathfinding. This was addressed later by Roger Sperry who provided the more refined “chemoaffinity hypothesis” that suggested the molecular identity of an axon was useful in identifying attractant or repellant signals that guide its direction of growth and to some degree aid in regrowth after injury through local detection of graded chemical cues or contact-mediated communication via expression of receptors found on a growth cone (Sperry, 1963). In the decades since, Sperry’s hypotheses have been validated through the identification of many of the membrane-bound receptors found enriched along growth cones and

filopodia and their associated ligands expressed during development (Harris et al., 1987; Sanes and Yamagata, 2009; Williams et al., 2010).

Neuronal guidance factors can be subdivided into at least four highly conserved families of ligand and associated receptors including: 1) netrins/DCC/UNC5 (Sun et al., 2011), 2) slits/Robos (Wong et al., 2002), 3) ephrins/Ephs (Klein, 2004) and 4) semaphorins/plexins (Tamagnone and Comoglio, 2000) although several morphogens (BMPs, Shh, Wnts and others) may additionally serve as attractive or repulsive cues for axonal guidance as well (Zou and Lyuksyutova, 2007). These cues can be encountered by axons when found along a diffusional gradient diffusely spread across the extracellular environment, integrated into components of the extracellular matrix environment and/or incorporated into intermediary cell surfaces (Tessier-Lavigne and Goodman, 1996; Huber et al., 2003; Mortimer et al., 2008; O'Donnell et al., 2009; Chédotal and Richards, 2010; Kolodkin and Tessier-Lavigne, 2010). Virtually all guidance molecules mediate the regulation of the structure and stability of the underlying cytoskeleton, especially targeting actin within the motile peripheral domain of the growth cone, via a variety of molecular mechanisms including activation of Rho family GTPases that further activating additional effectors such as kinases and actin binding proteins (Song and Poo, 1999; Suter and Forscher, 2000; Luo, 2002; Koh, 2007; O'Donnell et al., 2009). However, a second well-described effect of guidance receptor activation is exerted through induction of Ca^{2+} -dependent signaling that indirectly regulates membrane recycling through influences in the balance of endo- and exocytosis that preferentially extend the membrane of the growth cone toward chemoattractants and away from chemorepellants (Song and Poo, 1999; Tojima et al., 2014). For a more comprehensive review of general mechanisms of axon guidance, refer to the following reviews (Raper and Mason, 2010; Gomez and Letourneau, 2014).

Netrin-1 was first purified from the brain tissue of chicks and found to effectively guide elongating commissural axons as an attractant signal towards the floor plate of the developing spinal cord (midline of the neural tube) in *in vitro* studies (Serafini et al., 1994, 1996). DCC mediated growth towards gradients of secreted netrin-1 was found to be a potent attractant signal for retinal ganglion cells exiting the retina through the optic nerve head (de la Torre et al., 1997). Alternatively, expression of UNC-5, another netrin-1 receptor repels axons outgrowth (Chan et al., 1996; Keleman and Dickson, 2001). Co-expression of DCC and UNC5 allows the membrane-bound proteins to bind about their cytoplasmic tails, downregulating DCC and converting the effect of DCC-mediated guidance to UNC-5/DCC mediated repulsion (Hong et al., 1999; Keleman and Dickson, 2001).

A study utilizing forward genetic screens in *Drosophila* identified over 250 genes with possible implications in axon guidance (Seeger et al., 1993). Importantly, mutations in two of these identified genes produced inverse routing errors during axonal pathfinding at the midline: mutated roundabout (Robo) receptors produced aberrant contralateral projections; commissureless (Comm) induced severe deficits in commissural axon pathfinding as axons failed to cross the midline (Seeger et al., 1993). Slit, a large extracellular matrix protein produced by midline glial cells in *drosophila* was later confirmed as a chemo-repulsive ligand for the growth cone-bound Robo receptor (Kidd et al., 1999) and has been found to be an evolutionarily conserved signal utilized from invertebrate to mammalian development (Brose et al., 1999). In addition to elongating axons, Comm is also expressed by cells at the midline. Paradoxically, axons that need to cross the midline are inhibited by the presence of slit-mediated repulsion. In order to overcome this effect, a second gene (Comm) expressed by and concentrated along midline glia downregulates the expression of Robo in commissural axons mitigating the

repulsive signaling of slit proteins during their passage across the central commissure. Then, as axons reach the contralateral side of the brain, where Comm expression is reduced, Robo is again upregulated to reinstitute a slit-mediated repulsion (Kidd et al., 1999; Georgiou and Tear, 2002). Binding of slit to Robo induces a conformational change in the membrane-anchored Robo protein that primes the receptor for metalloprotease-mediated cleavage, subsequently altering the signaling capacity of the receptor and preventing recurrent attraction towards the midline (Coleman et al., 2010).

Ephrins represent a third family of guidance molecule and are comprised of two classes: Ephrin-A that is anchored to the membrane through a GPI anchor and ephrin-B that also is linked through a transmembrane protein with a short cytoplasmic PDZ-binding domain (Wilkinson DG, 2001). Many Ephrins, uniquely among guidance molecules, can undergo bidirectional signaling through interaction with their respective tyrosine kinase receptors, Eph-A and Eph-B, a phenomena extensively reviewed elsewhere (Egea and Klein, 2007). Generally, ephrin signaling promotes repulsive growth cone behaviors following cell-cell interaction such as occurs in the commissural migration of retinal ganglion cell axons (Nakagawa et al., 2000) although some cases of attractive behaviors have been documented such as occurs following ephrin association with the Ret tyrosine kinase receptor in developing motor axons (Bonanomi et al., 2012).

The fourth major family of guidance molecule, semaphorins, can function as both diffusible or contact-mediated cues that bind to an array of surface receptors, primarily through interaction with plexin molecules (Perälä et al., 2012), to form multimeric protein complexes that generally serve as a repulsive signal during axonal migration (Mann et al., 2007). However, the effects of semaphorins can be complicated as their co-receptor, neuropillins (Nrp), generate attractant signaling. Thus, the effect of semaphorin-mediated signaling represents a balance in

the expression of particular guidance receptors in axons in order to reach appropriate terminal locations. Such a balance is exemplified in the graded and overlapping expression of EphA5 and Nrp1 among others, that must be sensed by axons arriving within the developing olfactory bulb (Imai et al., 2009; Schwarting and Henion, 2011).

Synaptic Exuberance and Pruning

The chemoaffinity hypothesis only partially explains the assembly of the nervous system. As axons reach their distal target nuclei, target-derived signals (including traditional guidance molecules, neurotrophins and cell-adhesion molecules) initiate a behavioral change in the axon that induces altered growth cone dynamics (including prolonged pauses in motility and expansion of the growth cone) and an elaboration of the axon through interstitial collateral branch formation (Szebenyi et al., 1998; Krylova et al., 2002; Bak and Fraser, 2003; Gutierrez et al., 2005; Bilimoria and Bonni, 2014). Often, such divergence of an axon produces supernumerary afferent projections that connect it to more target neurons than are maintained in the mature connectome, suggesting early exuberance must be followed by subsequent synapse elimination in order to further increase the specificity of neuronal connections (Lohof et al., 1996; Hensch, 2004; Schuldiner and Yaron, 2014). For example, as many as a 25-50% of synaptic connections in the neonatal human brain are pruned by puberty in a process that refines the mature connectome and underlies the maturation and consolidation of cognitive faculties (Huttenlocher, 1979; Huttenlocher and de Courten, 1987). Generally speaking, such progressive and selective elimination of excessive innervation has been described as a process of “synaptic competition” in which afferents “compete” for limited availability of local resources, territory or

relative synaptic competency (Bi and Poo, 2001; Buffelli et al., 2003; McCann et al., 2007; Davies, 2008). Synaptic competition has been demonstrated in a variety of brain regions, especially sensory systems that undergo a sensitive period of development in which an initial innervation within a circuit must be honed into an ever more precise pattern of connectivity (Hensch, 2004), including but not limited to: cortical targets of thalamocortical projections to the somatosensory cortex (Rios and Villalobos, 2004), corticofugal projections (Curfs et al., 1995), visual cortex (Innocenti et al., 1977) as well as within the thalamus (Hong and Chen, 2011), brainstem nuclei (Holcomb et al., 2013), and in the cerebellum (Watanabe and Kano, 2011). Perhaps the most well-studied area of the nervous system exemplifying synaptic competition among exuberant presynaptic innervation occurs in the peripheral motor end plate along muscle fibers by motor neurons at the so-called neuromuscular junction, or NMJ (Wu et al., 2010; Tintignac et al., 2015). The following sections will outline the stages of development culminating in targeted synaptic refinement that have been identified in several key brain regions.

The Neuromuscular Junction

Work in developing systems with ultimate ‘one-to-one’ or ‘few-to-one’ connectivity offers a clear advantage in investigating the selective strengthening and pruning events that sculpt immature circuits during competition; the endpoint of competition is more readily identifiable in these systems. In part, the NMJ has become one of the most utilized systems for studying synaptic competition because of its large size and readily accessible location in the periphery. These features make it amenable to both *in vitro* and *in vivo* electrophysiologic

(Colman et al., 1997; Favero et al., 2007; Vilmont et al., 2016), genetic (Buffelli et al., 2003) and microscopic investigation (Ball et al., 2003; McCann and Lichtman, 2008) in a variety of species from drosophila to vertebrates (Lu and Lichtman, 2007; Wu et al., 2010). As myogenesis occurs during embryonic development each newly formed motor endplate is prepatterned with dense assemblages of acetylcholine receptors (AChR) (Zhang et al., 2009). In mice, by the day of birth, collections of motor neurons arrive and as many as 10-20 unique afferents have formed small bouton-sized terminals (Brown et al., 1976; Tapia et al., 2012). During postnatal development in this system, two distinct phases of competition resolve supernumerary innervation of the NMJ such that a single motor neuron provides dominant monoinnervation across the entire endplate. First, a subset (2-3) of the initial pool of 10-20 motor neurons increase in size and other inputs withdraw from the motor endplate (Bishop et al., 2004). Secondly, during a protracted period that may last for up to two weeks of postnatal development, the remaining inputs then compete until a single input predominates each endplate (Brown et al., 1976; Balice-Gordon and Lichtman, 1993; Walsh and Lichtman, 2003).

The process of selective refinement does not occur randomly at the developing motor endplate, but instead requires synaptic transmission wherein the most active inputs, as defined by either the frequency of synaptic release or the net total secreted neurotransmitter, aids in the establishment of dominance. This principal requirement of synaptic activity was beautifully demonstrated via *in vivo* experiments where it was shown that inputs indirectly compete such that less active inputs retreat and vacated sites along the motor endplate can be overtaken by more active inputs (Turney and Lichtman, 2012). Moreover, the requirement of synaptic activity in selective refinement of supernumerary innervation at the NMJ has been further demonstrated in a collection of studies that provided additional clues to the underlying mechanism. Chronic

inhibition of synaptic signaling through either irreversible blockage of AChR through bath application of the antagonist α -bungarotoxin (Balice-Gordon and Lichtman, 1994) or through the overexpression of postsynaptic inwardly rectifying potassium channels that inhibit the generation of action potentials (Favero et al., 2009) could dramatically stall synaptic pruning. Alternatively, driving synchronous activation across all supernumerary inputs undergoing synaptic competition too stalls selective pruning and induces the maintenance of polyinnervation in muscle fibers (Colman et al., 1997; Busetto et al., 2000; Favero et al., 2010). Thus, a central requirement for the selection of appropriate inputs during competition is a measurable disparity in synaptic strength and spike timing among competing inputs and in fact an artificial enhancement in these disparities can hasten the removal of less active inputs (Thompson, 1983; Keller-Peck et al., 2001; Favero et al., 2012).

Several key molecules have been identified that have a strong molecular influence on competition in this system (Tintignac et al., 2015). These signaling molecules released from competing terminals can have dramatic effects on NMJ development by affecting the competitive vigor of afferent co-competitors and also through selective reinforcement of a single input. A molecular mechanism proposed in the late 1970s by O'Brien et al. pointed at indirect competition between MNs along developing rat muscles (O'Brien et al., 1978). In some elegant experiments, it was demonstrated that activation of MNs led to the release of not only ACh but also proteinase enzymes that, at sufficient concentrations along a developing muscle fiber, act to non-selectively disrupt synaptic adhesions (O'Brien et al., 1978). Interestingly though, sustained levels of activity in a single afferent can counterbalance this effect via the delivery of new synaptic machinery through microRNA regulated retrieval of dense cored vesicles. This process bolsters the generation of new synaptic and adhesion sites under active terminals (Wei et al.,

2013) and perhaps partially explains the effect of synaptic takeover that underpins the ultimate fate of monoinnervation at the motor endplate (Walsh and Lichtman, 2003). Agrin, a proteoglycan that serves as a proteinase inhibitor is also secreted from afferent terminals and may play a part in protecting active terminals from the secretion of proteinase enzymes (O'Brien et al., 1978; Biroc et al., 1993). Moreover, agrin efficiently induces the clustering of AChR along the membrane of muscle fibers (Dulcort and Changeux, 1995). This is achieved through the interaction of agrin with the postsynaptic, and membrane-bound, muscle-specific tyrosine kinase, MuSK, which phosphorylates several targets including rapsyn to initiate postsynaptic density formation (PSD) (review by Sanes & Lichtman, 1999). Thus, the agrin-mediated cascade may help to organize the random and prepatterned assortment of motile AChRs (described as randomly 'walking' receptors before association with PSDs) on the surface of early postnatal myotubes into dense clusters directly under active terminals (review by Sanes & Lichtman, 2001).

Climbing fiber innervation of Purkinje cells

The peripheral, activity-dependent competition and pruning process required for the development of the NMJ has strong similarities to another well described monoinnervating central connection, the climbing fiber (CF) to Purkinje cell (PC) terminal (Lorenzetto et al., 2009). The cerebellar CF represents a powerful, excitatory input that undergoes similar stages of synaptic competition to the NMJ (Watanabe and Kano, 2011). This neural circuit was initially studied by Ramón y Cajal (Ramón y Cajal, 1911; de Castro et al., 2007). At birth in mice, CF axons generate as many 100 en passant boutons onto the somata of PCs, during what has been

termed the ‘creeper stage’. Between P4-P7 and during what is termed the ‘pericellular stage’, only about 10% of these initial CF arbors remain and the soma is innervated by ~4-5 unique and larger nest-type inputs (Sugihara, 2005). The afferents are of moderate synaptic strength and evoke similarly sized postsynaptic currents during the short-lived pericellular stage (Hashimoto and Kano, 2003) as synaptic competition begins to resolve polyinnervation during final stages of development, termed the ‘capuchon and dendritic stages’, during the second postnatal week. The capuchon stage is hallmarked by the emergence of 2-3 inputs that increase in size and strength (Hashimoto and Kano, 2005). During this period of active competition, the remaining competitors also begin to shift position along the soma of the PC where the ‘winning’ input, that generates the largest postsynaptic current, translocates to the base of the apical dendrite of the PC. This input grows with and innervates the PC dendrite while the remaining smaller inputs are sequentially removed from the soma and pruned to produce a strong mono-innervating connection (Hashimoto et al., 2009). Similar to the NMJ, the process of selective refinement of excess CFs is dependent on disparate levels of activity between inputs. A dramatically sharp period between P15-16 mice appears to define the largest period of active NMDA-receptor mediated synaptic competition in this system wherein MK-801 administration antagonized NMDA receptors and inhibited removal of excess innervation (Kakizawa et al., 2000). Similarly, administration of harmaline to drive synchronous activation of competing CFs too has been shown to inhibit synapse elimination (Andjus et al., 2003).

The CF:PC system, like the NMJ, has been a useful model to identify molecular underpinnings of synapse removal. Here a cytoskeletal protein called Arc has been implicated in stability and maintenance of somatic CF inputs during development through selective endocytotic induction of AMPARs (Chowdhury et al., 2006). Arg3.1, the gene encoding Arc, is

upregulated following activation and Ca^{2+} entry through enriched P/Q-type channels in PCs (Mikuni et al., 2013). Once translated, Arc preferentially binds the calmodulin-dependent protein kinase II β (CaMKII β) that is enriched at recently activated synapses (Okuno et al., 2012). Repetitive Ca^{2+} influx produces a persistent association between Ca^{2+} /calmodulin and CaMKII β that induces autophosphorylation of CamKII β and prevents association with Arc, thereby stabilizing the most active synaptic sites (Yamauchi, 2005). This activity-mediated mechanism alters the balance of activity among competing inputs and can indirectly induce the selective maintenance of some and the removal of others.

Retinal Ganglion Cell Innervation of the Dorsal Lateral Geniculate Nucleus

A major projection of the mammalian retina, the dorsal lateral geniculate nucleus (dLGN), represents another key model system for understanding the process of selective circuit formation in the central nervous system. Here, retinal ganglion cells (RGCs) that have partially crossed the optic chiasm will arborize, innervate and terminate widely across midbrain visual centers including the superior colliculus (McLaughlin et al., 2003a) and importantly for this discussion within the dLGN (Guido, 2008). The majority (85-90%) of afferents within the dLGN are of RGCs originating from either eye owing to the partial decussation of the optic nerve fibers across the optic chiasm, at least in mice (Guido, 2008). The resulting pattern of binocular innervation within the dLGN is comprised of two underlying forms of organization fundamental to visual processing; afferent projections from each eye form non-overlapping territories in the mature dLGN (Godement et al., 1984; Muir-Robinson et al., 2002) and critically, the spatial

nasal-temporal arrangement of the retina is topographically mirrored in the dorsal-ventral axis of the dLGN (Torborg and Feller, 2005).

Development of the dLGN can be divided into distinct phases that occur before animals begin processing much of the visible world. The first phase of development relies on chemotactic guidance wherein RGC axons must navigate to appropriate territories within the relay nuclei of the visual system. This is achieved after RGC axons have left the eye wherein neuroepithelial Sema5a expression generates a repulsive signal that concentrates axons tightly within the optic tract. Axon growth is promoted towards the ventral diencephalon via a graded expression of ephrin-A that is sensed by axonal membrane-bound Eph-A receptors (Oster et al., 2003; Flanagan, 2006). Ephrin-B2 is expressed by midline radial glia (Williams et al., 2003), and contralaterally projecting RGCs that do not express EphB1 will cross the optic chiasm, arriving at the dLGN by E16 in mice. Two days later the non-crossing ipsilateral projections arrive by E18 (Godement et al., 1984). Soon after, axons extensively arborize in response to molecular cues encountered during the immediate perinatal period until approximately P4 (Godement et al., 1984). Chemotactic guidance to the thalamic dLGN produces an initially overlapping innervation pattern representing inputs from both eyes (Guido, 2008). A second phase of development involves the structural refinement of exuberant RGC axonal projections in order to produce the final arrangement of retinotopic organization (Penn et al., 1998). During the end of the first postnatal week, between P4-8, selective pruning begins to reduce the extent of exuberant connectivity (Godement et al., 1984; Bickford et al., 2010). These remaining inputs are subsequently reinforced between P8-12 when an increase in vesicle release sites results in much larger (up to ~50X) synaptic currents (Chen and Regehr, 2000). Ultimately, following the onset of sensory experience (P12-14), the innervation pattern of the dLGN largely resembles that of

the adult such that most relay neurons are driven by monocular innervation from between one to three RGCs.

Roles for neural activity were revealed by early experiments reducing whole eye input by ocular removal or pharmacological treatment (e.g., continuous delivery of TTX or glutamate receptor antagonist; (Garraghty et al., 1988; Shatz and Stryker, 1988; Hahm et al., 1991)). The proposed necessity for sources of activity within developing circuits was further reinforced as contemporary researchers began identifying patterns of spontaneous activity wherein neighboring RGC projections fire nearly synchronous bursts of action potentials (APs), separated by minutes, that propagate in waves across the entire retina (Meister et al., 1991). These waves were further preserved across the dLGN (Mooney et al., 1996). Thus, the next step in understanding the development of the visual system required more strategic manipulation to probe for possible underlying Hebbian mechanisms, whereby such spatially relevant patterns of repetitive presynaptic firing may selectively favor the maintenance of more correlated inputs while uncorrelated activity may target others for selective removal in a fashion that followed Hebb's postulate (Hebb, 1949).

The important question to be answered was whether the pattern of spontaneous activity was crucial for the generation of eye-specific layering in the visual system. Initial attempts at unilateral pharmacologic manipulation of RGCs in developing ferrets targeted the generation of patterned spontaneous activity and produced significant interruption in ocular layering organization in the downstream dLGN (Penn et al., 1998; Stellwagen and Shatz, 2002). Importantly, more targeted genetic approaches that disrupt the generation of spatiotemporally correlated activity between neighboring RGC axons from the same eye (through genetic abolishment of $\beta 2$ subunit of the nicotinic acetylcholine receptor) too generated significant

alterations synaptic refinement without the necessity to account for surgical effects (Grubb et al., 2003; McLaughlin et al., 2003b). Thus, correlated and patterned RGC firing during the period of early postnatal development has been repeatedly found to represent a powerful requirement for selective refinement of initially exuberant RGC terminal fields necessary to generate topographically organized, eye specific segregation of inputs to the dLGN, essential for higher-order visual processing to enable the perception of binocular vision once relayed to the cortex (Torborg and Feller, 2005).

Calyx of Held Innervation of the Medial Nucleus of the Trapezoid Body

The auditory system too serves as an important model for studying the formation of a circuit necessary for the computation of aural cues such as sound localization (Grothe et al., 2010). The primary aim of my dissertation research has been to describe the structural development of the calyx of Held (CH) nerve terminal and elucidate the competitive interactions that occur during the formation of this structure.

The CH was first described in seminal sparse Golgi staining experiments performed just prior to the 20th Century by Hans Held (Held, 1893). Soon after, Ramón y Cajal, H. Waldeyer-Hartz and others described examples of neuronal structures with observable pre and post-contact delineations between neurons in support of the Neuron Doctrine (López-Muñoz et al., 2006). In more recent decades, technological advances in cellular and molecular experimentation paired with the exaggerated morphology of the CH permit the direct manipulation and observation of the function and plasticity of a nerve terminal with hundreds of discrete synapses that provides fast, robust and high-fidelity stimuli to single principal cell targets in the medial nucleus of the

trapezoid body (MNTB) within the auditory brainstem (Forsythe, 1994; Meyer et al., 2001). Moreover, the CH:MNTB connection exhibits coordinated pre and postsynaptic maturation (Hoffpauir et al., 2010) and undergoes presynaptic competition during development with two distinct phases of presynaptic strengthening and pruning mirroring synaptic development of the NMJ, CF:PC and RGC:dLGN (Hoffpauir et al., 2006; Holcomb et al., 2013). Thus, the development of the CH:MNTB connection represents another strong model for synaptic development with a demonstrable resolution to monoinnervation by maturity that will remain the central focus of this dissertation. The remainder of this document will largely focus on describing the sequence of events pertinent to the formation and maturation of the CH nerve terminal that innervates the principal cell of the MNTB.

Development of the Calyx of Held

Charles Stockard first proposed a general concept of a 'sensitive period' to the field of developmental neurobiology in the 1920s in order to describe the confluence of permissible cues that exist during a limited period in development and allow for massive plasticity within circuits in the brain (Stockard, 1921). This idea was later reinforced in seminal animal studies utilizing reversible monocular deprivation studies, first in cats (Hubel and Wiesel, 1965; Wiesel and Hubel, 1965), and later in primates (Hubel et al., 1977). Further, this general concept of a sensitive period has proven useful in describing the development of other sensory systems as well, including the auditory system (Kandler et al., 2009). In these cases, development is controlled with tight spatial and temporal regulation wherein the confluence of genetic regulation

(Kang et al., 2013; Miller et al., 2014), onsets and changes in the pattern or onsets of synaptic activity (Blankenship and Feller, 2010) and the arrival of specific subsets of cells (such as radial glia, for example, that produce new neurons through asymmetric cell division (Noctor et al., 2004)) can all play vital roles in the genesis and reorganization of neural tissues during early development.

In rodents, the CH forms soon after birth and undergoes dramatic morphological transformations during a brief postnatal period in order to form a terminal capable of fast and reliable transmission of auditory information that percolates through the auditory system within 10 days of birth in mice, comparable to the stages of development in a human fetus occurring after about 28 weeks of gestation (Lecanuet and Schaal, 2002). The terminal's large size is reflective of its proportionally large number of active zones, which across all sites are capable of releasing more than a hundred synaptic vesicles during a single depolarizing event (Borst and Sakmann, 1996). Such robust signal transmission guarantees a single action potential is capable of generating a suprathreshold depolarization in the postsynaptic principal neuron to critically preserve timing information necessary for auditory processing (Trussell, 1999). Another important feature of the CH that is shared with much of the mature auditory system is a strong trajectory bias towards precise tonotopic organization. Tonotopy within brainstem auditory nuclei is largely established during early perinatal development, before animals are exposed to environmental auditory stimuli, suggesting development here arises from a sensory-independent set of mechanisms (Friauf and Lohmann, 1999; Kandler et al., 2009; Clause et al., 2014). However, much like in the development of the NMJ, CF:PC and RGC:dLGN connections, synaptic competition and selective refinement of supernumerary connections within the developing MNTB occurs during a brief postnatal period to establish precise connectivity

(Holcomb et al., 2013). A comprehensive review of the role and development of the CH:MNTB within the auditory system will be provided in the following sections.

Role of the Calyx of Held in Auditory Processing

The spiral ganglia neurons that form the auditory nerve relay peripheral auditory information from the inner ear to the brain via innervation of the cochlear nucleus (CN) in a way that preserves the tonotopic arrangement of the cochlea (Kandler et al., 2009; Muniak et al., 2013). Importantly too, this low to high frequency-dependent organization is further maintained throughout the ascending brainstem circuit by the onset of hearing in mammalian species (Kandler et al., 2009). The CN can be subdivided into the two primary nuclei, the dorsal and ventral cochlear nuclei (DCN and VCN). Within the VCN, a variety of neuronal populations exist with unique projections important for auditory processing including the so-called bushy cells. Of the two subtypes of bushy cells, the first are spherical bushy cells that provide excitatory ipsilateral input to neurons of the medial and lateral superior olive (MSO;LSO) as well as an additional projection to the contralateral MSO (Smith et al., 1993); the second class are referred to as globular bushy cells (GBC) which send a primary efferent projection to the contralateral MNTB giving rise to a CH but too less consistently project collateral innervation to both the ipsilateral superior olivary complex (SOC) targets including the periolivary nuclei, LSO, and lateral nucleus of the trapezoid body (LNTB) and contralateral targets such as periolivary nuclei including the MNTB, ventral nuclei of the trapezoid body and ventral nucleus of the lateral lemniscus (VNLL)(Spirou et al., 1990; Smith et al., 1991; Cant and Benson, 2003)(Fig 2).

Moreover, the arrangement of afferents and targets throughout the auditory brainstem preserve frequency-specific coding to maintain tonotopic organization.

Relevant for sound localization, the SOC is the first site to receive a convergence of binaural signaling. The shape and orientation of the two ears relative to each other produce sub-millisecond differences in the arrival time of bilateral signals. This allows the computation of differences in intensities, aural levels and arrival times of auditory cues with incredibly fast and reliable transmission (<300 Hz) (Taschenberger and von Gersdorff, 2000; Tollin, 2003; Grothe et al., 2010; Borst and Soria van Hoeve, 2012). Thus, by receiving tonotopically organized afferents from both ipsilateral and contralateral CN projections, the nuclei of the SOC are responsible for the initial integration and relay of highly organized bilateral auditory cues (Kandler et al., 2009).

Initial Genesis of the Auditory Brainstem

Soon after the closure of the neural tube during embryonic development, the rhombencephalon can be ultimately subdivided into 12 subregions, called rhombomeres (r). Each rhombomere has been shown to produce unique lineages of cells owing to differential expression of unique sets of transcription factors permitting the identification of discrete rhombomeres as early as E11-14 in mice, or approximately E29 in humans (Farago et al., 2006; O’Rahilly and Müller, 2007; Di Bonito et al., 2013). Importantly for the basis of this report, we will focus on two important auditory nuclei that develop specifically from this collection of rhombomeres: the ventral cochlear nucleus (VCN), which contains globular bushy cells and which arises from the caudal rhombic lip of r2-4 (Farago et al., 2006) and the MNTB, where the CH forms onto its

principal cells, and that likely arises from the proliferative dorsal rhombic lip of rhombomeres r4 and r5 between E9-14 in mice (Maricich et al., 2009; Di Bonito et al., 2013; Marrs et al., 2013).

Establishment of GBC-MNTB Connections

GBCs undergo neurogenesis beginning as early as E10 in mice and soon after project axons out of the VCN (Pierce, 1967). These axons arrive at the midline as early as E13.5 in mice (Howell et al., 2007) and subsequently cross into the presumptive contralateral SOC by E16 (Kandler and Friauf, 1993; Howell et al., 2007). During a similar gestational period between E16.5-E17.5, differentiating principal neurons within an amalgam of SOC cell groups in the ventrolateral brainstem begin to migrate medially across the brainstem to form a discernable MNTB that meets the arriving GBC axons (Hoffpauir et al., 2010; Marrs et al., 2013). Similar to commissural spinal neurons, GBC neurons rely on the expression of key guidance signaling to appropriately reach their newly arriving contralateral targets. In brief, GBC axons express a membrane bound receptor, DCC, that steers the outgrowth of axons along the ventral brainstem towards a gradient of netrin-1 secreted from and concentrated near cells at the midline (Nakamura and Cramer, 2011). Loss of either DCC or netrin1 results in an inability for GBC axons to appropriately reach the midline (Howell et al., 2007). Once axons have successfully navigated the midline however, a molecular switch occurs whereby DCC loses its sensitivity to netrin-1 and axons are further promoted in a trajectory away from the midline. Secreted Slit-1 secreted acts through Robo-1 and Robo-2 receptor mediated repulsion to effect axon guidance. Similar to knockout models of DCC (Fazeli et al., 1997) or netrin-1 (Serafini et al., 1996), conditional abolishment of Robo-3 produced errors in appropriate GBC axonal targeting and

resulted in aberrant ipsilateral connectivity via a failure to cross the midline (Michalski et al., 2013).

Microscopy studies in embryonic rodent tissues have demonstrated newly arriving GBC axons at E15 next experience a brief waiting period within the MNTB of about two days before axonal contacts onto principal neurons are established just before birth (Kandler and Friauf, 1993). Further confirming the existence of a waiting period prior to innervation, slice experiments that utilized *in vitro* preparations that maintained axonal projections from the cochlea to the brainstem demonstrated bushy cell axons could not elicit responses in principal neurons until E17.5 (Hoffpauir et al., 2010; Marrs and Spirou, 2012), although bushy cells themselves could be driven by spiral ganglion neuron (SGN) input as early as E15 (Marrs and Spirou, 2012). Spontaneous activity through this connection has been measured during the immediate perinatal period between E17 and P2 in the MNTB despite little if any growth occurring during this period (Hoffpauir et al., 2010). The initial contacts made by calyciferous axons onto between 5-20 discrete principal neurons per axon each remain small into postnatal ages as no large terminals have been detected earlier than P3 in mice (Hoffpauir et al., 2010; Holcomb et al., 2013).

Formation of a Large Terminal

The phenomena that trigger the explosive growth of the CH have only partially been elucidated. A genome-wide microarray analysis comparing gene expression in the MNTB and LSO identified the bone morphogenic proteins (BMPs) as enriched in the MNTB during calyx growth as well. Genetic deletion of BMP receptors using an Engrailed-2 (Egr2) cre line led to a

disruption in growth and competition of the CH as inputs failed to progress past the stage of protocalyceal poly-innervation (Xiao et al., 2013). Loss of BMPR1a/1b genes in this context may have impaired CH growth in a due to disruption in downstream signaling. First, loss in typical levels of BMPR1a and BMPR1b-mediated phosphorylation of the transcription factor SMAD likely alters the capacity for SMAD to modifying downstream gene regulation (Miyazono et al., 2010). Alternatively, loss of BMP association with BMPR1a/1b may induce a deficit in SMAD-independent regulation of axon dynamics that further disrupts CH formation (Zhong and Zou, 2014). Wildtype mice express the serine/threonine-protein kinases SAD-A and SAD-B both pre and postsynaptically in the MNTB. SAD-A/Sad-B double knock-out mice show significant deficits in calyx growth owing to an inability to regulate cytoskeletal phosphorylation (Alvarado-Kristensson et al., 2009; Lilley et al., 2014). Aiming for another possible molecular candidate, knock-out experiments targeting neural recognition molecule 2 (NB2), which plays a role in synapse stability and cell-cell adhesion, led to a minor disruption in calyx growth and stability (Toyoshima et al., 2009). Unfortunately, none of these perturbations entirely prevent the formation of a presynaptic terminal in this system or fully explain the rapid formation of the characteristically large CH.

At P3 in rodents, GBC axonal termini in the murine neonatal MNTB begin to rapidly transition into spoon-shaped terminals called the protocalyx (Morest, 1968a; Kandler and Friauf, 1993; Holcomb et al., 2013)(Fig 3). Robust expansion of the protocalyx coincides with the onset of patterned, experience-independent spontaneous activity that begins to percolate throughout the auditory system soon after birth (Beutner and Moser, 2001; Tritsch and Bergles, 2010). The spontaneous bursting activity pattern typical of the developing postnatal auditory system originates within the transient Kölliker's organ of the immature cochlea. There, a mass of

columnar supporting cells nearby to inner hair cells (IHCs) secrete ATP locally within the organ as IHCs concurrently become responsive to purinergic signaling. This spontaneous release and buildup of ATP readily depolarizes maturing IHCs and triggers bursts of action potentials that subsequently stimulate the dendrites of Type I spiral ganglion neurons. Moreover, irregular changes in local ATP concentrations across the developing cochlea produce differences in the pattern of activity among IHCs; neighboring IHCs more synchronously depolarize relative to more distant pairs of IHCs. Moreover, these differences in local firing among IHCs across the cochlea further produce differences in firing patterns among first order spiral ganglion neurons (Tritsch et al., 2007; Tritsch and Bergles, 2010).

In order to investigate the necessity for spontaneous activity in the development of the auditory system, manipulations that experimentally alter the temporal fine structure of spontaneous bursting activity in the developing cochlea have been shown to measurably affect the tonotopic organization of downstream nuclei in the auditory system (Clause et al., 2014). Thus, it is tempting to speculate that the onset of spontaneous activity found to temporally coincide with the expansion of the terminal may serve as a trigger for growth and the pattern of activity may be useful for the selection of appropriate connections such as occurs in the visual system (Wong et al., 1993; Ackman et al., 2012).

Morphology of the Protocalyx

Morphological surveys of developing protocalyces have revealed insights into the potential mechanisms of large terminal formation in this system. For example, much like the CF:PC system wherein the dominant input takes a position at the base of the apical dendrite

(Hashimoto et al., 2009; Watanabe and Kano, 2011), the development of the CH may be contingent on the location of initial innervation during its period of synaptic competition. At early postnatal ages, before the onset of hearing in mice (P3-9), MNTB principal cells exhibit a polarized morphology with an eccentrically located nucleus that is biased towards one end of the cell, defined as the nuclear pole. Moreover, in the first survey of CH development using serial electron microscopy it was noted the emerging protocalyces were excluded from growth over this nuclear pole (Hoffpauir et al., 2006). This observation was later confirmed in a more systematic analysis of CH formation across 92 cells (11 at P2, 29 at P3, 17 at P4, 29 at P6, and 11 at P9) that further demonstrated the prevalence of nuclear eccentricity in the developing MNTB. Further, the study reported a significant bias favoring growth of the largest protocalyceal terminal from multiple large competitions occurs on the non-nuclear pole of MNTB principal cells (Holcomb, 2018). This type of permissive growth towards a preferred somatic region along a postsynaptic soma closely resembles the established mechanism of selective reinforcement of competing terminals described in the CF development (Hashimoto et al., 2009; Watanabe and Kano, 2011).

As the protocalyx expands from initial en passant axonal projections into enlarged, spoon-shaped terminals, it characteristically produces abundant cytoplasmic extensions, defined as thin collateral stalks, in a process that parallels axonal arborization (Fig 3). These collateral structures radiate dozens of microns away from the immediate innervation territory of the developing terminal (Kandler and Friauf, 1993). Akin to developing axons and dendrites, calyceal collaterals can be tipped with growth cones and often support numerous branches as well. Importantly, these collateral features of the emerging CH persist as a common feature only during the neonatal period of calyceal development (Morest, 1968a; Kandler and Friauf, 1993).

Although the targets of calyceal collaterals are unknown, Ca^{2+} imaging *in vivo* demonstrated the collaterals generate presumptive synaptic signals, thus increasing the extent of presynaptic innervation territory beyond a single terminal target during early development. However, much is still unknown about these structures and their function during development and the subsequent chapter will aim to investigate their role during development wherein their function may serve to establish or maintain innervation territory during a chaotic period of rapid growth and synaptic competition.

Transient Period of Synaptic Competition

As described previously, developing axons in many parts of the nervous system produce exuberant branches within targeted territories. In order to achieve synaptic specificity reflective of more mature connectomes, an activity-dependent process of synaptic competition favors the stabilization of a subset of inputs and the removal of others (Hua and Smith, 2004; Ooyen, 2005; Schuldiner and Yaron, 2014). It has remained a fairly contentious issue in the literature as to whether the development of the CH can be described as proceeding according to this canonical program of early exuberance followed by synaptic competition or if, in fact, the connections form with extreme tonotopic precision owing to genetic or molecular guidance (Rodríguez-Contreras et al., 2006; Kandler et al., 2009). In part, this may be due to the rapid establishment of dominant monosynaptic innervation in this system that emerges between P4-6 (Hoffpauir et al., 2006; Holcomb et al., 2013).

The number of large, competing inputs innervating principal neurons in the MNTB has been assessed through minimal stimulation paradigms in which single inputs are recruited

through step-wise increases in external stimulation (Hoffpauir et al., 2010; Xiao et al., 2013). Initial physiologic studies revealed multiple large inputs, but at relatively low percentages (4 in 29 cells at (P4) (Hoffpauir et al., 2006) and 6 in 101 cells nearer the onset of sensitivity to airborne sounds (P7-12)(Bergsman et al., 2004). Systematic reconstructions from volume electron microscopy images has revealed a higher proportion of competing inputs (9/16 at P3; 6/18 at P4; 10/42 at P6) (Holcomb et al., 2013). In part this discrepancy could be explained in a few key ways. The generation of an acute brain slice for *in vitro* study inherently reduces the number of maintained synaptic connections through physical severance of many axons as well as potentially damaging any cells accessible to whole-cell patching in superficial layers near the cut plane. Alternatively, the protocalyx has been defined as large when its area of direct apposition is greater than $35 \mu\text{m}^2$, which, relative to the mature CH, is still considered a relatively small input that may have been overlooked in electrophysiologic surveys of large terminals (Holcomb et al., 2013). Similar to the NMJ, the progressive formation of the large CH nerve terminal passes through two distinct phases of strengthening and pruning to a single, large nerve terminal. However, these events in the MNTB occur more quickly (24-48 hours) relative to other classically described systems (Holcomb et al., 2013).

Early Stage Strengthening and Pruning

The first stage of calyceal strengthening and pruning occurs during the perinatal period from E17 until P3. Following arborization of newly arriving GBC axons within the MNTB, each principal neuron becomes innervated by 10-20+ small bouton-type or en passant GBC axonal endings (Holcomb et al., 2013). From among these superfluous connections, at most two or three

inputs per neuron are strengthened and permitted to grow large ($>35 \mu\text{m}^2$) between P3-4 (Kandler and Friauf, 1993; Hoffpauir et al., 2006). In this context, apposed surface area is used as a proxy metric for synaptic strength as studies have shown the number of active zones increases with age and in proportion with the size of the terminal (Taschenberger et al., 2002; Holcomb et al., 2013). During this early period wherein inputs are still small ($<35 \mu\text{m}^2$), single inputs to the principal neuron are only capable of eliciting proportionally small postsynaptic currents, suggesting release from only a small number of active zones per terminal (Hoffpauir et al., 2006). However, in order to maintain high frequency spike rates, the immature terminals employ multiple Ca^{2+} clearing mechanisms including ion channel exchangers and specialized mitochondria anchored to the presynaptic membrane by filamentous supports to form ancillary conglomerate structures referred to as mitochondria-associated adherens complex (or MACs) located adjacent to a presynaptic membrane nearest the readily releasable pool of synaptic vesicles to increase the efficiency of neurotransmitter release via efficient Ca^{2+} sensing and local ATP production (Rowland et al., 2000; Kim et al., 2005). These adaptations allow competing protocalyces to quickly become even more potent inputs and hence stronger competitors.

Late-Stage Strengthening and Pruning

During a second distinct phase of strengthening and pruning, the subset of remaining large protocalyces (2-3; $>35 \mu\text{m}^2$) compete to establish a dominant innervation territory along each principal cell. Importantly, the mode of competition in this context is indirect as terminals form largely non-overlapping territories on single postsynaptic principal neurons (Holcomb et al., 2013), although significant changes in the postsynaptic cell described in a subsequent section

may mediate a postsynaptic mechanism with which the competitive vigor of competing inputs may be assessed. This second phase of competition in this system lasts only a brief period (< 2-3 days) and involves both the selective removal of unneeded GBC innervation and the rapid emergence of a single, large and spoon-shaped protocalyx on most principal cells, at an accelerated growth rate that may exceed $200\text{ }\mu\text{m}^2$ per day at its peak during this period (P6 ~75%; P9 ~88% monoinnervated) (Bergsman et al., 2004; Holcomb et al., 2013). Interestingly though, although the developing MNTB has been imaged *in vivo* (Rodríguez-Contreras et al., 2008), the selective withdrawal of excess large innervation has yet to be directly observed and may in fact outpace even the rapid expansion of a protocalyx.

Maturation and Fenestration of the CH

Sound localization can only be achieved with precise synaptic processing across the auditory system and the CH:MNTB connection in this regard serves as an exemplary example of specialized adaptation. Following the opening of the ear canal and the onset of sensitivity to airborne sound (P10-12), the CH has perhaps achieved its greatest size. Across this large area of apposition, hundreds or thousands, depending on species, of discrete adherens junctions and presynaptic active zones provide increased mechanical stability and robust excitation (Morest, 1968a; Kandler and Friauf, 1993; Rowland et al., 2000; Sätzler et al., 2002; Hoffpauir et al., 2006). In order to maximize the temporal fidelity of this terminal, there are additional adaptations that must be implemented through complex morphologic and physiologic changes. After P10, protocalyces both structurally consolidate through the retraction of thin collaterals stalks and transform from a “hairy” spoon-like shape into the stereotypically smooth, fenestrated

or “finger-like” arrangement of axo-somatic contacts that encompass a substantial proportion (as much as 50%) of the postsynaptic soma. This new morphology allows for more efficient clearance of glutamate from the synaptic cleft during high frequency transmission (Ford et al., 2009). Despite the morphology of the CH permitting synchronous release of glutamate from throughout the terminal, the mature CH also exhibits a characteristically shorter duration AP (~25%) that allows little opportunity for presynaptic calcium influx (Taschenberger and von Gersdorff, 2000; Yang and Wang, 2006). In turn, this is reflected in a relatively low release probability at single active zones throughout the CH. Thus, to maintain synaptic activity during high frequency stimulation with high fidelity necessary for acute auditory processing, it is necessary for Ca^{2+} to become more tightly regulated to increase the probability of single release (Wang et al., 2008; Kochubey et al., 2009). This is achieved as immature protocalyces shift from a reliance on ωCgTx GVIA-sensitive N-type and $\omega\text{Aga-IVA}$ -sensitive P/Q type voltage gated Ca^{2+} channels to predominantly a P/Q-type current driven by the subunit Cav2.1 (Iwasaki and Takahashi, 1998) and Kv3 channels (Wang et al., 1998; Elezgarai et al., 2003) which cluster tightly with active zones nearby the readily releasable pool of vesicles (Iwasaki and Takahashi, 1998; Fedchyshyn and Wang, 2005; Wang et al., 2008; Chen et al., 2015). Together, these final presynaptic adaptations function to accelerate the repolarization of the membrane, shorten AP duration and increase synaptic fidelity to aid in the transformation of the CH into a fast and reliable relay for mature sensory processing.

Maturation of the Postsynaptic Principal Cell

The maturation of the postsynaptic principal cell mirrors the developmental changes seen presynaptically. Immature principal cells in the MNTB at birth have a high input resistance ($>1\text{G}\Omega$) and a relatively depolarized resting membrane potential ($-55\pm 7\text{ mV}$) making them extremely excitable and capable of generating action potentials as early as E17 in response to minimal synaptic current downstream of spontaneous neurotransmitter release ($>30\text{ pA}$) (Hoffpauir et al., 2010; Rusu and Borst, 2011). However, soon after birth (P2) the pattern of spontaneous activity measurable in this system dramatically shifts towards bursts of bursts (Tritsch and Bergles, 2010). Such repetitive activation makes these cells susceptible to tonic excitation that may lead to increased Ca^{2+} concentrations, especially through upregulated GluA2-deficient Ca^{2+} -permeable AMPA receptors at early postnatal ages (Lujan et al., 2019), known to directly induce gene expression in the developing nervous system (Flavell and Greenberg, 2008). Once protocalyces emerge between P2-3, these glutamatergic terminals begin to drive EPSCs in principal neurons characteristically approaching -1 nA . The CHs that emerge soon after, however, are capable of driving currents exceeding -6 nA by P4 (Futai et al., 2001; Hoffpauir et al., 2006). This rapid change in input strength means principal neurons too must rapidly evolve more mature firing properties in concert with the rapid growth of the presynaptic protocalyx. Postsynaptic cells during this period transition from tonic to phasic response patterns (Hoffpauir et al., 2010). The principal cells, in part, progressively gain this ability by P6 through a reduction in their intrinsic excitability as the resting membrane potential increases to -72 mV , the input resistance drops roughly 5-fold to $\sim 200\text{ M}\Omega$ and the expression of low voltage-gated potassium channels (Kv1.1 and Kv1.2) increases which together act to inhibit tonic postsynaptic responses and tune MNTB neurons towards single spikes (Brew and Forsythe, 1995; Dodson et al., 2002; Hoffpauir et al., 2010). Moreover, these postsynaptic changes may provide a means for each

principal neuron to tune its firing to the competing input that drives the strongest and most synchronous activity, similarly to the development of the NMJ (Favero et al., 2007).

The changes in biophysical properties of principal cells during this early postnatal period also mirror events occurring in other auditory nuclei including a loss of dendritic arbors in the presence of spontaneous bursts of activity (Rietzel and Friauf, 1998; Franzen et al., 2014). The cell body also significantly increases in volume, perhaps through partial resorption and membrane recycling (Hermida et al., 2010; Hoffpauir et al., 2010). The intrinsic charging capacity of the cell is clearly altered by this structural increase in cell size as membrane capacitance and resistance decreases dramatically whilst current threshold is significantly increased (Hoffpauir et al., 2010; Rusu and Borst, 2011). Undeniably, these changes in the intrinsic biophysical properties of the postsynaptic cells collectively raise the thresholds to favor only the strongest presynaptic inputs and may bely a functional pressure necessary for the selection of a single dominant input.

The principal cells of the MNTB also undergo glutamate receptor subunit switching to alter firing patterns. The early neonatal principal neuron initially relies on the expression of AMPAR subunits GluR1/2 but is largely dominated by NMDARs as well which together provide slow decay properties in response to immature EPSCs (Caicedo and Eybalin, 1999; Taschenberger and von Gersdorff, 2000; Joshi et al., 2004). Over the course of the first week of life, the expression of AMPAR begin to switch as GluR1 expression decreases with age (Hermida et al., 2006). Moreover, the dramatic increase in synaptic strength and firing rate experienced between P3 and the onset of sensory experience in the MNTB triggers an increased expression of GluR3/4 (Caicedo and Eybalin, 1999; Joshi et al., 2004) and a sharp reduction in NMDAR along the postsynaptic membrane (Caicedo and Eybalin, 1999; Joshi and Wang, 2002).

These adaptations provide substantially faster AMPAR-EPSC kinetics required for this synapse to preserve timing information relevant in auditory cues (Trussell, 1999) and collectively tune the maturation of the MNTB principal neuron in concert with the development of its largest presynaptic input (Hoffpauir et al., 2010).

Aim of the study

The CH is the largest terminal in the mammalian brain and has been shown to develop very rapidly. However, to date, very few studies have tried to elucidate the dynamics that define the development of this system in terms competitive growth, directly or indirectly, from among immature protocalyceal terminals. From the establishment of a broad GBC axonal innervation territories in the MNTB, to the emergence and motility of elaborate secondary/terminal arborization and the functional strengthening of maturing CHs, the goal has become to describe these processes from a more targeted perspective of synaptic competition during the first weeks of life. Here we study the fine changes in CH ultrastructure during early postnatal development, assess the interaction of competing CHs, and chart the emergence of a rapidly maturing, functionally fast and high fidelity (faithfully phase locking up to 1 kHz) central synapse that is paramount to encoding timing information with respect to binaural auditory cues using advanced forms of high-resolution microscopy to chart the formation of the calyx of Held.

**Chapter 2: Rapid Terminal Dynamics Within Target Territories During Neural
Circuit Formation**

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Introduction:

The central nervous system is the most complex of organs and self-assembles its basic organization at extremely high rates. In mice, for example, about 100 million neurons are born during an eleven-day period between formation of the neural tube at embryonic day (E) 9 and birth. During this time frame the nervous system expands to a volume of 90 mm³ (De Felipe et al., 1997), and forms interconnections in neocortex at a density of about a trillion synapses per cm³ (Drachman, 2005). Soon after neurogenesis, growth-cone led axons begin extending toward targets that can be tens of micrometers to centimeters distant from their cell body, and must do so rapidly, at velocities estimated at up to ~3 $\mu\text{m}/\text{min}$ in many parts of the nervous system (Chien et al., 1993; Halloran and Kalil, 1994; Witte et al., 1996; Phan et al., 2010). Within terminal territories however, axon extension rates generally decline as axons begin to densely arborize and form immature synaptic contacts (Harris et al., 1987; Halloran and Kalil, 1994; O'Rourke et al., 1994; Rodríguez-Contreras et al., 2008). In monitoring this process directly, tissue stability, photobleaching and phototoxicity have often limited sample rate and constrained temporal resolution of time lapse experiments (Chien et al., 1993; Halloran and Kalil, 1994; Witte et al., 1996; Schlosshauer et al., 2003; Phan et al., 2010; Özel et al., 2015). We questioned, given the dramatic CH growth rates estimated in a previous investigation (Holcomb et al., 2013), whether CH dynamics would occur more quickly than has been previously reported in other systems.

More recently light sheet microscopy (LSM), which achieves volume acquisition at high sample rates with minimal tissue damage, has provided an imaging platform ideally suited to visualize morphologic changes within complex, 3D tissue geometry (Keller and Stelzer, 2008; Ahrens et al., 2013; Tomer et al., 2015). We have developed procedures to image brain slices using lattice light sheet (LLS) microscopy, which offers high temporal and spatial (near isotropic at the diffraction limit) resolution between image planes on scales useful for subcellular imaging

(~1 Hz, < 1 μm), higher signal-to-noise and substantially reduced phototoxicity and bleaching through delivery of thin optical sheets (~100 nm) at low laser powers (Planchon et al., 2011; Chen et al., 2014; Heddleston and Chew, 2016).

Topography in neural connections from cell group to cell group is a fundamental feature of brain organization, underpins neural computation, and has been explored in numerous studies over the past 100+ years. Initially demonstrated and most easily studied in sensory systems, single neurons initially project broadly and then restrict projection territory, often in an experiential context. Mechanisms of synaptic competition are offered to explain these phenomena, but little explored is whether axon collaterals interact in space, away from the synaptic surface. We explored collateral process dynamics and their spatial interactions during formation of the calyx of Held (CH) in the auditory brainstem, which exhibits an extreme and easily quantified version of topography, called tonotopy, via mono-innervation of its target in the medial nucleus of the trapezoid body (MNTB). The CH offers advantages as a developmental model (Kandler and Friauf, 1993; Hoffpauir et al., 2006, 2010; Crins et al., 2011; Holcomb et al., 2013; Xiao et al., 2013), because it passes through largely stereotyped phases of synapse formation including competition and pruning *en route* to mono-innervation similar to, but more quickly than (within 48 hours) other well-studied systems such as the neuromuscular junction (Sanes and Lichtman, 1999; Tapia et al., 2012) or the climbing fiber innervation of Purkinje cells within the cerebellum (Hashimoto et al., 2009; Watanabe and Kano, 2011). CH axons resolve to mono-innervation of 2/3 of postsynaptic targets over a restricted 48 hour period (postnatal days (P)2-4) and increase in contact area at a rate of up to 200 $\mu\text{m}^2/\text{day}$ (Rodríguez-Contreras et al., 2006; Holcomb et al., 2013). Golgi and cell labeling studies first noted the existence of thin collateral processes of variable length, often tipped with growth cones, that extend from

immature terminals but are fewer and shorter by maturity (Morest, 1968a; Kandler and Friauf, 1993; Rodríguez-Contreras et al., 2008). An unspecified number of CHs innervate neighboring cells via collaterals, so these have potential to form permanent synaptic connections. In a *tour de force*, an *in vivo* two photon (2P) preparation was developed that revealed motility of CH collaterals. Notably, evidence from Ca^{2+} transients observed directly within these collaterals suggests a single calyx may functionally innervate multiple cells within the neuropil (Rodríguez-Contreras et al., 2008).

These observations imply important roles for collaterals as: potential seekers of additional innervation targets, defenders of established innervation from other CHs, and/or vehicles for selective communication organizing frequency tuning across this auditory nucleus. To address these issues, we combined our unique volume EM series collected across developmental age with novel application of LLS microscopy. From these structural data, we defined collaterals types based on length and whether they were growth-cone or filopodial-tipped. Long collaterals exhibited highly dynamic motility, and extended and retracted at higher rates than previously reported (Bray and Chapman, 1985; Halloran and Kalil, 1994; Bellar et al., 2013; Özel et al., 2015; Stanganello et al., 2015). Contact between collaterals was common as all CHs projected radiating collaterals densely throughout the MNTB neuroil. Furthermore, we show that a small population of postsynaptic neurons remain dually innervated into adulthood, and that occasionally these secondary terminals arise from collaterals extending from nearby CHs.

Results:

Collateral Number and Length Increase Transiently during Nerve Terminal Growth

Tissue from neonatal (P2, 3, 4, 6, 9) and mature (P30) mice was processed and imaged using serial block-face scanning electron microscopy (SBEM) to bracket initial growth of the calyx of Held (CH) nerve terminal at P3, resolution of competition to achieve monoinnervation onto most cells (88%) by P9, and a mature end point (Kandler and Friauf 1992; Holcomb et al, 2013). Light microscopy showed prominent collaterals from the CH during growth (Kandler and Friauf 1992; Morest 1968). We provide the first 3D volumetric views of calyx collaterals at high spatial and daily temporal resolution, to characterize them by internal structure and contact with neurons and glia as the neural circuit forms. CH terminals here were segmented and those with largely complete collateral networks were skeletonized to measure the length and number of these arborizations across postnatal development ($n = 2970$ collaterals, 34 CHs; Fig. 1A₁₋₅, C'). The number of collaterals increased with size of the CH, measured as apposed surface area with the postsynaptic membrane (Fig. 1B, $r^2 = 0.79$, $m = \sim 11$ collaterals/ $100 \mu\text{m}^2$). The density of short reaching collaterals increased between P6 and P9 (Fig. 1C), despite the majority (75% P6 – 88% at P9) of principal cells having established mono-innervation by these ages (Holcomb et al. 2013). Mature CHs (P30) form a consistent end point, whereby either no (80%) or one (20%) collateral innervates a nearby neuron and which volume EM reveals to be of moderate size ($\sim 64 \mu\text{m}^2$; 16 synapses within terminal).

We measured a broad range of collateral length ($\sim 1 - 70 \mu\text{m}$). Only 5/21 collaterals $> 40 \mu\text{m}$ extended outside of our image volumes such that their length could not be measured, so it is likely that few collaterals exceeded lengths beyond $70 \mu\text{m}$. Median and variance of collateral

length did not change from P3-6 (median values at P3, $5.88 \pm 8.17 \mu\text{m}$, $n = 245$; P4, $6.24 \pm 9.65 \mu\text{m}$, $n = 590$; P6, $4.84 \pm 9.53 \mu\text{m}$, $n = 270$; Holcomb et al. 2013), so the spatial extent of the collateral field is established early and increased in density with age. We combined these ages into a single histogram ($n = 1,352$, median $5.85 \pm 9.32 \mu\text{m}$; Fig. 1C) and used the median value to distinguish the short collaterals from long filopodia and growth cone tipped collaterals. Following the establishment of monoinnervation, P9 CH collaterals were composed predominantly by short filopodia ($95.6\% < 5.85 \mu\text{m}$) with few observable branches ($n=340$, median = $1.63 \pm 1.99 \mu\text{m}$), and the longest measured was $21 \mu\text{m}$ (Fig. 1A₄, C, D).

Both filopodial (finger-like) and growth-cone tipped collaterals were shown using light microscopy to emanate from the growing CH (Morest, 1968b; Kuwabara et al., 1991; Kandler and Friauf, 1993). We show that all short collaterals are filopodial tipped. A minority of long collaterals (124/ 634) end in growth cones (closed and open arrowheads, respectively in Fig 2A). Few growth cone tipped collaterals remained at P9. Two of five CHs by P9 retained a small number of growth cone tipped collaterals. These remaining growth cones formed areas of apposition along nearby axonal processes (2 of 5) or a dendrite of a nearby principal cell (not shown).

Arrangement of CH Collaterals Facilitate Selective Spatial Overlap

The developing MNTB is composed primarily of dense populations of principal cells and glia (Dinh et al., 2014; Brandebura et al., 2018). While collaterals enumerate and lengthen during CH growth, collateral fields from nearby CHs may occupy an overlapping territory within the

neuropil (Fig. 3A-C). This was especially true of observations from the first postnatal week (P3, P4 and P6) in which many of the longest CH collaterals were documented (Figure 2C, 3D) and principal cells were most tightly clustered within the nucleus (Hoffpauir et al., 2006; Holcomb et al., 2013). An intriguing possibility arises that collaterals may signal through contact-mediated communication between presynaptic endings. However, SBEM observation reveals nearby CHs can participate in selective contact with other inputs with either heavily overlapping collateral fields in some cases, or entirely segregated territories in others (Fig. 3E-F).

Internal Structure and Contacts of CH Collaterals

Since EM labels all cells and their intracellular structure, we next investigated differences in the morphology and targets of these three types of endings (short and long filopodial, long growth cone collaterals), and whether they formed synaptic connections. The main body of the CH contained a large collection of discernable synapses, defined by concentrations of synaptic vesicles near convex regions of the postsynaptic membrane, dark and thickened postsynaptic staining indicative of a postsynaptic density, and locally positioned mitochondria and reticulated membranous structures (Fig. 2B').

Most short collaterals extended within the immediate vicinity of the neuronal soma in regions of the neuropil with notable extracellular space (76% at P3, 63% at P4, 58% at P6; labeled “f” in Fig. 2C). About 1/3 of terminal collaterals extended along the somatic surface and may act to guide CH expansion along the cell body. Very few short collaterals (<10%) contacted neighboring neurons (somata, dendrites or axons) or glia. Short filopodia contained vesicles and

reticulated membrane (Fig. 2C, C'), whether the collateral extended among other neurites and the extracellular space or along a cell body, but did not exhibit features of synaptic contacts.

Long filopodial collaterals also terminated largely among other neurites and extracellular space (66% at P3, 81% at P4, 73% at P6). Unlike short collaterals, these collaterals more commonly contacted neighboring neurons and glia (~30% at P3, ~15% at P4 and ~13% P6) and supported numerous secondary branches (Fig. 2B''). Long filopodia contained many vesicles, reticulated membrane and mitochondria although the latter typically did not extend within 10 μm of the tip (Fig. 2D'). This intracellular profile also was independent of terminus target, and did not exhibit synaptic specializations (Fig. 2D, D').

Different still from filopodial collaterals, growth cone-tipped collaterals more frequently contacted neighboring neurons and glia at P3 (50%) and P4 (46%), but less frequently at P6 (15%). Conversely, growth cone contacts with other CH collaterals from nearby terminals increased during the same period (P3 19%; P4 43%; P6 45%). Growth cone collaterals and their filopodial extensions contained vesicles and reticulated membrane. Mitochondria were present up to the central domain at the base of the growth cone (Fig. 2E, E'), however no evident features of synaptic specializations.

At P9, CHs had very few long collaterals (4.4% of 340 $> 6 \mu\text{m}$; Fig. 1A₄, C, D). Nearly all were filopodial-tipped (12/15), although growth-cone tipped collaterals were longest. Such a preponderance of simple, short filopodia is suggestive of a net retraction or removal of the longest exploratory processes begins ~P9. Moreover, only 3% of the 340 collaterals at P9 (5

CHs) exhibited contact with adjacent cells or CHs. These may represent the limited number of CHs at P30 that extended a single collateral that contacted a neighboring cell (4/23 CHs; Fig. 1 A₅) (Morest, 1968a; Kandler and Friauf, 1993).

We next devised methods to observe these tissue dynamics *in vitro* using brain slices imaged by lattice light sheet microscopy.

Dynamic Imaging of the Developing MNTB

Calyx Collaterals.

The diverse morphology and density of the CH collateral arborizations demonstrated with SBEM at young ages, taken with previous indications that CH collaterals exhibit observable motility and progressive turnover *in vivo* (Rodríguez-Contreras et al., 2008), may bely a complex form of presynaptic organization/communication through collateral contact during MNTB circuit formation. We sought to employ lattice light-sheet (LLS) microscopy to volumetrically image CH development at a rapid time scale within living tissues (0.3 – 1.4 Hz) in order to characterize the behavior of CH terminal arborization. Parvalbumin Cre (PV-Cre) driven tdTom expression provides selective and strong labeling of bushy cell neurons within the ventral cochlear nucleus (VCN; Fig. 4A, A') as well as the associated axonal projections to the respective ipsilateral lateral superior olive and contralateral MNTB (Fig. 4A, A''). PV is not expressed until the second postnatal week by MNTB principal neurons (Kolson et al., 2016). To reveal postsynaptic targets within the MNTB we transfected the MNTB with HDAd to deliver a GFP construct into MNTB

neurons (Fig. 4A, A''). Moreover, the selective presence of retrograded GFP-labeled somata in the VCN confirms that tomato labeled axons of the ventral acoustic stria in the MNTB are originate from the GBCs (arrows Fig 4A'). Acutely prepared tissue was secured to a slatted metal holder following resection of the cerebellum (Fig. 4B) and 4D image volumes were collected from live slices using LLS microscopy under near physiologic conditions (Fig. 4C, D). Light sheet excitation was presented at a 45° orientation using a custom Bessel pattern with concurrent and fixed orthogonal image detection to produce three dimensional rhomboid volumes (Fig. 4C-C'). Following each imaging session, tissue viability was verified through verification of no obvious blebbing or tissue shrinkage and continued growth throughout each time-series. Repeated imaging sessions (20-90 minutes) were performed on each slice where possible throughout the 4 hour period of tissue viability following rapid decapitation. Subsequently, CH collateral dynamics were analyzed in an immersive virtual reality (IVR) environment using visualization and annotation software developed within our laboratory (syGlass; www.syglass.io) (see Methods). Data analyzed in this way provided insight into the dynamic behavior of each of our three classes of CH collateral structures.

Short Filopodial Collaterals.

We hypothesized that the short filopodial CH collaterals maintain a static boundary around immature innervation territories based on the perseverance of these numerous stunted structures throughout the first two weeks of life. The PV Cre driven cytoplasmic tdTomato fluorescence provided robust labeling of calyciferous axons and terminals although fewer labeled collaterals could be dynamically tracked along each developing CH relative to the more

numerous arborizations revealed with complete reconstruction with SBEM (Fig. 5 maximum intensity projection (MIP) illustrating a single CH with a number of finger-like collaterals protruding away from the primary terminal). A variety of motile behaviors were observed among dynamic CH collaterals. Approximately 29% of all collaterals with entirely captured extents (16 of 56 collaterals from 11 CHs) did not significantly change length ($> 2 \mu\text{m}$) during the observation period of any of our experiments. The remaining 40 collaterals extended ($n=32$), retracted ($n=6$) or both ($n=2$), by more than $2 \mu\text{m}$ (often doubling/halving overall collateral length) within respective observation periods. Moreover, the relatively short collaterals measured here often exhibited transient lifetimes (11 of 21 short collaterals with lifetimes completely captured within a single experiment persisted <5 minutes) but still exhibited significant extension and retraction dynamics (Fig. 5C-E). In one particularly well resolved experiment, adjacent collaterals from the same CH illustrate unsynchronized extensions and retractions of $4\text{-}6 \mu\text{m}$ that further demonstrate the limited extant common among these shortest collaterals (<30 minutes; Fig. 5B, B', B''). Importantly, the highest frequency volume acquisition rates ($\geq 1.3\text{Hz}$) during largely halted periods of motility revealed a near constant state of small changes ($150\text{-}300 \text{ nm}$) in collateral length (Fig. 5C-E). To mitigate jitter in measurement of growth trajectory, a threshold of $3 \mu\text{m}$ of monotonically directed length change was used to filter plots of collateral velocity. These rates varied between 0.1 and $18 \mu\text{m/minute}$ (Fig. 5C'). These resultant velocities at times rivaled some of the fastest measured filopodia and/or axonal growth cone extensions/retractions in studies of neuronal organotypic culture from a variety of model organisms (Bray and Chapman, 1985; Halloran and Kalil, 1994; Bellar et al., 2013; Özel et al., 2015; Stanganello et al., 2015).

Long Filopodial Collaterals.

Long collaterals ($> 5.85 \mu\text{m}$) extend to lengths sufficient to interact directly with a plethora of local principal neurons within a territory several times wider than the CH terminal itself (1 of 4 principal cells innervated at the soma by outgrowths from a single CH shown in Fig 2A). Fast LLS imaging revealed these long filopodia extend and retract as fast as short filopodial ($\sim 40 \mu\text{m}/\text{min}$ peak instantaneous velocity, $1.236 \mu\text{m}/\text{min}$ average velocity during imaging session at P9). However, the dense branching observed along the length of long collaterals also underwent dramatic turnover and complex motility that significantly increase the volume patrolled by a single collateral projection. This sequence was often characterized by the rapid formation of proximal branches that rapidly change length and position as though each is anchored at a motile branch point, capable of shifting position along the length of the primary collateral stalk. The emergence of interstitial branches allowed collaterals to contact many adjacent calls within the neuropil ($469.7 \mu\text{m}^3$ covered in approximately 10 minutes in the example illustrated in Fig. 6). Perhaps unsurprisingly, long collaterals were found to regularly come into direct contact with collaterals from other CHs due in part to arbor density and the extreme proximity among developing CHs. An example of direct interaction between collaterals depicted in Supplemental Fig 1 illustrates the transient duration of contact, generally lasting only a few seconds per instance, that inevitably led to a change in the trajectory of one or both collaterals participating in this interaction.

Long Growth Cone Collaterals.

Given the length of many growth cone tipped collaterals (GC collaterals), it was challenging to capture them and their CH of origin utilizing the reduced field of view

necessitated for fast LLS image acquisition. However, for some CHs, we were able to image long collaterals tipped with growth cones for at least a subset of the imaging session (10 and 22 μm long GC collaterals, outlined in green and purple respectively; Fig. 7A-A'). The growth cone presented in purple (Fig. 7A') exited the volume within one minute of the experiment; the growth cone in green exhibited no net change in collateral length although the shape of the terminal lamellipodium underwent significant reorganization (not shown). The growth cone dynamics reported here were inferred to originate from CHs situated outside of our restricted field of view in the LLS experiment. In support of this inference, further investigation revealed the PV-Cre driven tdTom expression in pre-hearing animals used in the present study was restricted to the axons of the ventral acoustic stria passing in and through the MNTB in the auditory brainstem. Thick tissue (300 μm) surveys using 2P microscopy yielded no apparent growth cones originating from non-calyceal axons within the bounds of the MNTB that were constitutively labeled with tdTom further reinforcing the specificity of our genetic targeting of bushy cell afferents within the ventral brainstem (Supplemental Fig. 1).

GC collaterals from CHs outside of the restricted ROI of the LLS volumes were more easily monitored without direct observation of their source (Fig. 7B). The motility of GC collaterals appeared stochastic with punctuated periods of extension, retraction and pausing while these dynamics were directly paralleled by highly variable fluctuations in the reflected rate of motility (Fig. 7 C-D). Local GC displacements of up to 20 μm were measured by monitoring the advancement of the lamellipodium over time. The average rates of motility peaked in correlation with the expansion of the CH itself between P2-5 (Fig. 7D; Holcomb et al., 2013; Rodríguez-Contreras et al., 2008). Interestingly, the fastest rates of extension ($>3 \mu\text{m}$) measured revealed instantaneous growth can be 20 times higher than the corresponding average growth

rate. In fact, the growth cones monitored in the current experiments exhibited unprecedented instantaneous motility as high as 20 $\mu\text{m}/\text{min}$ (Fig. 7D-E, F).

Similar to the long filopodial-tipped collateral interactions, growth cones were found to transiently contact other neurites and CHs. Generally, the linear growth of the GC is significantly altered following contact, wherein the GC undergoes dramatic lamellipodial collapse, collateral retraction and subsequent growth in along an altered trajectory (Supplemental Figure 2).

Discussion:

Neural circuit assembly involves the complex interplay of axonal path-finding, arborization and synaptogenesis to establish appropriate mature connectivity (Cohen-Cory and Lom, 2004; Garner et al., 2006). High resolution microscopic techniques in recent years have been developed that are ideally suited to investigate fine changes in circuit structure within developing tissues. We first applied SBEM to the MNTB to more fully characterize the nature of the postnatal establishment and transformation of the CH. As previously reported, protocalyces produce a complex assembly of collateral arbors that intriguingly persist only during development and ultimately consolidate by maturity to leave a highly fenestrated and often unbranched terminal (Kandler and Friauf, 1993; Rodríguez-Contreras et al., 2008; Holcomb et al., 2013). The collateral field present on protocalyces shown in the present study represent a collection of structures including simple, short collaterals as well as longer, more complex terminal collaterals with either complex filopodia or growth cone-tipped endings that may or may not be exclusively segregated. SBEM has also revealed a greater number of collaterals than could be observed with light microscopy, in part due to finer resolution aiding in the identification of these thin and highly abundant collaterals, and further helps to explain the

discrepancy in previously underestimated frequency of collaterals of less than 5 μm (Rodríguez-Contreras et al., 2008). In agreement with previous work, we found that CH collaterals are present for at least several days following the establishment of mono-innervation, until just after the onset of sensitivity to airborne sound in rodents ($\sim\text{P10}$) (Kandler and Friauf, 1993; Holcomb et al., 2013). It is tempting to speculate the consolidation of the CH through retraction of the collateral field may be related to the sculpting process that leads to the stereotypically fenestrated morphology of the mature CH during this period of development. The loss of CH collaterals likely marks the end of the critical period defined within this system (Nakamura and Cramer, 2013) especially when considering the reduction in secondary signaling capacity these collaterals would otherwise provide (Rodríguez-Contreras et al., 2008). We have found that CH collaterals form abundant associations with secondary principal neurons within a local volume around the CH, as well as with other collaterals from nearby CHs and/or glial cells (Supp Mov 1). Notably, the conspicuous lack of mitochondria along many of the sites of collateral apposition may be indicative of developmentally transient sites of communication via immature synapses, with most contacts being maintained for only short time periods (Lee and Peng, 2008). However, SBEM reveals a static view into the process of neural circuit formation and inherently prevents the possibility of monitoring the real-time dynamics requisite to the formation or maintenance of complex arborization within a self-assembling neural network.

Dynamic Tissue Reorganization.

Recent advances in imaging capabilities provided a unique opportunity to monitor tissue dynamics at high spatial and temporal resolution in living tissues. Acute brainstem slices here were applied to the LLS microscope successfully for the first time to reveal high rates of

structural reorganization and motility in living mammalian neural tissue. We found a subpopulation of simple calyceal collaterals, short filopodia, that do not extend great distances from the edge of the CH. Short filopodia were previously postulated to have limited lifespans on the order of an hour (Rodríguez-Contreras et al., 2008) but we observed similar extension and retraction dynamics could limit the existence of some collaterals to less than 5 minutes. Moreover, these often short-lived collaterals could achieve extension and retraction rates of nearly 20 $\mu\text{m}/\text{min}$. Notably, these dynamics were asynchronous among the collaterals from each CH and permitted each terminal to maintain a collateral field size of several to tens of microns, irrespective of the absolute size of the terminal.

Long calyceal collaterals were considerably more complex in both morphology and behavior. These collaterals were more exploratory in nature during live cell imaging and extended significantly further from the CHs in which they emerged in both fixed and live preparations. Previous work monitoring calyces *in vivo* reported peak collateral extension of approximately 20 $\mu\text{m}/\text{hr}$ with 2P imaging (Rodríguez-Contreras et al., 2008). Our fast LLS microscopy revealed GC collaterals could sustain an average motility of 1-2 $\mu\text{m}/\text{min}$ for tens of minutes at a time while expanding the bounds of the terminal territory. Often the long collateral dynamics remained highly variable throughout the longest imaging sessions (up to 90 minutes). Long filopodia had a less directed growth trajectory relative to the more linear motility of growth cone-tipped arbors. Complex branching of long filopodia further differentiated these structures as these collaterals rapidly extended branches that dramatically swept through tissue to cover relatively large volumes of the neuropil. This “patrolling” behavior creates a larger zone that defines the extent of the terminal territory and may be important for spatial organization as these structures have been shown to be sites of transient calcium signaling (Rodríguez-Contreras et al.,

2008). Moreover, for the first time we have observed interactions between collaterals from different CHs that may demonstrate a contact-mediated repulsive signaling behavior that prevented the advance of collaterals from encroaching CHs and may further act to organize the innervation territory for proper frequency tuning during neonatal growth and competition among developing protocalyces. Growth cone-tipped collaterals were found to exhibit a net extension in nearly all recorded observation sessions, which suggests these collaterals are responsible for actively exploring and seeking new innervation territory within the MNTB. It is possible this type of pathfinding and synapse forming behavior in collateral growth cones aids directly in the stereotyped establishment of proper frequency tuning through selective synapse formation with postsynaptic partners during the postnatal period of CH growth. Specifically, the initial surge in terminal growth between 2 and 3 days after birth coincides precisely with the arrival of spontaneous auditory activity derived from purinergic signaling in the neonatal cochlea (Tritsch et al., 2007, 2010). Thus, the onset of bursting activity could serve as a permissive developmental signaling regime for the expansion of the protocalyx and its associated collateral network in rodents via a Hebbian-like mechanism that promotes the growth of the CH along the soma of a MNTB principal neuron.

Peak Dynamics of Calyx Growth.

Importantly, exploratory extension and retraction of collaterals is generally thought to represent a transient phenomenon aiding in the precise establishment of mature connectivity (Ruthazer et al., 2010). Our previous work using SBEM revealed that peak expansion of the protocalyx occurs between P3 and P4 in mice (Holcomb et al., 2013). Similarly, in rats it has been shown that CH collaterals are most abundant and reach the greatest extent between P3 and

4 in agreement with the present study in mice (Kandler and Friauf, 1993; Rodríguez-Contreras et al., 2008). The high temporal and spatial resolution of the LLS was effective in revealing that high level of motility over short periods of time among calyceal collaterals, further enriching our understanding of these presynaptic processes. Multiple studies show that precise presynaptic innervation of appropriate targets is achieved through a complex interaction between temporally regulated chemoattraction (Mortimer et al., 2008; Poon et al., 2013) synaptic activity (Tashiro et al., 2003; Chen et al., 2013; Massobrio et al., 2013), structural organization of the stratum or microenvironment (Rajnicek et al., 1997; Weigel et al., 2012), and intrinsic transcriptional programs (Koundakjian et al., 2007; Lallemand et al., 2012) that all serve to regulate the extent and nature of neuronal connectivity. Many, if not all of these aforementioned mechanisms may have differential effects over time but likely serve to initiate the bulk of cellular reorganization necessary to sculpt the auditory system ahead of the sensitivity to external sound.

Critical Period for CH:MNTB Formation.

Several factors likely culminate in the postnatal MNTB to provide a permissive environment for large terminal growth. However, as this critical period concludes and many neural circuits achieve their mature connectivity, extracellular matrix (ECM) proteins begin occupying the spaces between cells. Some of these ECM proteins, such as sulfate proteoglycans, inhibit growth cone advance and filopodial number (Bellar et al., 2013) although others such as laminins promote the growth of axons expressing appropriate subtypes of integrin receptors (McKerracher et al., 1996). Establishment of the ECM may in part act to limit the capacity for CH collateral maintenance and end the period of dynamic growth and reorganization by limiting the amount of free extracellular space available within the microenvironment (Tashiro et al.,

2003). Just after P3 within the MNTB, ECM molecules including aggrecan, brevican and hapln1 slowly begin the protracted process to coalesce in the spaces between cells and reduce available area for presynaptic motility (Kolson et al., 2016). In addition to reduced motility through ECM formation, the microglia population in the MNTB also increases by the end of the first postnatal week (Cramer and Rubel, 2016). These infiltrating macrophages have been shown to inhibit neurite outgrowth and stimulate collateral retraction in other parts of the nervous system (Busch et al., 2009) and may further help to explain the reduction in collateral motility, length and number by the end of CH development.

Conclusion.

Recent advances in gentle and fast volumetric imaging allow for the observation of rapid tissue dynamics in thick mammalian tissue explants. We have found CH collateral arborization can be structurally reorganized over an unprecedented timescale of seconds to minutes and their dynamic interaction raises interesting signaling questions for future research. Additionally, acute brainstem slices are inherently difficult to maintain in culture limiting acute imaging sessions here to less than three hours per experiment to allow even neurites severed from their cell bodies to continue to grow before noticeable degeneration of the tissue (Harris et al., 1987). Recent advances in culturing techniques such as media supplementation with a targeted set growth factors (Kronander et al., 2017) paired with chronic LLS microscopy may reveal detailed insight into the initial formation of the protocalyx over longer periods in future studies. In conclusion, we have presented an *in vitro* model highlighting the complex cellular reorganization undergone by a developing neural circuit at high spatial and temporal resolution.

Materials and Methods:

Ethical Approval. The Institutional Animal Care and Use Committees of both West Virginia University and Janelia Research Campus, Howard Hughes Medical Institute, approved all procedures involving animals in accordance with the United State Public Health Service and the United States National Institutes of Health guidelines.

Animals. Mice were maintained in two facilities, West Virginia University and Janelia Research Campus, on a 12-hour light/dark cycle. Homozygous reporter mice (*rosa-tdTomato* or *Ai9*; Jackson Laboratory) were crossed with homozygous parvalbumin Cre-recombinase mice (*PV-Cre*; Jackson Laboratory) to produce offspring that we refer to as PV-tdTom. A separate colony of FVB (FVB/NJ; The Jackson Laboratory) mice were maintained at the vivarium of West Virginia University. Following overnight pairing for timed-mating, pregnant mice were monitored twice daily to ensure ages of pups following birth are accurate to within 12 hours.

Animal Surgery. P0 PV-tdTom mice were anesthetized by hypothermia for six minutes. Afterwards, a drop of Marcaine (0.5%) was placed on the scalp and animals were placed onto a clay-filled aluminum block that had been kept on ice, with the skull oriented into approximately the horizontal plane. A small incision was made in the scalp with a 26G needle to expose the skull. Coordinates for lambda and bregma were determined using an injection needle lowered near the skull and held in a Kopf stereotaxic frame (model 940 digital; David Kopf Instruments, Tujunga, CA). The following injection coordinates were used (in mm) relative to bregma in

order to target the (MNTB): medial-lateral, 0.3; anterior-posterior, 5.2; dorsal-ventral, 4.2. The injection needle was filled with a solution containing a recombinant adenovirus (rAd) optimized for fast, high-level expression of GFP driven with the pUNISHER cassette (Montesinos et al., 2011). A final volume of 0.5 μL (0.33 μL of rAd PUN EGFP at 2.39×10^8 viral particles/ μL stock mixed with 0.17 μL of 20% mannitol in storage buffer containing 10mM HEPES, 250mM sucrose, and 1mM MgCl_2 ; pH 7.4) was injected using a calibrated injection capillary (Blaubrand; intraMARK, Wertheim, Germany) at a rate of 0.1 $\mu\text{L}/\text{min}$. Once the solution was injected, the glass needle was kept in place for an additional 4 min to allow the dissipation of backpressure before slow removal. Animals were then placed under a heat lamp ($\sim 35^\circ\text{C}$) to recover. Animals were returned to their mothers and monitored to ensure pups began to nurse. Pups were sacrificed for imaging at ages P2-12.

Live-Slice Preparation and LLS Imaging. Following rapid decapitation, brains were dissected in ice-cold oxygenated low-calcium (0.1 mM) ACSF. Coronal brainstem slices that contained both the ventral VCN and MNTB (250-600 μm -thick) were prepared using a Compresstome VF-200 tissue slicer (Precisionary Instruments, Greenville, NC) and immediately transferred to an incubation beaker containing standard ACSF (at 21°C , continuously bubbled with 95% O_2 –5% CO_2) containing the following (in mM): 125.0 NaCl, 2.5 KCl, 1.0 MgCl_2 , 2.0 CaCl_2 , 10.0 glucose, 25.0 NaHCO_3 , 1.25 NaH_2PO_4 , 0.4 L-ascorbic acid, 3.0 myo-inositol, and 2.0 Na-pyruvate, pH 7.3–7.4 (310 mOsm). After 30 min of incubation, slices were mounted in 1% low melting point agarose onto a modified zebrafish holder (Simon Watkins, University of Pittsburgh) to allow gas exchange from above and below the slice. The holder was immediately

transferred to an imaging chamber and actively perfused with oxygenated ACSF (at 37°C) supplemented with additional potassium (25 mM).

Images were acquired using a lattice light sheet (LLS) microscope in the Advanced Imaging Core at Janelia Research Campus (<https://www.janelia.org/open-science/advanced-imaging-center/instruments/lattice-light-sheet-microscope>), at low power excitation (150 mW at 560 nm; 90 mW at 488 nm). Coherently interfering, dithered Bessel beams were presented through a custom 0.65 NA, 3.74 mm working distance water dipping lens (Special Optics, Denville NJ) and collected with a CFI Apo LWD 63x water dipping detection objective (1.1 NA, 3 mm working distance; Nikon Metrology, Brighton MI). Image volumes (42 μm , 83 μm , $\leq 37 \mu\text{m}$) were collected at high speed (0.1-1.4 Hz) with fine spatial resolution (104 nm X 104 nm X 369 nm) for durations of 30 minutes to three hours.

Image Processing. Image volumes appear shear-transformed due to the movement of the sample along the s-axis during acquisition. These data were first de-skewed to reorient image planes to conventional x, y and z coordinates. Data were next deconvolved with a Richardson-Lucy algorithm using an experimentally obtained point spread function at each wavelength with a custom Matlab script (Chen et al., 2014). Deconvolved data were cached into syGlass (Morehead et al., in preparation) for rapid presentation and analysis of 4D images. Trajectory mapping was accomplished by manual placement of reference points at the leading tip of target structures. Resultant 3D points were analyzed with Excel and Matlab scripts.

Confocal Imaging. PV-tdTom mice were anesthetized with an IP injection of Tribromoethanol (Avertin, 250 mg/Kg). Animals were perfused with normal Ringer's solution

(room temperature) followed by PBS containing 4% PFA (Fisher Scientific, City, State). The brain was removed from the skull and post-fixed overnight in 4% PFA (4°C). Brains were next transferred to cryoprotectant (30% sucrose in 0.075 M sodium phosphate buffer, pH 7.4; 4°C) for a second overnight incubation. Coronal sections of the brainstem were sliced to a thickness of 40 µm using a freezing microtome (model HM450, Microm, Waltham, MA). Free floating sections containing the MNTB were imaged in a Fluorodish (WPI, Sarasota, FL) on a confocal microscope (model LSM-710, Zeiss, Thornbridge, NY) with either a 10X or 40X objective in order to assess the accuracy and extent of viral transfection within the developing MNTB.

Serial Block-Face Scanning Electron Microscopy. FVB mice were perfused and processed for serial block-face scanning electron microscopy as described previously (SBEM; Holcomb et al, 2013). Briefly, animals were perfused with normal Ringer's solution followed by 0.15 M cacodylate buffer containing 2.5% glutaraldehyde (Polysciences) and 2% formaldehyde (Fisher Scientific) with 2mM calcium chloride. Brains were subsequently drop-fixed in the same solution for an additional 18 hours before sectioning and embedding. Resin embedded samples were shipped to the National Center for Microscopy and Imaging Research (University of California, San Diego) for image acquisition.

MNTB principal cells completely contained within image volumes along with their associated presynaptic innervation were manually segmented using IMOD (University of Utah) and digitally meshed to produce ultrastructural representations of the circuit. Furthermore, these models serve as a histological reference of the fine structure of the CH collateral field which were difficult to entirely recapitulate using lattice light-sheet microscopy. 3D reconstructions of presynaptic terminals using SBEM were subsequently skeletonized to produce splines

representing each arbor within the CH collateral network differentially representing fine branching structure of each collateral. The number of collateral arbors were compared to the size of each CH, defined as its apposed surface area or the segmented plane representing the area of direct contact between pre and postsynaptic membranes.

Statistical Analysis. A t-test was employed to assess the Pearson correlation coefficient between CH size and collateral number. Values reported in the text are the mean $\pm$ SD.

**Chapter 3: Two Types of Somatic Spines Provide Sites for Intercellular Signaling
During Calyx of Held Growth and Maturation**

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Abstract:

Dendritic spines have been described in developing and mature systems, but similar extensions from cell bodies are less studied. We utilized electron microscopy image volumes, uniquely collected across a range of early postnatal and month-old mice, to characterize and describe two types of somatic processes that extended into and under the developing calyx of Held (CH), which we named type 1 and type 2 spines. Type 1 spines occurred singly, were mostly vermiform in shape, and formed regularly spaced indentations into the CH. Type 1 spines appeared in concert with the earliest expansion of the CH by P3, peaked at P6 and returned to low density at P30. Type 2 spines were intertwined into a secondary structure called a spine mat, which has not previously been described in the CNS, and were more complex geometrically. Type 2 spines formed after the CH crossed a size threshold, reached maximum density at P9, and were absent from most CHs at P30. Both spine types, but a higher density of type 1 spines, were sites for synapse formation. Spine mats brought pre- and postsynaptic neurons and glial cells into contact, and were captured in stages of partial detachment and engulfment by the presynaptic terminal, suggesting trans-endocytosis as a mode of removal ahead of maturity. In conglomerate, these observations reveal somatic spines to be sites for chemical neurotransmission and chemical sampling among synaptic partners and glia as tissue structure transforms into mature neural circuits.

Introduction:

Neurons communicate via synaptic and non-synaptic mechanisms, often via specialized extensions of pre- or postsynaptic processes into or underlying the adjoining post- or presynaptic partner, respectively. A well-studied and prevalent structure is the dendritic spine which commonly protrudes from dendrites, displays variation in size and shape, and receives predominantly excitatory chemical synaptic input onto the spine head (Nimchinsky et al., 2002; Bourne and Harris, 2008; Gipson and Olive, 2017). In some cases, spines are surrounded by and therefore embedded into presynaptic terminals (Westrum and Blackstad, 1962; Petralia et al., 2015, 2018). These processes exhibit a range of morphologies, occur in many neural systems across invertebrate and vertebrate species, and are generally termed invaginating projections (for comprehensive reviews, see Petralia et al., 2015, 2018). An example of this arrangement with a complex geometry is the thorny excrescence on the dendrites of hippocampal pyramidal cells, which are enveloped by mossy fiber terminals of dentate gyrus neurons (Gonzales et al., 2001; Popov et al., 2011).

Similar to dendritic spines, invaginating projections can extend into presynaptic terminals from the somatic membrane and be sites for innervation. These structures have been found in the auditory brainstem and vestibular nuclei, where they have been termed somatic appendages. In the cochlear nucleus somatic appendages can embed into the large presynaptic auditory nerve terminals, called endbulbs, that form onto many cells (Cant and Morest, 1979; Tolbert and Morest, 1982; Spirou et al., 2005) and in the medial nucleus of the trapezoid body (MNTB) they embed into the large presynaptic terminal called the calyx of Held (CH) (Morest, 1968a; Sätzler et al., 2002; Hoffpauir et al., 2006; Holcomb et al., 2013).

In addition to single cellular processes, specialized clusters of intertwined processes have been documented extending from and overlying the postsynaptic somatic membrane underneath the large calyx of the chick ciliary ganglion, where each element is termed a spine and their collection is termed a somatic spine mat (Shoop et al., 1999, 2002; Coggan et al., 2005). Somatic spine mats were studied by high resolution electron tomography which permitted description of the complex, branched shape of individual spines. Spine mats form during embryonic development and persist in the mature nerve terminal. To date, this intriguing organization of somatic processes has not been identified at synaptic junctions in the CNS nor in mammals.

In this study we focus on extensions from the postsynaptic somatic membrane that embed into or underlie the presynaptic CH which, like the calyx in the chick ciliary ganglion, is a large axosomatic terminal that is a useful model system for studying neural circuit formation. This terminal grows rapidly in rodents after postnatal day 2 (P2) as innervation proceeds from multiple, converging axons with small inputs to a single large input onto about 75% of postsynaptic neurons by P6 (Kandler and Friauf, 1993; Hoffpauir et al., 2006, 2010; Holcomb et al., 2013). Both morphological and electrophysiological maturation of the calyx and MNTB principal cell occur in concert over this time period (Hoffpauir et al., 2010; Rusu and Borst, 2011; Holcomb et al., 2013; Sierksma et al., 2017).

In previous studies we noted, qualitatively, the presence of multiple extensions from the MNTB principal cell somatic membrane into the developing CH, and more complex arrangements of somatic processes (Hoffpauir et al., 2006; Holcomb et al., 2013). Using our unique

developmental series of volume electron microscopy images, we were able to reconstruct these processes in 3D. We describe single spines and their high prevalence of innervation. We also provide the first description of spine mats in the CNS, reveal that they are a common but transient feature of the developing CH:MNTB connection, that they appear to be removed by trans-endocytosis, and that they are also innervated. In conglomerate, single spines and spine mats provide multiple routes for intercellular communication. We suggest that as volume EM techniques become more routine, these small yet often complex structures will be more systematically studied and may be revealed as key features of other developing and mature neural systems.

Materials and Methods:

Ethical approval. All procedures involving animals were approved by the West Virginia University (WVU) Institutional Animal Care and Use Committee and were in accordance with policies of the United States Public Health Service.

Serial Block-face Scanning Electron Microscopy (SBEM). FVB mice of either sex (FVB/NJ; The Jackson Laboratory; or FVB/NCr; National Cancer Institute) were perfused and processed for serial block-face scanning electron microscopy (SBEM) as described previously (Holcomb et al., 2013) at P2, 3, 4, 6, 9 and 30. Briefly, each animal was perfused with normal Ringer's solution followed by 0.15 M cacodylate buffer containing 2.5% glutaraldehyde (Polysciences) and 2% formaldehyde (Fisher Scientific) with 2mM calcium chloride. Following dissection, brains were drop-fixed in the same fixative solution for an additional 18 hours before sectioning for *en bloc* staining (Deerinck et al., 2010) and embedding. Resin-embedded samples were sent to the National Center for Microscopy and Imaging Research (NCMIR, University of California, San Diego) for SBEM image acquisition (3View, Gatan, using FEI or a Zeiss Merlin scanning electron microscopes). Image volumes were located in the medial MNTB to minimize tonotopic bias and were sectioned at a thickness of 60 or 40 nm (P9). Resulting SBEM volumes varied by sample in section thickness (P3: 55 nm; P4: 57 nm; P6: 53 nm; P9: 36 nm; P30: 40 nm) and pixel dimensions (P3: 12 nm; P4: 10 nm; P6: 4.9 nm; P9: 4.8 nm; P30: 4 nm) (previously reported in Holcomb et al., 2013) and each volume contained between 18 and 70 complete cell bodies.

Segmentation and Three-Dimensional Reconstruction. Principal cells and their associated inputs fully contained within SBEM image volumes were segmented using the IMOD 3D reconstruction software (Boulder Laboratory for 3-D Electron Microscopy of Cells, University of

Colorado, Boulder; <http://bio3d.colorado.edu/imod/>) and subsequently exported to and visualized in 3D using syGlass (IstoVisio, Inc.; www.syglass.io). A total of 44 cells and 60 presynaptic terminals were segmented across our timeframe of interest (P3, 4, 6, 9, and 30; Table 1). Somatic appendages that extended into the calyx of Held nerve terminal were classified as type 1 spines occurring singly or type 2 spines that comprise spine mats. These structures were segmented across serial sections and reconstructed in 3D using the meshing tool in IMOD. Subsequently, reference points (located each at the base of somatic attachment and along the tip of each branch) and associated spine segmentations were converted to a 3D file format using the imod2OBJ command. Exported OBJ files were used in subsequent quantitative analysis.

Morphological features of spines. OBJ files, each containing the meshed type 1 and type 2 spines, and for type 1 spines the base and tip coordinates, were imported into the Rhinoceros CAD software (McNeel, Seattle, WA) for morphological analysis. A custom Python script, using the *rhinoscriptsyntax* module with built-in commands within Rhino, was run on each spine to quantify the surface area and volume of each spine. For type 1 spines, error correction was then applied by filtering objects with volumes less than 5% of the mean type 1 spine volume for that terminal; these objects were excluded because they resulted from errors in segmentation or the IMOD meshing process. Additional error checking by inspection in 3D VR (syGlass, IstoVisio) ensured that each unbranched type 1 spine possessed exactly one base and one tip point. Within Rhino, twenty contours representing the relative profile of each type 1 spine were then created at equidistant points along the vector created using the base and tip points. The center of each of these contours was then used to create a center curve for the type 1 spine, which was the measure for the length of the type 1 spine. Center curves that deviated more than 200% in length from the

direct linear distance from base to tip point were inspected visually. If the deviation proved to be an error in the contouring process (1.5%), the linear distance from base to tip point was used as an approximate length instead. This methodology could not be applied to the highly branched type 2 spines, so we do not report total lengths for these spines. The area of near juxtaposition between the pre- and postsynaptic plasma membranes was measured as the apposed surface area (ASA), a metric used previously to assess the relative maturation of the terminal growing over the PC soma (Holcomb et al., 2013), and included the tight association of type 1 spines beneath CH terminals.

Complexity analysis. In order to quantitatively analyze the complexity of type 1 and 2 spines, as well as compare complexity between these two process types, we computed the convex hull volume for each reconstruction. Spine mesh objects were imported into RhinoCAD, the convex hull was created for each individual process using the ConvexHull Rhino plugin (MelonSoft) implemented with a custom Python code. Following this step, the volume was recorded for both the original mesh, as described above, and the convex hull. We then calculated the ratios of convex hull volume to the original spine volume and plotted these ratios against the original volumes to quantify complexity as a function of spine size.

Statistics. Unless otherwise stated, values are reported as mean $\pm$ standard deviation. Statistical significance across multiple groups was evaluated with a Kruskal-Wallis Test with Nemenyi post-hoc test or ANOVA with a Pairwise Mann Whitney test depending on whether all data being compared was normally distributed. All statistical outcomes of multiple groups were

Bonferroni corrected. Single pairwise comparisons were performed with the Mann-Whitney U-test.

Results:**The shortest somatic processes are located under the calyx of Held**

The transition of neurons in the medial nucleus of the trapezoid body (MNTB) from a smooth somatic surface at P2 to a rougher texture with multiple somatic processes of varying lengths occurs during growth of CHs and establishment of mono-innervation (postnatal days (P)3-9), as previously observed (Holcomb et al., 2013). Therefore, we initially characterized features of the somatic surface qualitatively during this time frame as well as a post-maturational time point (P30). By P4, 3D reconstructions reveal the longest somatic processes to be outside of the locations for innervation (white arrows in Fig. 1A, A'). These long processes extended in all directions and may be curved or branched. The somatic surface in contact with the overlying CH (indicated by the ASA in translucent blue, green and orange in Fig. 1A, A') also exhibits somatic processes (red arrowheads in Fig. 1A, A'), although these are notably shorter. At P6, when the CH is larger, the density and length of long processes outside of the ASA appears to be greater and many are located along the edge of the CH (white arrows in Fig. 1B, B'). Shorter somatic processes within the border of the ASA appear similar in length but occur at higher density than at P4 and P3 and with regularity in their spacing.

Somatic processes underlying the CH are classified into two groups

We noted that a subset of somatic processes within the ASA occurred singly, whereas others emerged nearby one-another and intertwined over the somatic surface (red arrows in Fig. 1B, B'). For reasons developed in this and later sections, we named them spines and grouped them into two populations. Single spines extend mostly orthogonal to the cell body surface and directly embed into the CH (Fig. 1C, arrowheads; Fig. 1C' single spine outlined in red). Clusters

of intertwined spines form a composite structure, which we term spine mats after the homologous description of the spine mat in the chick ciliary ganglion (Shoop et al., 2002), that either extends into the CH or forms a dome-shaped tangle within a larger recess beneath the CH (Fig. 1C, arrows; Fig. 1C'', spine mat dome outlined in red).

We next asked if single spines could be quantitatively differentiated from the individual intertwined spines of spine mats (Table 1). At each age, multiple cells were reconstructed and analyzed along with their largest or, for cells contacted by competing inputs, their two largest terminals (Table 1). Overall, a total of 44 cells across ages were completely analyzed and quantified (28 PCs with single CHs, 16 with competing pairs), yielding a total of 3373 individual spines. To reconstruct spine geometry in 3D, the bases were defined as the inflection in the curvature of the membrane away from the somatic surface (red dashed lines in Fig. 1C', C''), and structures were segmented across every serial EM section. Notably, nearly all single spines were unbranched (2062/2076 spines). Short single spines had relatively equal width and length (Fig. 2A-A'', B-B''; 3D reconstructions and two EM images shown for each single spine). Longer single spines either immediately turned to follow the somatic surface (Fig. 2C-C'') or extended deeper into the CH, typically perpendicular to the cell body surface, and nearly reached the non-synaptic face of the CH (Fig. 2D-D''). Only a very small (8/2076) subcategory had an expanded head (Fig. 2E-E'', F-F'') that resembled dendritic mushroom spines. Single spine diameters ranged from 200-500 nm and lengths ranged from 0.011-3.51 μm ($n=2076$; Fig. 2G). Mean length increased by nearly 20% from P3-P6, then decreased at P9 (both increase and decrease were statistically significant; Suppl. Fig. 1, Suppl. Table 1) and P30 due to the absence of longer

spines (P3: $0.758 \pm 0.40\mu\text{m}$, P4: $0.835 \pm 0.44\mu\text{m}$, P6: $0.887 \pm 0.46\mu\text{m}$, P9: $0.610 \pm 0.27\mu\text{m}$, P30: $0.621 \pm 0.36\mu\text{m}$; overall 0.752 ± 0.41).

Spine mat complexity is best understood in 3D reconstructions. One of the more complex spine mats, as defined by the number of component spines (range: 2-34 spines), illustrated a range of differing spine geometries (19 spines illustrated in Fig. 3). Most spines were branched (examples are #2, 12, 15 in Fig. 3A). Unbranched spines had multiple bends and/or irregular swellings (#1, 9, 17 in Fig. 3A) or occasionally appeared vermiform, like single spines (#4, 8, 11, 16, 18, 19 in Fig. 3A). Variability in interdigitated spine morphologies precluded a direct comparison of length with vermiform single spines. The CH and an astrocytic process (blue overlay and uncolored cellular elements with asterisk, respectively, in Fig. 3B, C) also can extend processes into the spine mat creating sites for triadic physical contact among pre- and postsynaptic neurons and glial cells.

We next compared single and intertwined spines by fitting their surface with a three-dimensional convex hull (akin to throwing a thin sheet over the structure, Fig. 4D-F), and computed a complexity score as the ratio of the volume of the convex hull to the volume of the spine. Curved and branched structures should have smaller volumes than their convex hull volumes (red or blue structures and translucent overlays, respectively in Fig. 4D-F). Nearly all spines (one exception) larger than $0.6 \mu\text{m}^3$ were elements of spine mats (blue symbols in Fig. 4A-C). Spines selected at regular intervals across the total population ranked by size at each age (P3, 6, 9) illustrate that the largest somatic processes in the rank order are those that comprise spine mats (blue structures at the right of Fig. 4D-F). Where spine volumes overlapped, especially at P3 and P6, intertwined

spines were more complex than single spines (see insets illustrating single vs. intertwined spines $< 0.2 \mu\text{m}^2$: P3: 1.27 ± 0.2 vs. 1.69 ± 0.2 , P6: 1.06 ± 0.02 vs. 1.95 ± 0.6 , P9: 1.19 ± 0.2 vs. 1.49 ± 0.3 ; $p < 0.0001$ for all pairwise comparisons Mann-Whitney U-test (M-W); Fig. 4A'-C'). Comparisons across all spines at each age also differed significantly (Fig. 4). The small number of single spines with a higher complexity score than intertwined spines of similar volume represented vermiform spines that had a substantial bend along their length, thus increasing the convex hull volume. Both single and intertwined spines decreased in complexity with age (single spines: P3 vs P9, $p < 0.05$; P6 vs P9, $p = 0.05$; intertwined spines: P3 vs P9, $p < 0.001$; P6 vs P9, $p < 0.001$; K-W). Based on these quantitative differences between the two spine types we henceforth designated single somatic spines as type 1 spines and intertwined spines as type 2 spines.

Prevalence of type 1 somatic spines and spine mats is developmentally regulated

Previous studies have shown many of the morphological stages of CH formation occur largely during the first week of life and ahead of the onset of hearing in mice (Kandler and Friauf, 1993; Taschenberger et al., 2002; Hoffpauir et al., 2006; Holcomb et al., 2013). However, auditory neurons continue to undergo changes to intrinsic and synaptic properties until the system nears maturity as early as P30 in both brainstem nuclei (Müller et al., 2019) as well as within the auditory cortex (Carrasco et al., 2013). We next examined the prevalence of type 1 spines and spine mats across principal neurons of the MNTB at early postnatal ages (P3, 4, 6, 9) in relation to young adult mice (P30). 3D reconstruction shows that at P3, during initial expansion of the CH, indentations from type 1 spines are rare but occur both in the center and at the edges of the CH (orange in Fig. 5A). At P6 the density of indentations increased (Fig. 5B), when type 1 spines occur more evenly across the CH. Spine mats (pink, Fig. 5B; arrow indicates spine mat

expanded in Fig. 3) occurred in both central and peripheral regions of the CH but were not as evenly distributed. At P30, fewer type 1 spines were observed, and spine mats were a rare occurrence. Note that the P30 CH is fenestrated into stalks and swellings, and most swellings do not contain type 1 spines (Fig. 5C).

For a given age, type 1 spine number increased linearly with CH size, as defined by ASA (Fig. 6A). The slope of each linear fit increased between P3-6, remained similar at P9 and P30, but the intercept at P30 was less, indicating a reduction in type 1 spine number. Normalizing these counts as type 1 spine density revealed a significant age dependence between time points (Fig. 6B; $p < 0.0001$; K-W). These data confirm that type 1 spines are a feature of initial CH growth (only 1/12 CH had no type 1 spines), their density increased significantly during the period of postnatal terminal expansion (P3 vs P6; $p < 0.0001$; K-W), and density decreased by P30 to levels similar to P3 (Fig. 6B, yellow; P9 vs P30: $p < 0.01$, P3 vs P30: $p = 0.987$; K-W).

Spine mats occurred in lower numbers than type 1 spines across all sampled ages (note difference in ordinate scale in Fig. 6A, C), and were rarely found on CHs below a threshold size (2 of 60 CHs $< \sim 100 \mu\text{m}^2$ in ASA). As with type 1 spines, the number of spine mats was linearly related to CH size (Fig. 6C). The slope of this relationship was similar from P3-9, but the intercept was much higher at P9, indicating a significantly greater density of spine mats (Fig. 6D; P9: $p < 0.001$; Kruskal-Wallis Test) that peaked within this age range later than type 1 spine density. At P30, only one spine mat was detected across five terminals. Spine mats were comprised of variable numbers of type 2 spines (median = 6, mean = 6.8 ± 5.0). Cumulative distribution functions for each age revealed a transition to larger numbers of type 2 spines per

spine mat between P4-6 (shift in 50% value of cumulative distribution; Fig. 6F), and a likely reduction at P30 (4 type 2 spines comprise the single spine mat; Fig 6F).

Type 1 spine and spine mat prevalence do not differentiate competing inputs

In this and the remaining sections we consider structural features of type 1 spines and spine mats that provide clues to their functional significance. Given the synchronized increase in type 1 spine density during the growth of the CH onto PCs and the appearance of spine mats only after CHs have crossed a size threshold, we hypothesized that they may influence competition between terminals. We defined competing inputs using criteria of Holcomb et. al. (2013): 1) at least two distinct inputs with $ASA > 25\mu m^2$ and 2) relative size ratio of inputs < 5 . Sixteen pairs of competing CHs were identified between P3 and P6 (16/34 PCs). Both inputs in a competing pair were often associated with type 1 spines and spine mats (Fig. 7A). Although the number of type 1 spines was greater in larger terminals (predominantly negative slopes in Fig. 7B), the difference in spine density between terminals was significant only at P4 (Fig. 7C; $p < 0.05$; Pairwise MW). We then used the reconstructed terminal ASAs to calculate the percentage of ASA on type 1 spines available for innervation. This quantity was calculated both as an aggregate (Table 1, “% of Terminal ASA on Type 1 Spines”) and on a pairwise competitive basis (Table 2) and revealed no statistically significant difference in percentage of ASA on type 1 spines for either competing category (Table 1). When competing terminals were compared pairwise, only the paired terminals at P4 approached significance ($p=0.050$, K-W) with the largest terminal at this age consistently having a higher percentage of ASA on type 1 spines than its competitor (Table 2). Due to the restriction of spine mats to terminals $> 100\mu m^2$, they were

only found at P4 and P6 and did not differ among competing inputs (Fig 7D-E). Thus, neither type 1 spine nor spine mat prevalence consistently differentiated competing inputs.

Types 1 and 2 spines provide area for membrane apposition and are sites for innervation

Regions of membrane apposition are potential sites for non-synaptic communication. We inspected type 1 spines and spine mats and determined they were in contact with the presynaptic membrane along their entire surface. Instances of glial incursions occurred only when spines were located near the edge of the CH (Fig. 2B', B'', C', C'', Fig. 3).

We next considered that type 1 spines and spine mats could affect synaptic strength if these structures are consistently innervated. Individual examples of similar appendages being innervated have been presented previously (Sätzler et al., 2002; Hoffpauir et al., 2006; Holcomb et al., 2013). The prevalence of innervation was evaluated for a single terminal at P9 (29 spines, 32 synaptic contacts) in rat (Sätzler et al., 2002). We tabulated the fraction of type 1 and 2 spines from our large population across age, using accepted criteria for synapse identification in SBEM images (Sätzler et al., 2002; Holcomb et al., 2013; Xiao et al., 2013; Sanculi et al., 2020): 1) parallel orientation of the pre- and postsynaptic membranes, 2) presence of synaptic vesicles near the pre-synaptic membrane (< 200 nm) in more than one serial section, 3) contact of at least one vesicle with the pre-synaptic membrane, and added the 4) conservative requirement that these features are present in at least 3 serial sections (2 serial sections shown in Fig. 8A, A'). Since *en bloc* staining for volume EM can reveal postsynaptic densities (PSD), but not reliably, we did not use this feature as one of our criteria. An excitatory synapse onto the more flattened somatic region of the postsynaptic surface is shown for reference (arrow in Fig. 8E).

Similar to the analysis performed for competing inputs, the percentage of ASA on type 1 spines available for innervation on single dominant inputs was quantified (Table 1, “% of Terminal ASA on Type 1 Spines”). This percentage increased significantly from P3-P6 (P3 vs. P6: $p < 0.05$, K-W), then declined between P6-P30 (P6 vs P30: $p < 0.01$, K-W) to levels similar to P3. These results were similar when competitive and dominant inputs were combined. Quantification of synapse prevalence revealed most type 1 spines are functionally innervated at P3 and P4 ($> 65\%$) and this value reached a maximum of 95% of spines innervated by P30 (Fig. 8F), even though type 1 spine density had declined significantly (Fig. 6B). Most type 1 spines were innervated at their base, but synapses could also form along their length or near their tip (arrowheads in Fig. 8A-C). Most spines were innervated by one or two synapses (Fig. 8G). Some non-innervated spines had very few synaptic vesicles in proximity to their presynaptic membrane (red outline in Fig. 8D, E). The type 1 spine cytoplasm was largely devoid of heavy metal-stained structures. This “exclusion zone” for cellular organelles, first described here, could extend to a boundary between 50-200 nm below the postsynaptic cell membrane, and laterally away from the spine (dotted lines in Fig. 8A-E).

Since type 2 spines were interwoven into spine mats underneath the CH, much of their surface was not in direct contact with the CH (Fig. 9). Survey of spine mats across each age revealed 50% (535/1070) of type 2 spines exhibited sites of synaptic contacts. Synapses could occur at the base of type 2 spines (synaptic contact area illustrated in blue; Fig. 9I) but were most frequently located along their length (red arrowheads Fig. 9C-F, H; nearby somatic synapses indicated with arrows, Fig. 9A-B, E-F, H). In a small percentage of spine mats (10%), the CH extended

intertwining processes into the core of the spine mat; however, these processes rarely form synapses (not shown). We counted the number of synapses per spine and averaged that number for individual spine mats (each spine mat is a data point in Fig. 9J). The density of synapses per type 2 spine per spine mat was maintained between P3 (mean = 0.76 ± 0.12) and P6 (mean = 0.80 ± 0.11 ; $p=0.999$ K-W) but significantly increased by P9 (Fig. 9J; mean: 1.27 ± 0.06 ; $p = 0.01$; K-W) when the incidence of spine mats was highest (Fig. 6D). Inspection of the interior of type 2 spines revealed a higher prevalence of labeled cytoplasmic structures than type 1 spines. These labeled organelles were often reticulated membrane that could appear contiguous with reticulated membrane in the main compartment of the cell body (red arrows in Fig. 9B-D, pink and green spines). Other structures found in type 2 spines were primarily bar-shaped and appeared round when transversely sectioned. Unlike the exclusion zone observed under type 1 spines, larger organelles such as mitochondria were found to extend to the edges of the somatic membrane located at the base of type 2 spines (Fig. 9 A, E and F) and in a few cases extended into the spine (not shown).

Trans-endocytosis of spine mats into the presynaptic terminal

Spine mats at the earliest ages (P3, 4 and 6) exhibited a consistent morphology of tightly interwoven components within a shallow dome beneath the CH. Moreover, nearly all components of the spine mat, be they glial or neuronal (pre- or postsynaptic) can be tracked to a larger process or a cell of origin within the volume. However, concurrent with the dramatic increase in spine mat density at P9 was the appearance of spine mats comprised of multiple elements that are not attached to a cell of origin (green structures in all panels of Fig. 10; all attached type 1 and type 2 spines depicted in orange and pink respectively). All spines were

reconstructed under CHs for five cells at P9 located near the center of the image volume. Three clusters of non-attached elements in the leftmost terminal in Figure 10A illustrate sequential stages of a spine mat removal mechanism. Spine mats could be comprised of intertwined type 2 spines (pink), CH (blue) and glial (unlabeled) processes, and a small number of elements that were not attached (green) to a cell (3 type 2 spines and 1 non-attached structure in Figure 9B). Other spine mats could be associated with a larger number of non-attached elements relative to few remaining attached elements (fletched arrows in Fig. 10A, Fig. 10C). In these cases, the attached type 2 spines appeared thinner than most other type 2 spines (compare Fig. 10C to Fig. 10B), and they interdigitated into aggregations of detached spines of various sizes that were shifted deeper into the CH (Fig. 10C-C''). A third group of structures consisted of a complex of fully detached components that were enclosed entirely within the CH (simple arrows in Fig. 10A, Fig. 10D). To ascertain the generality of this observation, we surveyed an additional 20 cells in this image volume and found partially and fully detached elements associated with CH innervation on 13/20 cells. We then selected spine mats that appeared to be in different stages of dissociation and quantified the percentage of spine mat volume in contiguous detached elements, which formed a continuum of values (expressed as percentage: 4, 8, 13, 19, 26, 33, 39, 43, 48, 53, 57, 70, 75, 91, 94). This sequence of image volumes indicates a process of trans-endocytosis of entire spine mats into the CH which may serve to regulate their numbers, among other functions. As the percentage of dissociated elements increased, these elements were located further away from the presynaptic membrane toward the interior of the CH.

Discussion:

Summary

Using manual segmentation of SBEM images, we present the first systematic description and differentiation of two types (called type 1 and type 2) of specialized spines that emanate from postsynaptic somata and embed into the pre-synaptic terminal of the CH. We make the first observation of spine mats in the CNS and reveal that the incidences of type 1 spines and spine mats follow similar yet distinguishable developmental trajectories. Several other observations support functional roles for intercellular communication. Type 1 spine membranes are contiguous with the presynaptic membrane along their entire surface, type 1 and 2 spines form synaptic connections, and type 2 spines are trans-endocytosed into the presynaptic terminal. Type 2 spines, in contrast to type 1, contain reticulated membrane and other labeled structures, and intertwine with glial and presynaptic processes, with some exposure to extracellular space. Combined, these findings suggest roles for type 1 spines and spine mats in intercellular communication relevant to CH formation, growth and maturation.

Application of volume electron microscopy to study of somatic processes

Post-synaptic processes that form invaginations in their synaptic partners were discovered using electron microscopy, as this technique affords the requisite resolution to observe structures with dimensions of tens to hundreds of nm in diameter (for review see Spacek and Harris, 2004; Popov et al., 2011; Petralia et al., 2015; Sanculi et al., 2020) with good tracking of cell membranes, staining of intracellular contents, and visualization of all cellular elements. The intertwined nature of spine mats poses additional challenges; the first description of this structure required the use of high resolution electron tomography to resolve individual structural

components (Shoop et al., 2002). SBEM, as well as other modern volume EM techniques, offers the opportunity to track the distribution of small processes across the entire somatic surface by collecting many hundreds to thousands of serial sections. Acquiring a large tissue volume involves the tradeoff between the size of the volume and resolution. These image volumes have pixel dimensions that ranged from 5 to 12 nm, and as such cytoskeletal elements, particularly microfilaments, could not be identified reliably so these were not quantified. *En bloc* staining for SBEM provides reliable membrane staining, supporting our report of few vesicles and other membrane-enclosed objects within type 1 spines and a membrane-free zone at their base, but is less reliable in labeling protein clusters such as post-synaptic densities. An advantage of block-face imaging over serial section reconstruction is good alignment across images, which is essential to achieve accurate measurements of surface area, length, and volume from small and, in the case of type 2 spines, tortuous structures, particularly over long distances.

At least three categories of somatic appendages on MNTB neurons

We previously noted in our volume EM series the extension of processes from the somatic surface, coincident with initial CH growth between P2-3, that were longer than the somatic spines described here and that transformed the smooth somatic surface into a more “fuzzy” appearance (Hoffpauir et al., 2006; Holcomb et al., 2013). The location of many longer processes along the edge of larger CHs (most evident in Fig. 1B-B’) may impose a barrier to further growth and regulate the size of the terminal. These longer somatic processes were noted in early Golgi studies of the developing MNTB (Morest, 1968a, 1968b), and will be treated in a separate report that also encompasses dendrite formation. Type 1 spines have been observed previously in ultrastructural studies of large somatic terminals onto cat bushy cells and perinatal mouse

MNTB, and were referred to as somatic appendages or spine-like protrusions (Cant and Morest, 1979; Tolbert and Morest, 1982; Sätzler et al., 2002; Hoffpauir et al., 2006). Spine mats were not previously observed, nor were the more complex geometries of their constituent type 2 spines. Despite the later appearance developmentally of spine mats relative to type 1 spines, tight adhesion with the associated CH membrane argues against lateral diffusion and aggregation of pre-existing type 1 spines into spine mats, and instead may be indicative of differential mechanisms leading to the extension of type 1 and 2 spines.

Similarity of type 1 and type 2 spines with other post-synaptic extensions

Spinules. The thinnest extensions of postsynaptic membrane in the mammalian brain are called spinules and they emanate from dendritic spine heads to penetrate pre-synaptic nerve terminals (Spacek and Harris, 2004). Their diameters range from ~10 to ~150 nm and their lengths from ~30 to ~750 nm. Spinules are often surrounded by coated pits on the interior of the membrane of the pre-synaptic terminal, contain few organelles, receive few synapses, and, like spine mats, can be trans-endocytosed into the presynaptic terminal (Spacek and Harris, 2004). Although spinules can follow a developmental profile of expression in cerebellar mossy terminals (Eckenhoff and Pysh, 1979; Tao-Cheng et al., 2009; Zancan et al., 2015), in their dimension and lack of innervation, they differ from type 1 and 2 spines.

Dendritic spines. Dendritic spines are short protrusions from the dendritic shaft and are sites for excitatory synaptic transmission at the spine head and, to a lesser extent, along the spine neck (Araya, 2014; Bartol et al., 2015). The spine neck can be thin (0.1 - 0.4 μm diameter,

hippocampus) relative to the spine head (1 μm for mushroom spines (Bourne and Harris, 2008)). Typical spine lengths are 1-3 μm (Bartol et al., 2015; Gipson and Olive, 2017), and in cortex averaged 1.8 μm (maximum length 3.8 μm) and 0.1 μm^3 in volume (Kasthuri et al., 2015). Somatic type 1 spines were shorter on average, but lengths and volumes fell within the ranges described for dendritic spines (Suppl. Fig. 1). The geometry of the spine neck may isolate synapses electrically from other sites on the dendrite (Noguchi et al., 2005; Araya et al., 2006) however, type 1 spines in the MNTB lack a clear neck or head, and their diameters (mean: $0.32 \pm 0.09 \mu\text{m}$) were somewhat larger than the dendritic spine neck. Therefore, the simple morphology of type 1 spines lacks the functional specialization to isolate currents from the soma, which is typically modelled to be nearly isopotential with the spike initiation zone. Type 1 spines were innervated mostly at their base, but also along their length and at their tip, and type 2 spines along their lateral membranes underneath the CH. Due to the general similarities in geometry and innervation between dendritic spines and type 1 somatic spines, and in contrast to the smaller caliber and lack of innervation in spinules, we favored naming these somatic processes as type 1 somatic spines.

Thorny excrescences and toric spines. A comparable structure to the somatic type 2 spines is the thorny excrescence that forms along dendrites of CA3 pyramidal neurons. These branched spines cluster on the proximal dendrite of CA3 pyramidal cells, embed into mossy fiber terminals of dentate gyrus granule cells without glial intervention (Chicurel and Harris, 1992; Popov et al., 2011), and, like type 2 spines, begin to form after expansion of the presynaptic terminal (Amaral and Dent, 1981) These structures resemble type 2 spines in that they are clustered and branched, but they are not intertwined. Moreover, each branch of the process is innervated, and their entire

surfaces are tightly apposed to the mossy fiber pre-synaptic membrane. Unlike type 2 spines, thorny excrescences are a feature of mature neurons. Recently, complex spines called toric spines, because some have looped components, have been described on the soma and basal dendrites of space-specific inferior colliculus neurons in the barn owl (Sanculi et al., 2020). Unlike type 2 spines, toric spines were unbranched, had larger volume, and were innervated by multiple different inputs via up to tens of synapses. The spine mats underlying the CH share many features with spine mats in the embryonic and adult avian ciliary ganglion, which have complex geometry, are intertwined, and can be contacted by synapses (Shoop et al., 2002). The constituent processes of these spine mats were named somatic spines by Shoop et al. (2002) and, in keeping with that precedent terminology, we also name these structures spine mats and somatic spines, but distinguish the individual components as a separate type (type 2) from somatic spines that occur singly (type 1).

Potential functions of type 1 spines and spine mats

Developmental profile. The increased density of somatic spines until P9, irrespective of the size of the CH nerve terminal (Fig. 6), and lack of correlation with the relative size of competing terminals (Fig. 7), suggests that emergence of spines and spine mats reflects maturation of the postsynaptic principal neuron. An initial phase of physiological maturation, in which, among other properties, membrane input resistance decreases and spike threshold increases (Hoffpauir et al., 2010; Rusu and Borst, 2011), is concurrent with increased type 1 spine density, which adds membrane surface area and directly contributes to these functional measures. We cannot know the outcome of competition from static images produced by SBEM (smaller CHs with higher spine density grow into a dominant CH), and the correlation of density with size at P4 is

intriguing, but the lack of a consistent relationship at P3 and P6 precludes drawing firm conclusions. As spine mat density peaks between P6 and P9, the principal cell is reorganizing its axonal field to a narrower range of frequency laminae in the LSO (Kim and Kandler, 2003; Clause et al., 2014). These elements of downstream principal cell maturation may be communicated, as another element of coordinated pre- and postsynaptic maturation (Hoffpauir et al., 2010), via type 2 spine interaction with the maturing calyx and extending to local astrocytes via their contribution of processes to the spine mat.

Intercellular communication. A notable feature of the growing CH is its adherence to the postsynaptic membrane over large areas of membrane without intervening extracellular space or glial incursion (Sätzler et al., 2002; Hoffpauir et al., 2006). These membrane apposition areas are potential sites for intercellular communication via adhesion proteins, clustered into adhering junctions as most clearly revealed by transmission EM (Sätzler et al., 2002; Hoffpauir et al., 2006) or distributed along the membrane surface. We did not observe presynaptic membrane invaginations without type 1 spines, so the two structural elements form cooperatively and may also impart mechanical stability to the growing CH in an environment of rapid structural dynamics.

Both type 1 and type 2 spines have roles in intercellular chemical communication since they are sites for innervation. The postsynaptic composition of AMPA receptor subunits shifts during development (P7-21) toward incorporation of more GluA3 and GluA4 subunits for faster, high fidelity transmission (Joshi et al., 2004; Koike-Tani et al., 2005). Moreover, application of a selective open channel blocker of calcium permeable AMPARs revealed immature principal

cells (P4-5), and "maturing" (P14) cells, receive a relatively higher Ca^{2+} influx than mature principal cell synapses (P30) (Joshi et al., 2004; Lujan et al., 2019). The decline in type 1 and type 2 spine density after P9 may contribute to a reduction in synaptic sites and an alteration in AMPAR composition although it is tempting to speculate calcium permeable AMPARs drive Ca^{2+} signaling locally at spines beneath developing CHs. This first description of organelle exclusion within type 1 spines and extending into the underlying somatic cytoplasm merits further study using high resolution electron tomography and other super-resolution approaches to ascertain the prevalence of cytoskeletal elements, such as actin, that may provide clues to the function and dynamics of this cellular region. Type 2 spines, in contrast to type 1 spines, contain organelle structures and can be tortuous and very thin at many locations along their length, so the substrates may exist for multiple modes of communication from electrically and chemically isolated compartments. These signaling modes may mediate synaptic plasticity and local processing within the spine mat that communicates among the intertwined neuronal and glial processes as glial and neuronal elements transform structurally into a mature circuit (Rodríguez-Contreras et al., 2008; Holcomb et al., 2013; Dinh et al., 2014; Saliu et al., 2014; Brandebura et al., 2018).

Spine mats are not a prevalent feature of P30 CHs. Spine mat number could be reduced by each cell withdrawing its processes, or the structural elements could be engulfed. SBEM images that capture (1) spine mats comprised of attached and detached structures, where the fraction of detached structures ranges from near zero to near 100 percent, (2) detached structures in partial states of engulfment including those appearing to be in the final stage of pinching off, and (3) fully detached intertwined processes enclosed within the CH, provide strong support for a

mechanism of their removal via trans-endocytosis. This evidence is like that in support of trans-endocytosis of spinules that extend from dendritic spines in hippocampus into their associated presynaptic terminals (Spacek and Harris, 2004). Trans-endocytosis may serve both to regulate the number of spine mats, but also to move signaling molecules from glial and postsynaptic cells into the presynaptic terminal. We noted also a layer around the engulfed structure that excludes other cytoplasmic organelles (Suppl. Fig. 2). This direct exchange of cellular material between cell types may ensure tightly coordinated maturation through the second postnatal week when the ear canal opens demarcating the onset of hearing (Ehret, 1976; Romand and Ehret, 1990; Kamiya et al., 2001; Sonntag et al., 2009), structural refinement of the CH via fenestration, and tuning of synaptic transmission for more precise and faster kinetics (Taschenberger and von Gersdorff, 2000; Ford et al., 2009; Takahashi, 2015).

Future work will require high-resolution light microscopy methods to visualize type 1 and 2 spines along with associated presynaptic and glial membranes to, ideally, track their dynamics *in vitro* or *in vivo*. High resolution electron tomography, conducted over sufficiently large volume, would also shed light on the intra and intercellular elements of spines and the associated localized regions of the postsynaptic cell body.

Chapter 4: Role of Spontaneous Activity on the Growth of the CH**Introduction:**

Many nuclei of the central auditory circuit initially begin to assemble during later embryonic and early postnatal development in rodents (Gurung and Fritzsche, 2004; Clause et al., 2014). For example, stimulation of the auditory nerve fiber can elicit the first central responses in cells of the ventral cochlear nucleus (VCN) at E15 (Marrs and Spirou, 2012). During the same period of time (E14-15), globular bushy cell (GBC) axons are actively navigating across the midline towards contralateral targets including the principal neurons of the medial nucleus of the trapezoid (MNTB) (Howell et al., 2007). GBC axons that form terminal arbors within the MNTB will have established the first functional contacts with principal cells between E17-18, just ahead of birth (Marrs and Spirou, 2012). Beginning between postnatal (P) days P2-3, a limited number of the GBC bouton-type terminals begin to expand such that each principal cell (PC) is contacted by at least a single protocalyx, and some may be contacted by 2 or 3 enlarged terminals (Hoffpauir et al., 2006; Holcomb et al., 2013). Ultimately, competition resolves such that each principal cell is contacted by one dominant input between P6-9 that will persist and mature into the calyx of Held (CH) (Morest, 1968a; Kandler and Friauf, 1993; Bergsman et al., 2004; Holcomb et al., 2013).

Throughout the period between initial GBC axon extension (Howell et al., 2007) and establishment of the CH (P6-9) (Bergsman et al., 2004; Holcomb et al., 2013), regulated axonal endo- and exocytosis is vital to the secretion of and response to both signaling and guidance molecules during axon outgrowth as well as neurotransmitter release for transsynaptic communication (Itoufusa and Kamiguchi, 2011; Tojima and Kamiguchi, 2015). Moreover,

supporting cells of the neonatal cochlea secrete ATP that triggers spontaneous activity in hair cells (Tritsch et al., 2007) that percolate as spontaneous bursts of activity across multiple nuclei throughout the auditory brainstem of pre-hearing adolescent rodents (Tritsch and Bergles, 2010; Tritsch et al., 2010). Although activity-dependent mechanisms have also been speculated to influence the selection of “winning” inputs (and removal of others) from sites of competition necessary for tonotopic refinement in the auditory system (Sanes and Takács, 1993; Friauf and Lohmann, 1999), the exact mechanism that leads to the establishment of a single CH is still largely unknown (Yu and Goodrich, 2014).

Traditional methods for eliminating activity in the developing auditory system generally involved lesioning one or both cochlea (Kitzes et al., 1995; Russell and Moore, 1995; Franklin et al., 2008). The limitation therein is the difficulty in separating the effect of cell necrosis following chronic deinnervation of cells and or trauma in the cochlear nucleus from the lack of presynaptic neurotransmission. Genetic manipulations of hair cells have targeted the mechanotransduction channel, Ca^{2+} channels, and the vesicular glutamate transporter, in order to reduce or eliminate synaptic transmission onto spiral ganglion neurons. In these animals, no effect on CH formation was noted (Yousoufian, 2005; Noh et al., 2010; Hirtz et al., 2011). The effectiveness of these manipulations on spiral ganglion cell spike activity was not directly assayed. In the case that spiral ganglion neurons are mostly or completely silenced by these manipulations, a possibility remains that GBCs homeostatically alter their spike thresholds to generate spontaneous activity.

In order to inhibit action potential (AP) evoked release of neurotransmitter specifically from developing protocalyceal terminals, we took advantage of a conditional mouse line that expresses a GFP-tox fusion construct, RC::PFtox (Kim et al., 2009). Tox desensitizes the readily

releasable pool to Ca^{2+} influx via tetanus toxin light chain's (TeTxLC) ability to proteolytically cleave the synaptic vesicle-associated membrane protein (VAMP2) and inhibit assembly of the SNARE complex needed for association and fusion with the plasma membrane (Schiavo et al., 2000). Conditional expression of GFP-tox in the developing neonatal population of GBCs prevented the formation of large cup-shaped terminals at up to 40% of principal cells ($p=0.00164$, z-test), and a similar proportion produced irregularly-shaped terminals that cover significantly smaller proportions of the respective somatic surface ($p=1.269\text{E-}07$, z-test). We also employed a secondary strategy targeting GBCs through expression of a tox construct (HdAd28E4 Cpun TeTxLC syn mCherry) delivered into newborn mice. Expression of the viral cassette occurred quickly in transfected cells (within 3 days), reducing fusion competency during the period of initial terminal expansion and preventing evoked release event (Holcomb et al., 2013).

Methods:

Ethical Approval. All procedures utilizing animals were performed with approval from the Institutional Animal Care and Use Committee of West Virginia University in accordance with the United States Public Health Service and the United States National Institutes of Health guidelines.

Animals. Selective conditional expression of the fusion protein GFP-Tox was achieved by breeding RC::PFtoX homozygous female mice (a generous gift from Susan Dymecki (Kim et al., 2009) to male FLPo recombinase mice (Jackson Laboratory; 129 ROSA26Flpo). Resulting RC::PtoX female mice were subsequently bred to PV cre males (B6;129P2-Pvalb^{tm1(cre)}Arbr/J; Jackson Laboratory, Stock No. 008069).

Virus Injection. P0 RC::PFtox mice (identified as day of birth) were temporarily removed from their mother and deeply anesthetized via hypothermia for a period of six minutes. Pups were then placed onto a pre-chilled, clay-filled aluminum block, with the head oriented into approximately the horizontal plane. Coordinates for lambda and bregma were determined using an injection needle lowered near the skull and held in a Kopf stereotaxic frame (model 940 digital; David Kopf Instruments, Tujunga, CA). Before introducing the virus, a small (1-2 mm) incision was produced across the scalp with a separate 26G needle to expose the skull. The following injection coordinates were used (in mm) relative to bregma in order to target the (VCN): medial-lateral, 1.4; anterior-posterior, 5.1; dorsal-ventral, 3.2-2.9. The injection needle was filled with a solution containing a recombinant adenovirus (rAd) optimized for fast, high-level expression of both the fluorescent reporter mCherry as well as the 50 kDa light chain of tetanus toxin, a metalloprotease that cleaves the vesicle-associated membrane protein synaptobrevin (VAMP) driven by the efficient pUNISHER cassette (Montesinos et al., 2011). A final volume of 0.5 μ L (0.33 μ L of HdAd28E4 Cpun TeTxLC syn mCherry at 4.41×10^8 viral particles/ μ L stock mixed with 0.17 μ L of 20% mannitol in storage buffer containing 10mM HEPES, 250mM sucrose, and 1mM $MgCl_2$; pH 7.4) was injected using a calibrated injection capillary (Blaubrand; intraMARK, Wertheim, Germany) at a rate of 0.1 μ L/min. Once the solution was injected, the glass needle was kept in place for an additional 4 min to allow the dissipation of backpressure before slow removal from the skull. Animals were then placed under a heat lamp ($\sim 35^\circ C$) to recover. Pups were ultimately returned to their mothers and monitored to ensure pups began to nurse.

Immunohistochemistry. Postnatal mice of either sex were anesthetized with an IP injection of Tribromoethanol (Avertin, 250 mg/Kg). Animals were perfused first with 15 mL of

normal Ringer's solution (room temperature) followed subsequently by 30 mL of phosphate buffered saline containing 4% paraformaldehyde (PFA; room temperature; Fisher Scientific, Pittsburgh, PA). The brain was removed from the skull and post-fixed overnight in chilled 4% PFA (4°C). Brains were next transferred to cryoprotectant (30% sucrose in 0.075 M sodium phosphate buffer, pH 7.4; 4°C) for a second overnight incubation. Coronal sections of the brainstem were sliced to a thickness of 40 µm using a freezing microtome (model HM450, Microm, Waltham, MA). Free floating sections containing the MNTB were transferred to blocking solution (3% goat serum in PBS containing 0.1% Triton X-100) for a period of 1 hr. Then, tissue was incubated overnight at 4°C with gentle agitation in a solution containing the following primary antibodies diluted in 3% goat serum in PBS: chicken polyclonal anti-MAP2 (1:2,500, Encor Biotechnology Inc.); guinea pig polyclonal anti-VGluT 1 and 2 (1:2500; EMD Millipore); rabbit polyclonal anti-GFP (1:500, Molecular Probes); chicken polyclonal anti-neurofilament (1:10,000, EMD Millipore).

The following day, sections were repeatedly rinsed in PBS. Secondary antibodies diluted (1:500; Molecular Probes) in 3% blocking solution were applied to sections and incubated for two hours at room temperature with gentle agitation. Sections then undergo a final series of washes in PBS before free floating sections are transferred to a FluoroDish (WPI) cell culture dish and mounted on an inverted an inverted Zeiss LSM 710 confocal microscope equipped with a motorized stage (Zeiss Microscopy).

Segmentation and three-dimensional reconstruction. Principal cells and their associated inputs contained within confocal image volumes were extracted using Seg3D software (Scientific Computing and Imaging Institute, University of Utah). A total of 287 cells were analyzed using the following pipeline: The thresholding tool in Seg3D was used mask the

highest density of signal at each channel. Manual inspection and editing of the resulting masks produced reconstructions of the volume of each terminal as well as cell body following the manual cleavage of neurites. Masks in the VGluT channel were expanded uniformly by a single pixel to produce a temporary mask layer. The Boolean AND tool was used to merge the segmentation of the soma with the temporary “expanded calyx” mask to produce a complex two-dimensional sheet representing the area of apposition of the terminal. All subsequent quantification calculations were performed in Microsoft Excel (Microsoft, Redmond, WA).

Statistics. All data are presented as mean $\pm$ standard deviation or as relative within group proportion. Statistical significance across multiple groups was evaluated with a Kruskal-Wallis Test with Nemenyi post-hoc test with a Bonferroni correction to control for the number of groups. The difference between group proportions were compared with the multiple-proportion z-test.

Results:

Constitutive GFP-tox expression in the developing auditory brainstem.

In a previous study taking advantage of a novel “whole-head” preparation that maintained parts of the peripheral cochlea within a sectioned piece of brain tissue, we have revealed several events in CH development that occur during late embryonic ages (Marrs and Spirou, 2012). These included the observation that stimulation of spiral ganglion neurons elicited synaptic responses in the VCN beginning at E15 (Marrs and Spirou, 2012) and that it takes at most two additional days for efferent bushy cell projections to become functional in the MTNB by E17 (Hoffpauir et al., 2010). With the goal of blocking these early synaptic events specifically between bushy cells and MNTB neurons, we employed a transgenic mouse model reliant on two

recombination events for conditional expression of a tetanus toxin fusion protein construct (Figure 1) (Kim et al., 2009). These mice are referred to as RC::PFto_x mice. In the presence of Cre recombinase, the mice express an mCherry reporter. Breeding these mice to PV-Cre resulted in relatively restricted mCherry expression in the auditory brainstem as evidenced by clear labeling of cells within the VCN, including bushy cells (compare A and B in Figure 1), as well as within the axonal projections of the VCN along the ventral acoustic stria (VAS) to targets in the lateral superior olive (LSO) and medial nucleus of the trapezoid body (MNTB), among others (Figure 1 D). However, note that Flp-mediated recombination is still required for expression of the GFP-to_x construct (Figure 1 C).

Surprisingly, the penetrance of the RC::PFto_x was variable despite confirmation of appropriate allele expression. Our first litter of RC::to_x pups yielded several pups with the same genotype (PV-cre, FLPo, RC::to_x) but which exhibited this variable penetrance. Two pups had a severe motor ataxia evident by the end of the first postnatal week ('strong phenotype') while two littermates of the same genotype exhibited no motor impairment ('weak phenotype'). For ethical reasons and concerns affected that pups may have difficulty feeding, this litter was sacrificed at P9 and the MNTB was collected alongside an age-matched control litter of RC::PFto_x mice. In addition to genetically expressed fluorescence, immunolabeling was performed for Vglut1 and 2, markers for the CH, MAP2, a marker for the MNTB cell body, and neurofilament-2 (NF2), a marker for axons. The CHs identified in control tissue looked typical for this age, with nearly all principal cells (87%) supporting a single large cup-like terminal, in close agreement with estimates of CH growth and maturation reported previously (88% resolved to monoinnervation by P9; Holcomb et al, 2013)(blue and green channels, respectively, in Figure 2A). RC::to_x pups exhibited disrupted terminal formation. In the less penetrant example (Figure 2B), ~50% of the

imaged principal cells were contacted by large presynaptic terminals relative to RC::PFtoX controls, and nearly ~40% were not contacted by a large terminal ($p=0.00164$, z-test; Figure 2D). The organization of the local innervation varied further in ataxic triple transgenic pups wherein ~25% of principal cells were not contacted by a large terminal (Figure 2C,D). Many (~40%) of the large terminals that formed appeared atypically swollen and blebbed (red and blue channels). Only 14% of principal cells were contacted by a normal appearing terminal ($p=1.269E-07$, z-test; Figure 2D).

Heterogeneous tox expression in the developing auditory brainstem.

Constitutive expression of GFP-tox in some animals disrupted normal structural maturation of the calyx of Held onto many MNTB neurons. We previously investigated the early stages of calyx formation through a series of serial electron micrograph volumes collected from mice across the first postnatal weeks of life (including P2, P3, P4, P6, P9, P30) in order to chart the morphological features exhibited during terminal expansion. We demonstrated a transient period of synaptic competition (Holcomb et al., 2013; Jackson et al., 2020). As an alternative to variable penetrance of genetic manipulation, we next sought a more consistent method to silence GBC synaptic activity through viral transfection of a TeTx construct. This manipulation was designed to determine whether reduced synaptic activity would preclude the growth of a calyx terminal. Pups were injected on the day of birth (P0) with a viral vector (HdAd28E4 Cpun TeTxLC syn mCherry) that was delivered focally within the left VCN (Figure 3A). By P3, trace amounts of mCherry expression were detectable in the VCN (not shown). By P5, robust labeling of cells in the injected VCN (Figure 3B-C) was observed complete with efferent projections

along the ventral acoustic stria that entered the lateral superior olive (LSO) bilaterally, (Figure 3B) and the contralateral MNTB (Figure 3F). The injection into one CN transfected additional neurons in the contralateral VCN (Figure 3D), although these cells did not produce CHs within the ipsilateral MNTB (relative to the injection site; Figure 3E). Details of the injection process remain to be worked out so that the contralateral projection can be used as a within-animal control group.

Tiled z-stacks collected bilaterally from each MNTB were imaged at higher magnification to quantify these observations. No virally labeled (mCherry) CHs were identified within the ipsilateral MNTB although commonly large terminals were defined by clusters of VGLUT staining (two curved profiles in Figure 4A). Within the contralateral MNTB, relative to the injection site, we found a combination of mCherry positive (arrowheads) and mCherry negative (blue) terminals (Figure 4B). These tiled image volumes from each nucleus were then exported to Seg3D where channel data was independently thresholded and masked to produce an approximate reconstruction of each pre- and postsynaptic compartment. A combination of manual inspection and smoothing was then used to refine these 3D renderings into individual pre- and postsynaptic compartment reconstructions as well as define a planar border representing the area of apposition between the terminal and the postsynaptic PC soma. (note* In order to study primarily the effect of the synaptic silencing through constitutive TeTxLC expression, only principal cells that were contacted by at least one mCherry positive profile were analyzed in the “contralateral group”).

Quantification of the partial reconstructions produced from the previous experiment suggested a role for neural activity in CH growth and maturation. Focusing first on PCs that were contacted by only a single calyceal terminal, mCherry/TeTxLC expressing terminals in the

contralateral MNTB exhibited an average thickness of $1.1 \pm 1.01 \mu\text{m}$, which was nearly double the values relative to CHs within the ipsilateral MNTB ($0.49 \pm 0.42 \mu\text{m}$ thick ($p=2.77\text{E-}8$, KW; Figure 4C)). We also quantified the size of the terminal by measuring its apposed surface area (ASA) and overall volume. Ipsilateral (non virally-transduced) CH terminals exhibited significantly larger ASAs than mCherry/TeTxLC positive terminals in the contralateral MNTB ($349.69 \pm 268.56 \mu\text{m}^2$ ipsilateral vs $188.02 \pm 130.50 \mu\text{m}^2$ contralateral; $p= 4.33\text{E-}3$, KW; Figure 4D). Despite differing thickness and ASA, the overall volumes of the terminals were similar between groups ($206.94 \pm 249.35 \mu\text{m}^3$ ipsilateral vs $215.98 \pm 209.44 \mu\text{m}^3$ contralateral; $p = 0.403$, KW; Figure 4E). Finally, we noticed a nearly 25% reduction in the somatic volume of the postsynaptic principal neurons that receive innervation from mCherry positive CHs in the contralateral MNTB ($1615.26 \pm 444.78 \mu\text{m}^3$ ipsilateral vs $1208.59 \pm 312.48 \mu\text{m}^3$ contralateral; $p= 2.63\text{E-}6$, KW; Figure 4F).

Finally, the competitive outcomes of all MNTB PCs both ipsilateral and contralateral to the injection site were assessed similarly to the previous experiment. However, NF labeling additionally allowed the axons of each potential input to be differentially tracked within the volume. Counts of the number of unique inputs to each cell are summarized in Table 1. Most dramatic at P5 was the ~30% reduction in the number of PCs that host a single dominant CH ($p= 0.00289$, z-score). Moreover, over 40% of the principal cells imaged within the contralateral MNTB hosted two or more large terminals with unique axons.

Discussion:

Use of the so-called “toxigenetic” model, or selective genetic expression of tetanus toxin light chain (TeTxLC), within subpopulations of neurons allows for direct manipulation of patterns of activity in living and developing neural circuits. Expression of the 50 kDa metalloprotease TeTxLC inhibits neurotransmitter release through proteolytic cleavage of the synaptic vesicle-associated membrane protein (VAMP2) (Schiavo et al., 2000). In a number of studies, perturbation in VAMP2 expression through the introduction of TeTxLC or genetic deletion (*syb2* KO) results in a 50-90% reduction in action potential evoked events, although some basal level of spontaneous vesicular fusion still occurs (Schiavo et al., 2000; Schoch et al., 2001; Yamamoto et al., 2003). However, even in the context of a abolishment of synaptic communication via a conditional *muc-13* deletion and subsequent deficit in synaptic vesicle priming capacity, synaptogenesis has still been shown to occur (Varoqueaux et al., 2002). So, it is perhaps unsurprising, despite expression of TeTxLC, GBC axons were capable of producing large terminals.

Silenced axons in this dataset exhibited a significant disruption in terminal growth over the first postnatal week. We found through conditional expression of GFP-tox across the population of GBCs produced a variable phenotype that could result in up to a 40% reduction in the number of principal cells contacted by a large terminal. Moreover, many of the terminals that persisted appeared swollen and irregular. Previous studies investigating the ultrastructural characteristics of cells that have chronically expressed tox have found synaptic vesicles can aggregate as a result of disrupted exocytosis leading to an engorgement of presynaptic boutons (Kim et al., 2009). It is likely a similar phenomenon occurred in our mutated GBC efferents as revealed by the near complete overlap of VGLUT and GFP labeling in immunohistochemical

preparations. However, it is currently unclear why the penetrance of the phenotype in RC::tox animals varied within litters and poses challenges as a reproducible model.

In order to verify the phenotype observed in transgenic animals, we additionally utilized viral transfection as a secondary method of TeTxLC expression. Particular interest during quantification of histological preparations was paid to principal cells that were contacted by at least one silenced input or no inputs at all. Although unreported, a larger number of principal cells were surveyed in order to meet this criterion as a result. Nonetheless, viral-mediated silencing of a proportion of GBCs still resulted in a similar reduction in the number of principal cells that failed to be contacted by a large terminal (30% vs 40%). Among cells that supported at least a single mCherry positive input, there was a 30% increase in the number of polyinnervated principal cells (42% contralateral vs 13% ipsilateral). This could be suggestive of a persistence of competing inputs when both competitors have similar levels of activity. Similar studies at the neuromuscular junction have raised similar possibilities. Blocking all AChR at a developing motor endplate with an irreversible antagonist, such as α -bungarotoxin, or genetically inhibiting the generation of an action potential through the overexpression of inwardly rectifying potassium channels post-synaptically in mice have each been shown to inhibit normal synapse elimination (Balice-Gordon and Lichtman, 1994; Favero et al., 2009). On the opposite end of the spectrum, driving synchronous activity through all competing inputs also inhibited synapse elimination at individual muscle fibers in rats (Busetto et al., 2000). However, artificially enhancing the disparity in firing strength and frequency between competing axons lead to an immediate loss of the less active input (Keller-Peck et al., 2001). These data helped to establish an understanding that the relative imbalance of activity among competing inputs could serve as an activity-

dependent trigger for permissive growth of a single input or instructive for the removal of others (Kasthuri and Lichtman, 2003; Walsh and Lichtman, 2003).

Moving forward, the toxigenetic model offers a unique platform to model the role of synaptic activity in the development of neural circuits. Although large terminals could form despite significant perturbation to the exocytic capacity of these terminals, additional experiments are necessary in order to draw firm conclusions on the persistence of polyinnervation as a result of interrupted activity-dependent pruning. Our quantification relied on incomplete fluorescent staining that could only approximate changes in gross morphology and axonal identity. Moreover, verification and quantification of the persistence of spontaneous release events too will further our understanding of the effectiveness of our silenced model.

Chapter 5: Discussion and Future Directions

In many parts of the developing nervous system, more synaptic connections are initially formed than exist at maturity. Activity-dependent refinement of initial connections via selective pruning is a necessary process in order to increase the precision of circuit connectivity. In the case of sensory systems wherein this phenomena is perhaps most well-studied, the resulting arrangement of highly precise connectivity between brain regions is referred to as topographic organization (Udin and Fawcett, 1988). In neocortex and other brain regions, many synapses, or functional connections between neurons, are formed at the interface of a presynaptic bouton and a dendritic spine head (Berry and Nedivi, 2017). Bouton terminals typically contain one or two active zones. However, very large inputs are located in a limited number of brain regions (see NMJ, CF:PC, RGC:dLGN; and CH:MNTB described in Chapter 1), and can contain hundreds to over one thousand distinct vesicle release sites, which ensures release of multiple synaptic vesicles and increased reliability of synaptic transmission (Walmsley et al., 1998; von Gersdorff and Borst, 2002; Rudolph et al., 2015). Examples such as the CH, the largest nerve terminal in the mammalian central nervous system, offer key advantages in the study of neural development due to the exaggerated size and relative simplicity of the mature endpoint of 1:1 innervation ratio of the CH and MNTB principal neuron. The results described in Chapters 2, 3 and 4 provide meaningful new insights into the structural and functional changes that underlie the growth and maturation of this largest central nerve terminal. The major conclusions of those studies will now be discussed in the broader context of neural circuit formation including a brief discussion of remaining questions and presentation of future research directions.

Elaboration CH collateral arborization

Studies of the fine structure of the developing CH have occurred over the last few decades, yet little attention was paid to the collateral processes that emanate from the edges of the CH (Morest, 1968a; Kandler and Friauf, 1993). Building upon this work, the Spirou laboratory developed the first and largest collection of serial electron micrograph volumes of tissue collected from the medial, high-frequency MNTB of mice, taken at various early postnatal ages (Holcomb et al., 2013). Accurate, high-resolution reconstructions of both presynaptic terminals and associated principal neurons had to be painstakingly obtained by hand. However, these efforts charted the structural expansion of multiple large terminals that initially grew over each principal neuron at younger ages (P2-4) as well as the emergence of a single dominant input just days later (P6 & 9) (Hoffpauir et al., 2006; Holcomb et al., 2013). These 3D reconstructions, in parallel with physiologic measurements taken from similar ages, both indicate that principal cells are contacted briefly by multiple large protocalyceal terminals (Hoffpauir et al., 2006, 2010). Calycigenic axons also have numerous branches that may support transient synaptic activity (Kuwabara et al., 1991; Kandler and Friauf, 1993; Rodríguez-Contreras et al., 2006; Holcomb et al., 2013). In rare cases, the calycigenic axon branches and delivers two CHs within close proximity, typically within 50 μm , such that tonotopic precision is maintained (Kuwabara et al., 1991; Rodríguez-Contreras et al., 2006).

We proposed and other experiments support a transient period of synaptic competition that resolves to monoinnervation (Holcomb et al., 2013; Xiao et al., 2013; Milinkeviciute et al., 2019). We hypothesized that competition resulted from indirect competition between competing inputs as each growing CH established an apposed surface area (ASA) along distinct, non-overlapping regions of the MNTB cell somatic surface (Holcomb et al., 2013). However,

consideration of collateral growth of the CH requires reconsideration of whether more direct signaling occurs directly between competing inputs. More recently, the dynamics of axon branch and perhaps CH collaterals were observed *in vivo*, (Rodríguez-Contreras et al., 2008). In our most recent efforts, we sought to address fundamental questions about the emergence, function and motility of CH collaterals, using lattice light sheet (LLS) dynamic imaging in combination with a further analysis using our SBEM image series.

We assessed whether collaterals are a feature of later stages of CH growth, once the CH has anchored a position on the postsynaptic cell body, or are extended as soon as the protocalyx, the first expansion of the small terminal, begins to form. At P3 about one-half of MNTB cells in the SBEM volume are contacted by growing terminals of at least the protocalyx stage ($\sim 35 \mu\text{m}^2$), and about one-half (43%) of these cells are contacted by two or three large terminals (Holcomb et al., 2013). Therefore, we focused on these smallest terminals, and noted that they already extended collaterals of each type: short filopodia, long filopodia, long growth cone tipped collaterals. One important question is why would a terminal expend the requisite resources and energy to produce an extensive network of collateral arborization network that will largely be removed within days? We considered that these extensions could provide either or both of exploratory roles, seeking out additional territories for innervation, or defensive roles in preventing competition from other calycigenic axons. Notably both potential functions are underway at the protocalyx stage due to the presence of all collateral types.

The length of CH collaterals places a limit on their sampled volume. Nearly all long collaterals are 60 μm or less, with a few growth cone-tipped collaterals reaching 100 μm or longer. Except for the longest collaterals, the exploratory volume could be within the tonotopic medial-lateral specificity of the MNTB (total distance $\sim 350 \mu\text{m}$ medial-lateral at early postnatal

ages in mice (Kolson et al., 2016; Weatherstone et al., 2016), especially since collaterals had a range of orientations and thus were typically not aligned along the frequency axis. Interestingly, protocalyces at P3 had already extended long collaterals, and the increase in number of collaterals with CH size did not alter the distribution of collateral lengths, but did add additional long collaterals which presumably increased the patrolled volume by these structures. Initial exuberance and overconnectivity of presynaptic afferents has been a well-described phenomena in a neurodevelopmental context in a variety of brain regions (Innocenti and Price, 2005; Portera-Cailliau et al., 2005; Gallo, 2011). Thus, if longer CH collaterals were capable of forming synaptic contacts, then this system could serve as a fail-safe to ensure that each PC is innervated and each GBC axon delivers at least one CH into the MNTB. A constraining endpoint of the system is that each calycigenic axon produces primarily one (90%) or occasionally two (10%) CH terminals, and in the latter case maintains tonotopic organization (Spirou et al., 1990; Kuwabara et al., 1991; Smith et al., 1991; Rodríguez-Contreras et al., 2006). Furthermore, approximately 10% of MNTB neurons are contacted by two CHs at P9, an arrangement that may persist into adulthood.

SBEM permitted us to evaluate the structures that were contacted by long CH collaterals. Nearly 20% of long growth cone tipped collaterals, which constituted 124/634 long collaterals, were found to physically contact multiple principal cells, which are densely packed, along the length of the collateral and occasionally at their tips. Careful inspection of SBEM images revealed no evidence of synaptic contacts along CH collaterals. However, immature synapses can form transiently even without the full complement of synaptic proteins (Lee and Peng, 2008). We did observe what appeared to be synaptic vesicles within long filopodial and growth cone tipped CH collaterals. Together with observed Ca^{2+} signals along the length of calycigenic

axon branches (Rodríguez-Contreras et al., 2008), we suggest that CH collaterals may provide transient innervation to multiple principal cells. Growth cone tipped processes are linked to synaptogenesis in other systems (Chilton, 2006; Gomez and Letourneau, 2014). Notably, at least a subset of mature CHs (1 in 6 at P30) maintain a single collateral branch onto a second nearby principal cell via a smaller terminal. Thus, we hypothesize an exploratory role for long CH collaterals to identify additional innervation targets, in case a growing CH loses competition onto its initial cell target and must form a new CH onto another cell, is associated with one of the few GBC axons forming two CHs, or is establishing a functional CH collateral that persists in the mature circuit.

SBEM permitted more detailed views into CH collaterals than afforded by previous light microscopy studies and revealed that 50% of collaterals were $\sim 6\mu\text{m}$ in length or shorter (median length: $5.85 \pm 9.32 \mu\text{m}$, $n = 1,352$ of 2970 collaterals, $n = 34$ CHs). These collaterals were filopodial in morphology, mostly devoid of internal structures, and extended laterally from the edge of the CH without contacting the somatic surface and mostly terminate within the extracellular space without direct contact with adjacent cells. In static images, we were unable to ascertain if short collaterals could lengthen to become more complex. However, given the relative constancy of the distribution of collateral length between P3-6, we propose that at least a subpopulation remain short. Such structures may establish a static perimeter that could provide a leading edge to CH growth, or subsequently attach to the cell body surface as part of CH expansion.

We next investigated whether CH collaterals contacted other CH collaterals, either from the same or from different cells. These contacts could represent a substrate for preserving innervation territory and perhaps mediating competition onto the same MNTB cell. Long

collaterals were often branched; and collateral branches were typically filopodial in shape. In SBEM images, we found nearly half (55.7%) of long collaterals make physical contact with at least one additional collateral from a nearby CH or a nearby principal neuron (Chapter 5 Figure 1). In static images, however, we were unable to assess the functional dynamics that resulted from these contacts. Given the rapid estimated growth rates for the CH during the early postnatal period (Holcomb et al., 2013), a steady fraction of short collaterals should be become long to maintain the length distribution across CH size.

Dynamic Motility and Potential Function of CH Collaterals

Our next steps towards evaluating the behavior of CH collaterals involved direct observation of collateral motility using the LLS microscope. This microscope achieves diffraction limited resolution in all three axes (Langhorst et al., 2009) with the low power presentation of an ultrathin plane of illumination produced by dithering a custom array of non-Gaussian Bessel beams (Chen et al., 2014). We developed a preparation that allowed a fresh section of perinatal tissue to be mounted within an oxygenated perfusion chamber for an acute imaging session. The rapid volumetric image acquisition rate varied between experiments but recorded collateral dynamics at a maximum frequency approaching 1 Hz. This approach eclipsed the sampling rate achieved in the only previous study of dynamic motility of filopodial processes in the developing MNTB (Rodríguez-Contreras et al., 2008). That preparation uniquely permitted *in vivo* imaging through a ventral cranial window; but the temporal resolution of the data was limited by a relatively low acquisition rate of 2-photon raster imaging. In that study, changes in collateral length were observed at minutes to tens of minutes intervals, and only a subset of processes were labeled, so interactions among neurites could not be monitored.

Notably, LLS imaging revealed more rapid rates of process motility within axon terminal zones than has previously been observed (Rodríguez-Contreras et al., 2008). We also noted clear differences in the types of motile behavior observed among collateral subtypes, and addressed several open questions from observations on the static EM images. We were able to image short collaterals, but due to the lower resolution of LLS relative to our ultrastructural reconstructions, we did not detect as many as were found using SBEM. Nonetheless, dynamic imaging of these structures revealed, as we predicted, that short filopodia typically did not extend to become long collaterals. We proposed that short filopodia provided a fixed structure at the edge of the CH, but in fact these short filopodia could have short lifespans and in some cases persisted for as little as 5 minutes (11/21 short filopodia). We did not observe them interacting with long collaterals, and our sample did not include closely located regions of competing CHs onto the same cell. From these data we conclude that short collaterals may be more associated with expansion at the edges of the CH rather than playing a protective function, but additional imaging sessions may reveal interactions between different CHs.

Long collaterals, both filopodia and growth cone tipped, exhibit a variety of movement patterns. Extension could be directed as a series of rapid linear extensions with short pauses, or collaterals could frequently change direction due to either juxtacrine interaction among labeled and unlabeled elements in the tissue or tracking of chemical gradients. Rapid extension was faster than any reports in the literature on developing nervous system, consistent with the speed with which the nervous system is assembled. These rates rarely surpass the proposed limiting rate of cytosolic and cytoskeletal extension, defined as slow axonal transport ($1\mu\text{m}/\text{min}$) (Brown, 2014). Typically, it is thought that long distance migrations of axons through along fiber bundles are the only times axons achieve these speeds, such as occurs as axons extend through the corpus

callosum and cross the midline (1.3 $\mu\text{m}/\text{min}$) (Halloran and Kalil, 1994). However, within targeted brain regions, axonal arborization dynamics within terminals territories rarely too can achieve impressive rates of motility. Retinotectal axons within target territories may elongate at impressive rates that exceed slow axonal transport (5 $\mu\text{m}/\text{min}$)(Witte et al., 1996). However, in our experiments, long collaterals exhibited the fastest rates of extension. CH collateral growth cones achieved average values of motility of 2.1 $\mu\text{m}/\text{min}$ during periods in which they transiently exhibit instantaneous growth rates exceeding 10 $\mu\text{m}/\text{min}$.

In a few instances we had a sufficient number of labeled processes and a favorable field of view to identify direct contact between long filopodia from different CHs. The most protracted interaction involved one filopodial collateral advancing upon and contacting another, withdrawing and re-contacting several times. At the end of this sequence, the advancing collateral retracted, underwent a unique windmill motion and extended in a different direction (Chapter 2, Figure 6). It is tempting to speculate that surface-mediated contact between these collaterals indicates a generalized repulsive signaling between CHs. Similar findings of repulsive axo-axon signaling via surface expression of adhesion molecules (such as Down-syndrome cell adhesion molecule 2, Dscam2; Ig superfamily protein, Turtle) have been implicated in the developmental axonal segregation of ocular dominance columns of the medulla mediated directly through axo-axonal contact (Millard et al., 2007; Ferguson et al., 2009). Effectively, these descriptions of repulsive interactions represent the initial evidence for direct signaling between growing CHs in the developing MNTB. Additional experiments will be required to more fully elucidate the valence of long collateral interactions and explore the molecular mechanisms that underlie collateral behaviors.

Postsynaptic specializations during growth of the CH

Despite a rich literature representing over a century of study investigating the shape and function of dendritic spines as sites for presynaptic innervation (Yuste and Bonhoeffer, 2004; Yuste, 2015), new structures are continuing to be discovered that broaden our understanding of the potential shape and function of postsynaptic spines (Sanculi et al., 2020). As noted in the previous section, little attention has been paid to the investigation of CH collaterals in the MNTB, but even less is known about the specialized somatic spines that have been observed in developing and mature tissue. We identified two types of somatic spines, which exhibit similarities in morphology to dendritic spines in their overall size (Harris et al., 1992), but differ by having larger caliber typically without an expanded head or constricted neck (type 1 somatic spines) or by being longer and branched (type 2 somatic spines). Type 1 spines were solitary and were entirely bound within invaginated pockets embedded into the presynaptic face of the overlying nerve terminal. Moreover, this arrangement ensured tight association with the presynaptic membrane along the entire surface of the type 1 spine, which provided additional sites for mechanical stability (Rowland et al., 2000) and synaptic innervation. The second type of spine, referred to as type 2 spines, exhibited a more complex morphology revealed by a complexity analysis reported in Chapter 3, but more importantly could be distinguished as a unique component in an intertwined tangle of spines that collectively reside within a shared invagination in the presynaptic face of the overlying terminal. We thus provide the first description spine mats in the CNS, as they have only been described previously at the somatic surface of ciliary ganglion neurons in the peripheral nervous system of the chick. Those neurons also are innervated by a calyx-type nerve terminal (Shoop et al., 2002).

Population dynamics and the persistence of CH collaterals and somatic spines

MNTB neurons are characteristically smooth at P2, prior to growth of large terminals onto their cell bodies (Holcomb et al., 2013). The first signs of CH growth at P3 are accompanied by a coordinated emergence of CH collaterals. We thus investigated in SBEM images whether the smallest protocalyces were characterized by extension of type 1 spines into the presynaptic face of the growing terminal. Recall that spine mats were present only when CHs exceeded about $100 \mu\text{m}^2$ of apposed surface area, so we did not investigate their presence in this context. In only the two smallest protocalyces in our sample was there an absence of type 1 spines (input A: $35 \mu\text{m}^2$; input B: $38.2 \mu\text{m}^2$). Thus, CH growth can likely initiate without type 1 spine formation, but the two events quickly become coordinated. These events imply early signaling between the growing CH and localized regions of the postsynaptic membrane.

Both CH collaterals and somatic spines increased in density until P9, one day ahead of the opening of the ear canal and the onset of experience-dependent activity. After P9, calyx collaterals, nearly all type 2 spines and hence spine mats, and the vast majority of type 1 spines are removed as the CH achieves its mature synaptic properties and fenestrated morphology (von Gersdorff and Borst, 2002; Ford et al., 2009). A general pattern of coordination among these events seems evident, but closer examination reveals temporal differences in the expression of these pre and postsynaptic structures.

The appearance of type 1 spines and CH collaterals appears highly synchronized. Type 1 spine density reaches its maximum at P6, as does the number of CH collaterals exhibiting the full representation of long filopodial and growth cone tipped structures. Type 1 spine density is maintained at P9, as is the number of CH collaterals, but long collaterals are few in number. P9

is the age at which we estimate the mature composition of mono and multi-innervated MNTB neurons is achieved. Spine mat density begins to increase one day after type 1 spine density and peaks at P9. CH collaterals, except a single thick branch from a minority of CHs that innervates a neighboring MNTB cell, and spine mats are nearly absent at P30, and type 1 spines are at very low density. Thus, the full complement CH collateral lengths are most directly tied to CH growth and establishment of the final CH:MNTB neural circuit. Type 1 spine expression tracks this same period, but persists into the next phase of CH fenestration. Spine mat expression peaks at the beginning of the phase of CH fenestration. We propose that CH collaterals play roles in synaptic competition, but we did not find differentiating features among competing CHs to suggest a similar role for type 1 spines and spine mats. CH collaterals and type 1 spines may be initiated by the same signaling processes between pre and postsynaptic cells, and these events may have downstream triggers for the formation of spine mats. Presumably, these molecular and cellular cues for process formation are active only during this narrow time period and are no longer required in the mature circuit. An accompanying schematic reveals the relative timing of each of these events (Chapter 5, Figure 1).

Importantly, we found little evidence of type 1 spines and no examples of spine mats outside of areas associated with presynaptic innervation. Thus, they are likely specialized specifically for communication with the presynaptic terminal. Transmission EM studies have revealed the CH:MNTB junction incorporates hundreds of discrete release sites and adherens junctions during development (Sätzler et al., 2002; Hoffpauir et al., 2006; Holcomb et al., 2013). Postsynaptic densities and adherens junctions are not reliably revealed by *en bloc* staining for SBEM, so in particular the presence of adherens junctions could not be assessed along the direct apposition between type 1 spines and the presynaptic membrane. Nonetheless, we suggest that in

addition to providing mechanical stability, adherens junctions and regions of direct membrane apposition as around type 1 spines may also serve a communicative function via surface contact-mediated interaction via transmembrane adhesion receptors. These are primarily comprised of cadherins, protocadherins, L1-CAM in the MNTB, but may also include nectins and nectin-like families of proteins that are found in many parts of the nervous system (Niessen and Gottardi, 2008; Indra et al., 2013; Kolson et al., 2016). The large diversity and non-uniform expression of adhering proteins expressed within the MNTB may be sufficient to differentiate targets across the nucleus for tonotopic organization (Whitlon et al., 1999).

In addition to contact-mediated signaling, direct signal exchange may also be mediated through connexin 36-associated gap junctions shown to be expressed using light microscopy at the synaptic interface of the CH and PC (Rubio and Nagy, 2015). However, our SBEM image volumes were not of sufficiently high resolution to reveal these structures. Future experiments utilizing immuno-EM or peroxidase labeling against connexin 36 in SBEM tissue blocks can reveal additional information about specialized signaling through somatic spines in the developing MNTB.

Signaling capacity of somatic spines

Before considering signaling through somatic spines, it is worthwhile to briefly consider the sequence of events that leads to their appearance. Notably, the formation of type 1 spines is synchronized to initiation of CH growth. Our hypothesis, to be tested in future experiments, is that emergence of bursting activity is the trigger, and if true that must induce molecular signals that reorganize membrane and cytoskeletal elements that constitute the spines. Some leads can be gleaned from the literature on dendritic spines, but the role for neural activity remains unclear

(Yuste and Bonhoeffer, 2004; Freire et al., 2010). In cerebellum, for example, despite loss of granule cells and their axons in Weaver mutant mice, Purkinje cells grow dendrites studded with spines. This phenotype suggests that intrinsic programs can promote the emergence of spines (Rakic and Sidman, 1973; O'Brien and Unwin, 2006), although climbing fiber activation could stimulate spine formation. It is not surprising that inducing reorganization of the actin cytoskeleton is essential to building spines, and has been shown via manipulations in the level of expression of the small GTPases Rac and Rho (Yuste and Bonhoeffer, 2004; Woolfrey and Srivastava, 2016). Overexpression of Rac1 is sufficient to produce shortened but persistent, supernumerary spines on Purkinje cell dendrites (Luo et al., 1996) and expression of a dominant-negative Rac-1 protein led to the progressive loss of spines (Nakayama et al., 2000). RhoA overexpression, in contrast, produced smoothened dendrites, an effect mediated by the activation of the downstream kinase ROCK (Nakayama et al., 2000). As the CH expands over the MNTB somatic surface, local contact via membrane proteins such as adhesion molecules, or neural activity, could induce type 1 spine formation. Future manipulations of CH activity should include an anatomical survey for the presence of type 1 spines.

When considering a functional role for the emergence of somatic spines between the developing CH and MNTB cell, we propose three modes of communication via somatic spines: (1) chemical synaptic transmission, (2) adhesion molecules at apposed locations of the pre and postsynaptic cells, and (3) trans-endocytosis of spine mats. The GBC to MNTB synapse is glutamatergic at least from E17, the earliest age that it has been monitored (Hoffpauir et al. 2006). Several studies have indicated the presence of NMDA receptors at early stages of CH growth, which indicates roles for activity induced postsynaptic Ca^{2+} -signaling to mediate

competition and maintenance of synaptic contact (Taschenberger and von Gersdorff, 2000; Futai et al., 2001; Hoffpauir et al., 2006). We consider these topics further in the next sections.

Role of chemical neurotransmission in the establishment of the CH. A variety of manipulations have been implemented previously to disrupt neurotransmission in the developing auditory system via the brute force approach of unilateral or bilateral cochlear ablation (Kitzes et al., 1995; Russell and Moore, 1995; Franklin et al., 2008) which produces significant cell loss in the cochlear nucleus, or via genetic perturbation of inner hair cells. The latter has been accomplished via Otoferlin knock-out (Roux et al., 2006; Longo-Guess et al., 2007), Cav1.3 KO (Brandt et al., 2003; Erazo-Fischer et al., 2007), VGLUT3 KO (Ruel et al., 2008; Seal et al., 2008) or knock-out of the $\alpha 9$ subunit of nicotinic acetylcholine receptors (Clause et al., 2014). However, manipulation of inner hair cells leaves open the possibility of homeostatic up-regulation of spontaneous activity in spiral ganglion neurons or GBCs. Thus, the small effect of these manipulations on CH growth does not have clear interpretation. As described in preliminary experiments of Chapter 4, through selective expression of TeTxLC directly in GBCs, synaptic vesicle release was presumably inhibited. Despite the retained capacity for terminals to form, less pruning occurred in these TeTxLC-positive animals such that supernumerary terminals were maintained more consistently than in TeTxLC-negative controls and their geometry was altered. In order to more fully validate this phenotype, paired pre and postsynaptic voltage clamp experiments will be required to characterize the level of intrinsic spontaneous activity that persists, as well as to characterize the biophysical maturation of deprived MNTB neurons.

The maturation of the principal cells is synchronized with the growth of the CH. For example, increases in the synaptic strength of terminals (as a byproduct of insertion of additional

release sites) is correlated highly with an increase in postsynaptic surface area and expression of low threshold potassium channels, which reduces intrinsic excitability of the neuron (Hoffpauir et al., 2010; Van Hoeve et al., 2010; Sierksma et al., 2017). Our preliminary data suggests that expression of TeTxLC presynaptically prevents the increase in somatic volume that occurs in control animals. A smaller cell size should result in a more excitable cell due to higher input resistance, which would be an appropriate homeostatic response to reduced synaptic input. Further electrophysiologic experiments will be required to more formally characterize the proposed delay in postsynaptic maturation due to abolishment of spontaneous and evoked activity. The altered morphology of the CH following expression of TeTX should, in our future experiments, be studied along with characterization of CH collaterals and somatic spine formation as one strategy to assay the primacy of physiological signaling to induce the growth and maturation of the CH:MNTB connection.

Spine mats offer a complex geometry for segregation of signaling routes among cells, as well as extensions from presynaptic and glial compartments wherein tri-synaptic relationships can form (Müller et al., 2009; Reyes-Haro et al., 2010). The complex extracellular space, perhaps aided by diffusion barriers due to adhering junctions, may facilitate aggregation of signaling molecules secreted locally within the available extracellular space. The frequent observation of reticulated membrane within type 2 spines further suggests signaling capacity that is similar to that associated with the spine apparatus within mushroom shaped dendritic spines (Deller et al., 2003). Ca^{2+} signaling at this site could affect the volume of spines and weights (Jedlicka et al., 2008; Holbro et al., 2009) of the few chemical synapses onto the outer surface of the spine mat. In the only other known occurrence of spine mats, diffusion of acetylcholine (ACh) through extracellular space to bind to receptors along the component somatic spines was proposed as a

mechanism for extra-synaptic neuromodulation (Shoop et al., 1999). Gene expression analysis in MNTB (Kolson et al., 2016) revealed temporal increase of was *Chrna4* (cholinergic receptor, nicotinic, alpha polypeptide 4), message at P6 relative to P0, and subsequent decrease at P14. Cholinergic signaling affects large somatic terminal formation in the ventral nucleus of the lateral lemniscus (Baumann and Koch, 2017), so it may generally be important for the establishment of large, calyx-type terminals in the auditory brainstem.

Signaling through adhesion proteins. Signaling via adhesion molecules is a broad topic (Aplin et al., 1999; Cavallaro and Dejana, 2011). Work from our lab has identified cadherins, including N-cadherin, protocadherins as possible substrates within the MNTB (Kolson et al., 2016). These associations may form directly between CHs and type 1 spines, but also between MNTB cells and astrocyte processes within spine mats. A recent report demonstrated evidence for a novel process of nectin-mediated material exchange, whereby nectin-1 expressing neurons trans-endocytose segments of membrane from cells containing nectin-4 (Generous et al., 2019). Thus, experimental manipulations in the expression of nectin-1 may provide potential opportunities to disrupt spine mat trans-endocytosis during the development of the MNTB.

Signaling via trans-endocytosis of spine mats. This mode of intercellular signaling offers multiple categories of molecular signals, but is more difficult to clarify, as spine mat elements contain membrane, and likely membrane bound receptors, cytoskeletal elements, diffusible cytoplasmic constituents including proteins, and organelles (Spacek and Harris, 2004; Petralia et al., 2015). Any and all of these cellular elements may provide a communication route between these synaptic partners. The onset of trans-endocytosis in the CH:MNTB system is at P9, just

after the MNTB neuron has refined its projection territory to the lateral superior olive (LSO) (Kandler et al., 2009; Clause et al., 2014), and may represent a retrograde signal of those downstream maturation events. Trans-endocytosis at sites of synaptic communication was first described as the extension of thin elements called spinules that extended from dendritic spines and were engulfed by the presynaptic terminal (Spacek and Harris, 2004; Petralia et al., 2015). Recent studies using super-resolution light microscopy have demonstrated trans-endocytosis to be prevalent, occurring at nearly 25% of synaptic sites in layer 4 of the primary visual cortex (Campbell et al., 2020). Labeling for an early endosomal marker, like the small GTPase Rab5, may reveal evidence for a transition towards spine mat trans-endocytosis beginning between P6 and P9 while Rab7 may label fully detached spine mats (Rink et al., 2005). Our images suggest that lysosomal structures may serve as the final destination for trans-endocytosed materials. However, additional experiments will need to be performed to more carefully characterize this process.

Clues to molecular signals carried by trans-endocytosed structures may be gleaned from structures that can be similar in caliber (100 nm) but longer (50-100 μm), called cytonemes. First described in the wing imaginal disc cells in *Drosophila*, cytonemes are specialized filopodia that transport signaling proteins directly between cells (Ramírez-Weber and Kornberg, 1999). Cytonemes have been described in both neuronal (Casas-Tintó and Portela, 2019) and non-neuronal cell types (Kornberg and Roy, 2014a, 2014b) but in all cases exist as thin, actin-supported filopodia that are responsible for juxtacrine signaling. Cytoneme subtypes can be defined by their response to different attractant signals, such as the *Drosophila* homolog of the vertebrate bone morphogenic protein (BMP), Decapentaplegic (Dpp); or the epidermal growth factor protein, Spitz (Roy et al., 2012). Once guided to appropriate signaling targets, cytonemes

may exert their effect through surface expression of membrane bound proteins. For example, hedgehog (Hh) proteins, enriched along cytonemes protruding from support cap cells, are secreted locally onto nearby niche cells (Rojas-Ríos et al., 2012). Newly delivered Hh protein activates the downstream transcription of multiple bone morphogenic genes including Dpp and Glass bottom boat (Gbb) in escort cells and provides a mechanism directly regulate local stem cell proliferation (Rojas-Ríos et al., 2012). Alternatively, cytonemes may in other cases deliver membrane bound molecules via trans-endocytosis (Parker et al., 2017). Finally, cytonemes are also capable of forming “gap junction-like” pores when contacting a second cell, producing a cytoneme variant called a tunneling nanotube. Tunneling nanotubes have been shown to bridge cells up to 100 μm apart, and were responsible for direct trans-endocytic exchange of cytoplasmic material between nervous cells (Vidulescu et al., 2004). Moreover, these cytoneme variants create web-like linkages between cells that can be stabilized and maintained in neural circuits and have been implicated in the pathophysiologic transfer of both amyloid A β peptide and tau during the progressive development of degenerative Alzheimer’s disease (Wang et al., 2011; Tardivel et al., 2016). We do not have direct evidence for this type of intercellular communication, but our SBEM images were not of sufficiently high resolution to capture even transient expression of patent intercellular bridges. Future work in the MNTB will need to establish the molecular composition of the membrane-bound constituents of somatic spines, and provide very high resolution images of the intercellular border, in order to investigate cytoneme-like function in the development of the CH:MNTB.

General conclusions

The establishment of the CH:MNTB functional connection has emerged as a model system to study neural circuit formation. The large size of the CH and its rapid emergence, the relatively simple to assay endpoint of 1:1 connectivity, and stereotypical stages of development allow researchers to ask very fundamental questions about the mechanisms that give rise to a mature neural connection. The ultrastructural information revealed here through the perspective of our time series of SBEM image volumes and lattice light sheet dynamic imaging affords a unique opportunity to directly observe novel aspects of tissue reorganization that constitute neural circuit development. For example, we have characterized previously described single somatic spines and CH collaterals, provided clues to the dynamics and function of CH collaterals, and added a new element, the spine mat, to the unfolding story of neural circuit formation. The synchronized onset of pre and postsynaptic structural changes, and differential temporal profiles of the developmental events that we describe here, offer a baseline to study their underlying mechanistic signals. We hypothesize that the trigger is an altered pattern of spontaneous activity. Future experiments linking physiological and molecular perturbation with high resolution light microscopy will provide necessary platform to extract a more complete description of the sequence of structural plasticity characteristic of the formation of the CH:MNTB connection. We propose that lattice light sheet imaging with new combinations of cellular labels and molecular perturbation can achieve these goals. Although the CH system is among the few that achieve mono or few:one innervation ratios, insights gathered will likely apply to those other systems and aspects of CH development will likely have generalizable relevance to neural circuits formation throughout the CNS.

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Chapter 1, Figure 1. Growth cone motility is highly regulated to balance asynchronies in endocytosis and exocytosis, as well maintain dynamic stability of cytoskeletal elements. Permissive cues of chemoattraction tilt balance of these forces to steer outgrowth of the growth cone towards the attractant signal

Chapter 1, Figure 2. The organization of the auditory system has evolved to maintain tonotopic information relevant for frequency discrimination

Chapter 1, Figure 3. Ultrastructural reconstructions of developing GBC terminals in the neonatal MNTB (Holcomb et al., 2013) have been useful in investigating the stereotypical formation of a large nerve terminal. During the first postnatal week, 2 or 3 bouton-type terminals present from birth are permitted to grow large. Convergent inputs compete to establish a the largest apposed surface area possible across the somatic surface of its target, the PC of the MNTB. However, following the opening of the ear canal ($\sim$ P10), the large spoon-like protocalyx will undergo its final structural transformation, achieve the mature fenestrated morphology.

Chapter 2, Figure 1. Structural characterization of CH collateral network. **A**, Collaterals of CHs of different sizes and developmental stages. A₁-A₄, Short filopodia (chevrons) and longer filopodial (open arrowheads) and growth cone-tipped (closed arrowheads) thin collaterals extend in large numbers from CHs at neonatal ages. A₅, Collaterals are largely absent by maturity, P30. **B**, The absolute number of collaterals increases with CH size until P6; collaterals consolidate immediately prior to the onset of detection of peripheral auditory cues. **C**, Distribution of collateral stalk length excluding added length of preterminal collateral arborization. Bin size: 1 μm . Dotted gray lines: 25th, 50th and 75th quartiles. **Inset**, Thin collaterals were skeletonized and their length measured from their origin at CH terminal to their distal tip. CH from A₂. **D**, Length of all collaterals fully contained within the SBEM volume plotted with respect to the ASA of each developing CH. 50th percentiles indicated for long filopodial-tipped (circular symbols linked by black dashed lines) and growth cone-tipped collaterals (filled triangles linked by black solid lines) (color code referencing age matches scheme used in **B**). Scale bars: **A**, **C**, 20 μm .

Chapter 2, Figure 2. Structural elements of the developing calyx of Held (CH). **A**, P3 calyx. Its afferent axon (ax) and smaller diameter distal axonal extension (ax') exit the image volume. Interstitial branches of different lengths (open arrows) emerge from the axon. Three categories of thin collaterals extend from the CH: short neurites with filopodial terminations (two examples, chevrons), long neurites with blunt filopodial terminations (four examples, open arrowheads), and long neurites with growth cone terminations (five examples, filled arrowheads). Only one of four different MNTB neurons innervated by this CH is depicted. Inset, Close-up of yellow boxed area reveals most collaterals emerging from the edges of the CH. Symbols indicate origins of collaterals in **A**. **B-E**, Electron micrographs of regions boxed in panel **A**. cb, cell body of MNTB principal neuron; m, mitochondria; s, synapse; white arrowhead, synaptic vesicle; CH, calyx of Held; gc, growth cone; arrows, reticulated membrane; f, filopodia. **B**, CH apposed to cell body of MNTB principal neuron; presynaptic structures include mitochondria and synaptic vesicles clustered that near a convexity in the somatic membrane, forming a likely synapse. **C**, Growth cone-tipped collateral that does not contact a neuron has little intracellular structure except for sparse distribution of synaptic vesicles. **D**, Distal section through a filopodia-collateral where it apposes the cell body of the principal cell and is filled with synaptic vesicles. **E**, Proximal and distal sections through a growth cone-tipped collateral. In this case the growth cone extends in apposition to cell body and contains synaptic vesicles but lacks mitochondria. Scale bars: **A**, 20 μm ; **B-E**, 0.5 μm .

Chapter 2, Figure 3. Interactions between collaterals originating from different terminals. A-C, During early postnatal development CH terminals growing along nearby principal neurons extend collaterals within a common neuropil. D, Most collateral extensions emanating from CH terminals reside within a local proximity of <10 μm from the somatic surface of the principal cell being innervated. E, Neighboring principal cells may support CHs that do not make direct contact along the surface of any collaterals. F, Other neighboring pairs intimately intertwine within a shared volume between innervated neurons. Scale Bar: 5 μm.

Chapter 2, Figure 4. Methods for tissue labeling and fluorescence imaging in acute coronal slices through the brainstem. **A**, P5 acute brain slice. Synaptic connections from the ventral cochlear nucleus (VCN) to the contralateral medial nucleus of the trapezoid body (MNTB) are largely preserved in a coronal slice through the auditory brainstem. Parvalbumin driven cre recombination leads to strong fluorescence (red, tdTomato) in the auditory nerve (AN), VCN cell bodies, VCN efferents projecting to the contralateral trapezoid body and presynaptic CH terminals within the MNTB. A subset of neurons and glia in the MNTB were labeled with EGFP (green) through recombinant adenovirus (rAd pUN GFP) introduced directly into the MNTB at P0. Inset: Slice plane. **A'**, close-up of large rectangle in **A**: AN fibers and bushy cells within the VCN show tdTomato labeling. A subset of bushy cells were retrogradely labeled via the CH terminal by pUN EGFP virus (yellow; filled arrowheads). **A''**, Expanded view of small rectangle in **A**: trapezoid body axons (red) and CHs (arrows) are strongly labeled with tdTomato. EGFP is expressed in a subset of principal neurons (fletched arrow), non-principal neurons (closed arrowhead) and glial cells (open arrowhead) within the MNTB. Box: approximate size of imaging field for lattice light sheet (LLS) microscopy. **B**, Tissue slices were embedded in 1% low melting point agarose mounted on a slatted metal frame and subsequently affixed to a sample holder (labeled in **D**). **C**, Tissue positioned within a perfusion chamber and supplied with heated and oxygenated ACSF. The green plane indicates excitation sheet presentation through the sample orthogonally to the detection objective. The orthogonal arrangement of objectives results in collection of parallelepiped 4D volumes following each time point (arrow indicates direction in which sample is advanced; image volume presented at larger than actual scale relative to objectives). **C'**, Custom Bessel patterns were optimized for imaging of fluorescent probes present in the sample (560 nm shown).

Chapter 2, Figure 5. Short collateral dynamics. **A**, Perspective view of labeled calyx shows presence of type I collateral branches. (Fluorescent signal presented with absorption model filter.) **B-B''** Manual tracking of the tip of individual collaterals reveal dynamic extension and retraction of each collateral outlined in **A**. Note a near synchronous extension and staggered retraction of each collateral. **B'-B''**, Selected frames (correlated red and black arrows in **B**) depict length changes in two short collaterals from **A**. (Note a near synchronous extension and staggered retraction of each collateral.) **C-E'**. Upper panels (**C**, **D**, **E**) described net changes in collateral length (each trace represents a unique collateral) as linearly defined as the distance from the CH to the tip of each collateral. Lower panels, (**C'**, **D'**, **E'**) depict the rate of change in collateral trajectories observed during live imaging experiments. Scale Bar: **B**, 2 μm .

Chapter 2, Figure 6. Long filopodial collateral dynamics. **A**, Small volumes within the MNTB are dominated by fluorescently labeled CH collaterals. **B**, Selected frames from a P3 tissue section highlight the emergence, translocation and retraction of several filopodial arbors emanating from the leading segment of a filopodial collateral branch. (sub volume from **A**). **C-D**, Average and maximum velocities of filopodia-tipped collaterals demonstrate reduced speeds relative to growth cone-tipped collaterals but surprising increase in motility as long collaterals begin to retract just prior to the onset of hearing (P9). Scale Bar: 5 μm .

Chapter 2, Figure 7. Type III collateral dynamics. **A**, Perspective view of two labeled CHs exhibit intertwined collateral networks. Note at least two type III collaterals in the center of the frame (arrowheads). (Fluorescent signal presented with absorption model filter.) **A'**, Zoomed view from **A** provides clearer depiction of growth cones emanating tens of microns from their CHs or origin. **B**, Time lapse images from P3 reveal motility of type III collateral (cyan). **C'**, Time lapse images from P5 reveal similar motility of type III collaterals (red and yellow). **D**, Plot of the velocity (dotted) and displacement (velocity) versus time of the type III collateral in **B**. Peak velocities for this growth cone reached above $80 \mu\text{m}/\text{min}$ with sampling frequency of 1.3 Hz. **E**, Plot of the velocity (dotted) and displacement (velocity) versus time of the type III collaterals in **C**. Peak velocities for this growth were generally less than those found at P3. Note, long branch retracted between frames (arrow). **F**, Average (red/green) versus maximum (pink/black) velocities reveal differences in motility across age. The fastest movement of type II collaterals occurred at P3. Scale Bars: **B,C**, $10 \mu\text{m}$

Chapter 2, Supplemental Figure 1. PV-cre driven expression of tdTom in the ventral auditory brainstem. Top-down view of thick coronal section (300 μm) sectioned in the coronal plane imaged with two photon microscopy. Inset, Rotated view about the MNTB. Scale Bars: 200 μm

Chapter 2, Supplemental Figure 2. Neighboring CH terminals make frequent contact. **A**, Two neighboring CHs that innervate adject PCs exhibit extensive overlap of collaterals. **B**, A closer inspection reveals a complex association between arbors arising from different cells. **C**, Live LLS time-lapse reveals contact between growth cones is transient (< 30 seconds). **D**, Growth cones were similarly observed contact the spoon like terminal growing along a less immediate neighbors (>20 μm). Scale bars: **A**, 5 μm ; **B**, 2 μm

Chapter 3, Figure 1. Somatic processes within and peripheral to territory of calyx of Held (CH) nerve terminals. **A-A'**, 3D reconstructions reveal that multiple calyceal terminals (region of membrane apposition with cell soma outlined and shaded in blue, green and orange, in descending order of size respectively) converge on a single principal cell (PC) soma at P4. Single somatic processes, called type 1 spines, visualized as bumps on the somatic surface (examples indicated by red arrowheads), extend into a matching invagination within the presynaptic face of the terminal (not shown). Long, branched somatic appendages (white arrows) extend from the somatic surface outside of the borders of each growing calyx. **B-B'**, Somatic processes that underlie larger CH terminals occur singly (type 1 spines, red arrowheads) or intertwined into spine mats (red arrows). Many of the longest somatic processes occur at the edge of the calyx apposition territory (blue). **C**, Single electron micrograph reveals the CH nerve terminal (labeled CH and shaded blue) contacting the cell body (cb, gold) of a P6 principal neuron. Numerous type 1 spines (white arrowheads) and spine mats (white arrows) extend into the CH. **C'-C''**, Boxed areas are expanded to show a single somatic type 1 spine (C'; dotted inset) and outline the component spines, called type 2 spines, that form a spine mat (C''; solid inset). Red traces indicate the borders of each spine structure as traced across serial sections for subsequent 3D meshing and quantification. Scale Bar: **A-B'**, 1 μm ; **C**, 2 μm ; **C'-C''**, 0.5 μm .

Chapter 3, Figure 2. Varying morphology of somatic type 1 spines from principal neurons. **A-G**, 3D reconstructions (left column) and two image planes (middle and right columns; image planes indicated by dashed lines on 3D structure) through each type 1 spine. **A-B**. Short type 1 spines have similar base (**A''** and **B''**) and length dimensions. In **B'**, **B''**, asterisk indicates nearby type 1 spine near the edge of the CH that contacts an astrocytic process (solid arrow). **C-D**. Long tubular type 1 spines could extend tangentially (**C'**, **C''**) or through nearly the entire thickness of the calyx (**D'**, **D''**), which can exceed 2 μm . Spine in **C** is also at edge of apposed surface area and contacts glial process (solid arrow). **E-F**. The most complex type 1 spine geometries include expansion and bending of the spine head. Asterisks indicate nearby type 1 spines. **G**, Despite heterogeneity in overall type 1 spine morphology, spine caliber remained consistent with 96% of type 1 spines maintaining a diameter between 0.2-0.5 μm (P3: 340 ± 100 nm; P4: 320 ± 60 nm; P6: 300 ± 90 nm; P9: 310 ± 80 nm; P30: 320 ± 100 nm). Scale Bar: **A-F''**, 0.5 μm .

Chapter 3, Figure 3. Spine mat comprised of 19 type 2 spines from a P6 principal neuron. **A**, 3D reconstruction of spine mat, with individual type 2 spines shown in different colors. Each spine also shown in exploded view surrounding the spine mat. Individual type 2 spines can branch and generally exhibit more complex geometry (e.g., #2, 5, 12, 15, 17) than type 1 spines. White line on each spine indicates location of attachment to the principal cell body. **B-C**, Single EM sections at two locations through the spine mat illustrate complex intertwining of 15 of the 19 type 2 spines. Colors and numerical labels correspond to the 3D reconstructions illustrated in **A**. Note in addition to various spines, both presynaptic calyceal processes (blue) and glial processes (asterisk, not colored) intermingle within spine mats. Scale Bar: **A**, 2 μm ; **B**-**Bii**, 1 μm .

Chapter 3, Figure 4. Analysis of Spine Complexity using Convex Hull Fitting. **A-C**, The ratio of convex hull to corresponding spine mesh volume was calculated for type 1 (blue) and type 2 (red) spines and plotted against the spine volume across ages (P3,6,9). With the exception of a small number of cases for type 1 spines which represent highly curved fingerlike projections that lack true complexity (see panel **D**, 6th spine from the left for an example), type 2 spines consistently showed greater complexity scores than type 1 spines at P3 and P6. Insets (**A'-C'**) zoom into smaller values, where the majority of the data are located, reveals divergence of values above small volumes indicative of simple spines ($< 0.2 \mu\text{m}^3$). Note that at P9 relatively fewer type 2 spines exceed $0.5 \mu\text{m}^3$. (**D-E**) Representative type 1 (blue) and type 2 (red) spines encompassing the range of sizes for each at the three ages analyzed (P3,6,9) are presented in order of increasing size (spine volumes above). Convex hulls (grey veils surrounding spines) were included for each object and in every case, produced larger resultant volumes (below). Based on these differences in size range and complexity difference of small spines, there was a statistically significant difference between single spines and intertwined spines at all ages (average complexity score for single vs. intertwined spines – P3: 1.32 ± 0.3 vs. 2.64 ± 1.1 , P6: 1.31 ± 0.3 vs. 2.50 ± 1.4 , P9: 1.27 ± 0.2 vs. 1.52 ± 0.3 ; $p < 0.0001$ for all comparisons within age using the Mann-Whitney U-test (M-W); Fig. 4A-C). Scale bar: **D-F**, $1 \mu\text{m}$.

Chapter 3, Figure 5. Stereo views of single CH terminals oriented *en face* illustrate age-dependent changes in type 1 spine and spine mat density during development of the CH. **A**, Large terminals ($>25\mu\text{m}^2$ in ASA) identified as early as P3, reveal relatively few invaginations associated with somatic type 1 spines (yellow). **B**, By P6, larger densities of type 1 spines (yellow) and spine mats (assorted colors) are found throughout the area of pre- and postsynaptic association. Arrow in **B** corresponds to the spine mat presented in **Figure 3**. **C**, The mature CH exhibits greater fenestration than immature terminals, a much lesser density of type 1 spines, and only rare occurrences of spine mats (none occur in this terminal). Mature CHs extend further around the perimeter of principal cell bodies and partially obscure the view of the inner surface. Scale Bar: **A-C**, 5 μm .

Chapter 3, Figure 6. Type 1 spine and spine mat occurrence and composition across developmental age. **A**, Number of type 1 spines increased with size of the calyx of Held (CH). For a given terminal size, type 1 spine number increased with age from P3 to P6, the latter of which was similar to P9, and decreased at P30. Slope of line increased with age from P3 to P6 and did not change further through P30 (P3, $m = 0.100$, $r^2 = 0.92$; P4, $m = 0.181$, $r^2 = 0.96$; P6, $m = 0.267$, $r^2 = 0.97$; P9, $m = 0.268$, $r^2 = 0.94$; P30, $m = 0.243$, $r^2 = 0.72$). **B**, Density of type 1 spines per $10 \mu\text{m}^2$ of somatic surface area significantly increased between P3 and P9 ($p < 0.01$, K-W) and returned to returned to initial densities at P30 similar to P3 ($p = 0.99$, K-W). **C**, Spine mat number scaled similarly with size between P3-6, and the slope was similar but with larger y-intercept at P9 (P3, $m = 0.0086$, $r^2 = 0.6828$; P4, $m = 0.0095$, $r^2 = 0.502$; P6, $m = 0.0132$, $r^2 = 0.4326$; P9, $m = 0.0136$, $r^2 = 0.1133$; P30, $m = 0.0005$, $r^2 = 0.4527$). Across age, CHs $< 150 \mu\text{m}^2$ generally lacked spine mats, as did most cells at P30. **D**, Similar to type 1 spine density, spine mat density per $10 \mu\text{m}^2$ of somatic surface area significantly increased between P3 and P9 ($p < 0.01$, K-W) before spine mats again become significantly rarer by maturity at P30 ($p < 0.01$, K-W). **E**, Distribution for number of type 2 spines in each spine mat, combined across age because of similarity in distributions (mean: 6.8 ± 5.0 ; median: 6.0; mode: 4.0). **F**, Cumulative distribution of spine mat composition (as defined by the number of component type 2 spines) reveals the narrowest distribution at P3 and widest at P4, P6 and P9. At P30, only one spine mat was found. Dashed line illustrates the 50th percentile in the cumulative distribution function and reveals a shift to more type 2 per mat between P6 and P9 (gray and green arrows, respectively) relative to younger ages.

Chapter 3, Figure 7. Competition among CH terminals. **A**, Two representative examples of competing CHs with unique axons (ax) at P3, P4 and P6 (largest input labeled in blue, second largest input in green; as measured by ASA). Somatic spines (red) and spine mats (pink) situated within invaginations in the associated competing input are illustrated without the soma to reveal their locations across the presynaptic face of each terminal. **B**, The largest input of the competing pair tends to cover more type 1 spines. **C**, Type 1 spine density, however, is not consistently greater in the largest CH as only at P4 does the larger input contain significantly more embedded type 1 spines ($p < 0.05$, K-W with Nemenyi test). **D**, **E**, No spine mats were found in competing terminals at P3. At P4 and P6 there is no consistent relationship between spine mat number or density and CH size. Scale Bar: **A**, 5 μm .

Chapter 3, Figure 8. Type 1 spine ultrastructure and innervation. **A-E**, Synapses (arrowheads) identified in SBEM images by dense clusters of vesicles near the presynaptic membrane across multiple sections and located adjacent to membrane in at least one section. Type 1 spines are largely devoid of stained organelles. This organelle exclusion zone extends several hundred nm into the cell body (dotted lines). Straight dotted lines indicate base of type 1 spines. **A-A'**, Adjacent sections reveal synapses located at base (lowest arrowhead) and near tip of a single type 1 spine (middle and top arrowheads). Asterisks indicate nearby type 1 spines. **B-C**, Synapses were most frequently identified at the base of type 1 spines. **D-E**, A subset of type 1 spines contained no visible synapses and exhibited a thin layer (transparent red band) adjacent to the presynaptic membrane devoid of vesicles. Typical somatic synapses were used as a reference to characterize inputs to type 1 spines. cb, cell body; CH, calyx of Held. **F**, Most type 1 spines were innervated throughout development with at least one visible synapse. By P30, nearly all type 1 spines were innervated. **G**, Cumulative distribution functions show that multiple synapses could occur onto a single spine at each age. Scale Bar: **A-D**, 0.5 μ m.

Chapter 3, Figure 9. Innervation of type 2 spines within spine mats. **A-H**, Synapses (arrowheads) found along type 2 spines were identified using the same criteria as type 1 spine inputs (red arrow indicates traditional somatic synapses along more flattened region of cell membrane for comparison). **B-D**, Postsynaptic organelles including reticulated membranous structures (fletched arrows) could infiltrate into type 2 spines. **I**, Three dimensional rendering of the spine mat segmented in **A-H** reveals the close association of type 2 spines typical of spine mats. **Ii**, The rendering above presents a top-down perspective looking through the presynaptic terminal to illustrate the occurrence of seven synapses found along the surface of the spine mat. **Iii**, 90° rotation of the spine mat reveals the attachment site (solid white overlay) of three of the six type 2 spines of the spine mat. **J**, Synapse density onto type 2 spines is similar from P3-6 and increased at P9 (P9, $p < 0.01$, Pairwise MW). Scale Bar: **A-I**, 0.5 μm .

Chapter 3, Figure 10. Evidence in Support of Trans-Endocytosis of Type 2 Spines. **A.** Three of five CH terminals analyzed at P9 (**i-iii**) each show evidence of type 2 spine detachment (green) from the somatic surface and suspension of spines within the CH. Spine mats in these CHs (**i-iii**; pink) can exist in various stages of type 2 spine detachment including “initial detachment”, “partial detachment” (fletched arrows) or “full detachment” (unfletched arrow) as illustrated in **B-D** respectfully. **B.** Unlike earlier ages, P9 spine mats may include membrane bound inclusions that cannot be traced back to a cell of origin despite most type 2 spines maintaining a clear attachment to the somatic surface (gold) and the macrostructure of the mat (pink and green) exists in a vertically compressed morphology close to the somatic surface. **B’-B’’**, Two of three component type 2 spines (pink) from this spine mat are illustrated in cross-section about their attachment to the underlying soma (gold). **C.** Spine mats observed transitioning to a trans-endocytotic state is hallmarked by several distinctive features including: inclusion of attached type 2 spines of diminished caliber (pink); overall morphology of the spine mat extends vertically away from the somatic surface (gold), and many component spines exist detached from the somatic surface (green). **C’-C’’**, Selected 2D planes highlight the heterogeneity in spine size in a transitioning spine mat relative the large caliber type 2 spines evident where each is somatically attached (**B’-B’’**). **D.** A trans-endocytosed spine mat is composed entirely of untethered spines (green) that reside wholly within the presynaptic terminal (detached mat in green resides above the presynaptic membrane shown in blue) while most other type 1 and 2 spines remain situated within invaginations of the presynaptic membrane (under the blue surface). **D’-D’’**, Detached spines in a trans-endocytosed mat are smaller than attached spines (see red spines immediately below) and the macrostructure is compressed into a sphere-like assemblage of component spines. Scale Bar: **A**, 5 μm ; **B-D’’**, 0.5 μm .

Chapter 3, Supplemental Figure 1. Comparison of single spine lengths across developmental ages. Mean values for single spine length per age increased from P3-6. Spine length declined by P9 and this shorter length is maintained until at least P30. Error bars are mean $\pm$ SD; n-values for each age indicated on plot, total n = 2,076. Statistically significant pairwise differences in length were found between all ages with the exceptions of P3-P4, P4-P6, and P9-P30 (Kruskal-Wallis H-test with Nemenyi post-hoc test and Bonferroni correction (K-W)).

Chapter 3, Supplemental Figure 2. Transendocytosis of somatic spine mats and local degradation within the CH. **A-B'**, Two sections through a spine mat at P9 show several spines attached to the soma intermixed with apparent detached spines. Clusters of synaptic vesicles are located near the type 2 spines. In contrast, an organelle-free zone surrounds the detached elements within the CH (dotted red lines in **A'**, **B'**). **C**, Fully detached spines, with organelle free zone, located at axon junction with the CH. This example may reflect spine mat degradation into a lysosome or multivesicular body. **A'-C'**, Colored labels: blue, CH; gold, cell body; type 1 spine, red (at left in **B'**); type 2 spines, pink; detached spines, green; organelle-free zone, dashed red outline. Scale Bar: **A-C'**, 0.5 μ m.

Postnatal Day (P)	Number of Terminals	Number of Type 1 Spines	Type 1 Spine Length (μm)	Type 1 Spine Volume (μm^3)	Per Type 1 Spine Surface Area (μm^2)	Per Terminal Type 1 Spine Surface Area (μm^2)	% of Terminal ASA on Type 1 Spines	Number of Type 2 Spines	Number of Spine Mats
Single Dominant Terminal									
P3	6	125	0.792 ± 0.42	0.063 ± 0.06	1.07 ± 0.69	26.016 ± 14.13	10.7 ± 4.4	72	11
P4	9	436	0.866 ± 0.57	0.077 ± 0.07	1.168 ± 0.77	60.944 ± 26.45	20.5 ± 4.8	176	34
P6	3	247	0.967 ± 0.51	1.505 ± 1.08	1.505 ± 1.08	140.928 ± 73.74	37.4 ± 10	110	24
P9	5	658	0.609 ± 0.27	0.703 ± 0.45	0.703 ± 0.45	100.39 ± 36.54	17.2 ± 4.1	571	89
P30	5	144	0.621 ± 0.36	0.043 ± 0.05	0.76 ± 0.62	22.335 ± 21.11	4.63 ± 3.6	4	1
Largest Competing Terminal									
P3	6	42	0.709 ± 0.36	0.067 ± 0.07	1.006 ± 0.67	7.045 ± 4.36	14.4 ± 17	0	0
P4	4	147	0.845 ± 0.45	0.078 ± 0.07	1.181 ± 0.77	43.392 ± 17.75	20.075 ± 7.58	98	7
P6	3	117	0.823 ± 0.42	0.078 ± 0.08	1.189 ± 0.78	46.383 ± 6.79	30.333 ± 8.07	43	7
Second Largest Competing Terminal									
P3	6	29	0.673 ± 0.21	0.046 ± 0.03	0.797 ± 0.37	3.85 ± 2.73	8.417 ± 7.25	0	0
P4	4	37	0.701 ± 0.31	0.053 ± 0.04	0.912 ± 0.48	8.436 ± 5.07	8.978 ± 2.653	3	1
P6	3	85	0.741 ± 0.28	0.078 ± 0.07	1.204 ± 0.71	34.101 ± 24.52	26.75 ± 10.285	49	8

Chapter 3, Table 1: Summary of morphological characteristics and terminal association of type 1 spines across all ages studied (P3-P30). Prevalence of type 2 spines and spine mats is also quantified.

	Terminal	Terminal ASA (μm^2)	Number of Type 1 Spines	Type 1 Spine Length (μm)	Type 1 Spine Volume (μm^3)	Per Type 1 Spine Surface Area (μm^2)	% of Terminal ASA on Type 1 Spines
P3	1a	68.8	32	0.73 ± 0.4	0.068 ± 0.05	1.05 ± 0.6	48.8
	1b	33.5	7	0.60 ± 0.2	0.057 ± 0.05	0.88 ± 0.5	18.4
	2a	68.5	5	0.77 ± 0.3	0.073 ± 0.07	1.04 ± 0.7	7.6
	2b	42.5	8	0.65 ± 0.3	0.049 ± 0.04	0.84 ± 0.4	15.8
	3a	57.3	5	0.67 ± 0.4	0.086 ± 0.20	0.93 ± 1.1	8.1
	3b	35.5	2	0.64 ± 0.01	0.036 ± 0.02	0.68 ± 0.2	3.8
	4a	142	7	0.66 ± 0.4	0.052 ± 0.03	0.90 ± 0.5	2.2
	4b	121	7	0.71 ± 0.2	0.042 ± 0.02	0.77 ± 0.3	4.4
	5a	53.4	5	0.73 ± 0.1	0.044 ± 0.02	0.89 ± 0.3	8.4
	5b	38.1	0	0	0	0	0
	6a	140	15	0.83 ± 0.6	0.064 ± 0.07	1.05 ± 0.7	11.3
	6b	43.2	5	0.62 ± 0.2	0.034 ± 0.02	0.70 ± 0.3	8.1
P4	1a	259	43	0.68 ± 0.3	0.048 ± 0.04	0.84 ± 0.5	13.3
	1b	74.6	7	0.53 ± 0.2	0.028 ± 0.01	0.58 ± 0.2	5.4
	2a	254	42	1.18 ± 1.1	0.099 ± 0.09	1.35 ± 0.9	21.9
	2b	84.3	11	0.71 ± 0.3	0.042 ± 0.03	0.79 ± 0.4	10.2
	3a	117	17	0.76 ± 0.3	0.076 ± 0.05	1.17 ± 0.7	15.1
	3b	48.2	5	1.12 ± 0.6	0.070 ± 0.05	1.12 ± 0.6	11.6
	4a	193.4	45	0.86 ± 0.5	0.089 ± 0.07	1.29 ± 0.8	30
	4b	178.7	14	0.82 ± 0.4	0.069 ± 0.04	1.16 ± 0.5	7.8
P6	1a	150.9	35	1.24 ± 1.6	0.083 ± 0.10	1.25 ± 1.1	28.9
	1b	131.4	34	0.75 ± 0.3	0.066 ± 0.20	1.06 ± 0.5	27.4
	2a	240.8	47	0.78 ± 0.3	0.068 ± 0.06	1.10 ± 0.6	21.5
	2b	157.7	41	0.78 ± 0.3	0.089 ± 0.08	1.31 ± 0.9	34
	3a	104	30	0.85 ± 0.4	0.085 ± 0.06	1.29 ± 0.6	37.2
	3b	50.9	7	0.70 ± 0.2	0.073 ± 0.03	1.14 ± 0.5	15.6

Chapter 3, Table 2: Pairwise comparison of morphological characteristics and terminal associations of the two largest competing terminals per cell.

Group 1	Group 2	p-value
P3	P4	0.105782
P3	P6	0.000681
P3	P9	4.81E-06
P3	P30	0.000256
P4	P6	0.170715
P4	P9	3.75E-14
P4	P30	8.55E-11
P6	P9	3.75E-14
P6	P30	1.58E-14
P9	P30	0.972052

Chapter 3, Supplemental Table 1: Pairwise statistical comparison in single spine lengths between developmental ages using the Kruskal-Wallis H-test with Nemenyi post-hoc test and Bonferroni correction. All pairwise comparisons were statistically significant except for P3-P4, P4-P6, and P9-P30.

Chapter 4, Figure 1: RC::PFtoxin provides conditional expression of a GFPtoxin fusion protein for ‘synaptic silencing’. **A**, RC::PFtoxin contains two stop cassettes, one flanked by directly oriented loxP sites (P-pentagons) and the other, by FRT sites (F-squares) that prevent expression of mCherry and GFPtoxin (**A_i**). **B**, Cre-mediated stop cassette recombination permits mCherry expression (**B_i**) while GFPtoxin expression is prevented by the remaining FRT-flanked stop cassette. **C**, Cre- and Flp-mediated recombination results in constitutive GFPtoxin expression (**C_i**). **D**, RC::PFtoxin crossed with PV-cre results in strong mCherry expression in neurons of the cochlear nucleus in postnatal animals with clear labeling of projections along the ventral acoustic stria (VAS) towards targets in the ipsilateral lateral superior olive (LSO) and the contralateral medial nucleus of the trapezoid body (MNTB). Scale Bar: **A_i**-**C_i**, 250 μ m; **D**, 500 μ m.

Chapter 4, Figure 2: Resultant litters of RC::tox animals from a PV background exhibit variable phenotypes revealed by disparate motor impairment and impaired CH growth by P9 revealed by immunohistochemical analysis. **A**, Homozygous RC::PFto_x animals produce stereotypical cup-like innervation to the developing PC of the MNTB, with contiguous VGLUT+ compartments that envelop the PC soma. **B**, 'Weakly phenotypic' RC::tox animals produce fewer cup-like terminals in the developing MNTB. **C**, 'Strongly phenotypic' RC::tox animals exhibited gross malformations in CH structure relative to controls. (**B-C**), Cell bodies are labeled with anti-microtubule associated protein 2 (MAP2, green), presynaptic terminals are labeled with anti-vesicular glutamate transporters 1 and 2 (VGLUT, blue), and paradoxically anti-GFP (red). Arrowheads indicate overlapping GFP and VGLUT signal is restricted to presynaptic compartments. **D**, Reconstruction of the presynaptic terminals revealed inhibition of synaptic activity significantly reduced the formation of stereotypical CH terminals. Scale Bar: **A-C**, 20 μ m.

Chapter 4, Figure 3: Unilateral inhibition of synaptic activity ahead of the growth of the CH. **A**, On the day of birth, the left VCN was targeted for stereotaxic delivery of a replication incompetent adenovirus for rapid expression of the enzymatically active light chain of tetanus toxin for selective abolishment of synaptic vesicle release (HdAd28E4 Cpun TeTxLC syn mCherry). **B-Biv**, Within days of transfection, robust mCherry labeling suggests strong TeTxLC expression in labeled cells. **C-Civ**, By P5, cells across the injected VCN express mCherry. **D-Div**, Projections from the contralateral VCN were also retrogradely transfected. **E-Eiv**, Despite bilateral expression of mCherry, no labeled CHs were observed in the MNTB ipsilateral to the injection site. **F-Fiv**, Numerous mCherry labeled endings were found in the MNTB contralateral to the injection site. **B-F**, Cell bodies are labeled with anti-microtubule associated protein 2 (MAP2, green), presynaptic terminals are labeled with anti-vesicular glutamate transporters 1 and 2 (VGLUT, blue), and presynaptic afferents are shown in red (mCherry). Scale Bar: **A**, 200 μ m; **B**, 500 μ m; **C-F**, 250 μ m.

Chapter 4, Figure 3: Unilateral inhibition of synaptic activity provided within animal controls for the evaluation of CH growth downstream of 'synaptic silencing'. **A**, Non-transfected afferents to the ipsilateral MNTB produced cup-like terminals free of mCherry expression although punctate mCherry labeling likely represent commissural GBC axons from the transfected VCN. **B**, Within the contralateral MNTB, relative to the stereotaxic injection, enlarged mCherry+ signal colocalizes with VGLUT labeling suggesting the formation of at least some enlarged terminals. **C-F**, Reconstruction of the morphology of the CH in each MNTB were evaluated to assess the effect of TeTxLC expression on terminal morphology. **C**, Silenced terminals formed bloated terminals that almost double the thickness of the stereotypically thin and cup-like terminals

in the ipsilateral MNTB (TeTxLC-). **D**, Silenced terminals occupied nearly half the somatic area occupied by CHs in the ipsilateral MNTB (TeTxLC-). **E**, Abolishment of presynaptic activity resulted in a reduction in postsynaptic cell volume. **F**, Despite silenced terminals inability to form a stereotypical morphology, no difference in terminal volume was detected between brain hemispheres. **A-B**, Cell bodies are labeled with anti-microtubule associated protein 2 (MAP2, green), presynaptic terminals are labeled with anti-vesicular glutamate transporters 1 and 2 (VGLUT, blue), and presynaptic afferents are shown in red (mCherry). Scale Bar: **A-B**, 20 μm .

Chapter 5, Figure 1: Summary of the major events pertaining to the growth of the CH.