Review

Formation and maturation of the calyx of Held

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ABSTRACT

Sound localization requires precise and specialized neural circuitry. A prominent and well-studied specialization is found in the mammalian auditory brainstem. Globular bushy cells of the ventral cochlear nucleus (VCN) project contralaterally to neurons of the medial nucleus of the trapezoid body (MNTB), where their large axons terminate on cell bodies of MNTB principal neurons, forming the calyces of Held. The VCN–MNTB pathway is necessary for the accurate computation of interaural intensity and time differences; MNTB neurons provide inhibitory input to the lateral superior olive, which compares levels of excitation from the ipsilateral ear to levels of tonotopically matched inhibition from the contralateral ear, and to the medial superior olive, where precise inhibition from MNTB neurons tunes the delays of binaural excitation. Here we review the morphological and physiological aspects of the development of the VCN–MNTB pathway and its calyceal termination, along with potential mechanisms that give rise to its precision. During embryonic development, VCN axons grow towards the midline, cross the midline into the region of the presumptive MNTB and then form collateral branches that will terminate in calyces of Held. In rodents, immature calyces of Held appear in MNTB during the first few days of postnatal life. These calyces mature morphologically and physiologically over the next three postnatal weeks, enabling fast, high fidelity transmission in the VCN–MNTB pathway.

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1. Introduction

The calyx of Held is a unique and highly specialized synaptic structure in the mammalian auditory brainstem. Its precise pathway and morphological distinctiveness have permitted detailed mechanistic studies of neural circuit assembly. Neurons of the ventral cochlear nucleus (VCN) receive cochlear input via the auditory portion of the VIIIth cranial nerve. VCN globular bushy cells project to the contralateral, but not ipsilateral, medial nucleus of the trapezoid body (MNTB), where they form the calyx of Held (Held, 1893; Kuwabara et al., 1991; Tolbert et al., 1982). The globular bushy cell axons are large diameter, glutamatergic fibers and the calyx encapsulates the somata of MNTB principal neurons with many finger-like processes that contain hundreds of synaptic active zones (Banks and Smith, 1992; Grandes and Streit, 1989; Satzler et al.,

2002; Taschenberger et al., 2002). Some of these axons branch and innervate multiple, adjacent MNTB neurons (Rodriguez-Contreras et al., 2006; Smith et al., 1991), although each MNTB neuron is innervated by a single calyx emanating from a single VCN neuron (Hoffpauir et al., 2006). MNTB neurons receive additional non-calyceal excitatory and inhibitory inputs (Bergsman et al., 2004; Green and Sanes, 2005; Hoffpauir et al., 2006; Rodriguez-Contreras et al., 2008; Smith et al., 1991). The source(s) of inhibitory inputs to MNTB is not known, however; they may arise from the ipsilateral VCN, the ventral and/or lateral nuclei of the trapezoid body, or from the MNTB itself (Awatramani et al., 2004; Green and Sanes, 2005; Kuwabara et al., 1991).

The VCN–MNTB pathway is essential for sound localization. In the mature brainstem MNTB principal neurons provide predominantly glycinergic inputs to the adjacent lateral and medial superior olives (LSO and MSO), which are components of the circuitry necessary for the computation of interaural intensity (level) and time differences (IIDs and ITDs), respectively (Brand et al., 2002; Caird and Klinke, 1983; Glendenning et al., 1992; Goldberg and Brown, 1969; Pecka et al., 2008; Sanes, 1990; Smith et al., 1993; Spitzer and Semple, 1995; Yin and Chan, 1990). MNTB neurons also project to the superior paraolivary nuclei (Banks and Smith, 1992; Sommer et al., 1993) and to the ventral nucleus of the lateral lemniscus (Smith et al., 1998; Sommer et al., 1993). The specialized

structural and functional properties of the calyx of Held ensure high fidelity transmission of temporal information that is required for determining these interaural differences.

Because of its large size and accessibility for electrophysiological recording, the calyx of Held has been widely used as a model system for synaptic transmission in the mammalian central nervous system. The calyx of Held has also drawn attention from researchers because of its distinctive anatomical and physiological characteristics. These characteristics have been extensively described in the mature auditory brainstem and the extraordinary precision of the circuitry has invited very interesting developmental investigations as well. Here we review the development of the VCN–MNTB pathway and the maturation of the calyx the Held.

2. Embryonic origins of VCN and MNTB

During embryogenesis, the cochlear nucleus complex, which is comprised of the VCN and dorsal cochlear nucleus (DCN), is derived mostly from the lower/caudal rhombic lip, a *Math1* (*Mouse Atonal homolog 1*)-positive germinal zone in the dorsal hindbrain spanning rhombomeres 2 (r2) to 8 (Wang et al., 2005). In rodents, cochlear nucleus neurons undergo their final mitotic division between embryonic days 10 and 14 (E10-14; Martin and Rickets, 1981; Pierce, 1967), but within the VCN the majority of neurons are born on E12 and E13 (Rose et al., 2009; Wang et al., 2005). Genetic fate mapping experiments have shown that VCN neurons originate in r2-4, with the anterior VCN arising mostly from r2 and r3 and the posterior VCN arising mostly from r4, while DCN neurons have an r5 origin (Farago et al., 2006). VCN neurons are generated in the cochlear extramural stream and their formation is *Math1*-dependent (Fujiyama et al., 2009; Maricich et al., 2009; Wang et al., 2005). Additionally, rhombomere specific deletions of the axon guidance receptor *Robo3* have provided evidence to suggest that all MNTB-projecting globular bushy cells of VCN are derived from r3 (Renier et al., 2010).

Fibroblast growth factor (FGF) signaling and *Math5* have also been implicated in the formation of the cochlear nucleus. Mice with decreased levels of *Sef*, an FGF antagonist, displayed varying levels of malformation in the cochlear nuclei and, conversely, *Sef* overexpression in embryonic chicks resulted in a reduction in the size of the cochlear nuclei (Abraira et al., 2007). *Math5* is expressed in the developing and adult VCN and MNTB and *lacZ*-positive VCN neurons are smaller in *Math5-lacZ* mutant mice (Saul et al., 2008). Additionally, *Sef* and *Math5* transgenic mice have auditory brainstem responses (ABRs) that reflect abnormal processing in the cochlear nucleus (Abraira et al., 2007; Saul et al., 2008).

MNTB neurons are derived from r3 and r5 (Maricich et al., 2009) and arise from the rhombic lip around the same time as cochlear nucleus neurons (E10.5-12; Hoffpauir et al., 2009; Rose et al., 2009), with lateral (low-frequency) neurons arising earlier than medial (high-frequency) neurons (Altman and Bayer, 1980). The generation and survival of superior olivary complex (SOC; which includes the MNTB) neurons also requires *Math1* (Maricich et al., 2009; Rose et al., 2009). Furthermore, it is likely that the different types of MNTB neurons are generated in different proliferative zones of the neuroepithelium and that they migrate and join to form the presumptive nucleus; a similar process occurs during the formation of the cochlear nucleus (Altman and Bayer, 1980; Fujiyama et al., 2009). Mechanisms and patterns of cellular migration in the mammalian auditory brainstem remain largely unknown because of the difficulty in using traditional techniques to perform the necessary experiments in embryonic rodents. Increased knowledge of spatiotemporal gene expression during these events, insights from other brain regions, and advances in mouse genetics will most likely increase our understanding of these key developmental processes.

3. Growth of VCN–MNTB projections

3.1. Timing of VCN axon growth and collateralization

Growth of VCN axons and formation of connections with their target MNTB principal neurons is a relatively protracted process that begins shortly after VCN neurons are born. Proper formation of VCN–MNTB circuitry requires guided axon growth from VCN towards the midline, growth across the midline, and collateral branching into MNTB on the contralateral, but not ipsilateral side. Once the VCN axons have branched and projected into the correct MNTB, VCN axons innervate the topographically appropriate region of the nucleus, preserving the tonotopy that originates in the cochlea. In the mouse, axon outgrowth from VCN neurons begins at around E13 (Howell et al., 2007). Most VCN axons reach and cross the midline by E14.5. It is believed that these axons branch to innervate the contralateral MNTB at E17 (Hoffpauir et al., 2009, 2010). Before calyx formation, MNTB neurons are innervated by multiple, non-calyceal VCN inputs. However, only one of these inputs matures into a calyx for the majority of MNTB neurons (Hoffpauir et al., 2010). Once calyces have formed, there is no large-scale, activity-dependent refinement or reorganization in the VCN–MNTB projection, suggesting that multiple molecular and cellular mechanisms ensure precise development (Kandler et al., 2009; Rodriguez-Contreras et al., 2006). Spontaneous activity generated in the cochlea before the onset of hearing may also play a role during the pre-hearing phase of development (Tritsch et al., 2007). This lack of refinement is unique compared to other sensory systems and other auditory pathways. For example, spiral ganglion axon terminals in the cochlear nucleus become more refined during postnatal development, which increases precision in cochlea-cochlear nucleus pathway (Leake et al., 2002). The projections from MNTB principal neurons to LSO (Kim and Kandler, 2003; Sanes and Siverls, 1991) and MSO (Kapfer et al., 2002; Werthat et al., 2008) undergo similar pruning.

3.2. Axon guidance molecules

Axon guidance in the auditory brainstem seems to be similar to axon guidance in other well-studied areas of the central nervous system, including the spinal cord, in which midline guidance has been extensively studied. During axon outgrowth, *netrin-1* mRNA is expressed at the midline of the brainstem and *DCC* (*Deleted in Colorectal Cancer*) mRNA is expressed in VCN neurons, with robust DCC immunoreactivity in VCN axons (Howell et al., 2007). Furthermore, VCN axons fail to reach the midline in *netrin-1* and *DCC* deficient mice (Fig. 1B). This observation provides evidence to suggest that DCC-positive VCN axons are attracted to netrin-1 secreted from the midline during embryonic development (Howell et al., 2007). Midline attraction is an essential step in the formation of the VCN–MNTB pathway, as it is a purely crossed projection. Once at the midline, decussating axons must decrease their attraction to the midline in order to extend past the midline to the contralateral target.

Similar to the spinal cord, *slit1*, *-2*, and *-3* mRNAs are expressed by midline cells and mRNAs for *Robo1*, *-2*, and *-3* are expressed in the cochlear nucleus (Howell et al., 2007; Renier et al., 2010). A recent study examined transgenic mice with *Krox20*-driven deletion of *Robo3*, in which the null mutation is limited to cells derived from r3 and r5. These mice have a completely *ipsilateral* VCN–MNTB projection (Renier et al., 2010; Fig. 1C). Interestingly, calyces of Held still form in the MNTB of these mutant mice, but they are on the wrong (ipsilateral) side of the brainstem. As expected, this abnormal circuitry results in irregular ABRs. There is no difference between the peripheral hearing thresholds of mutant and wild type mice, and no difference in the amplitude and latency
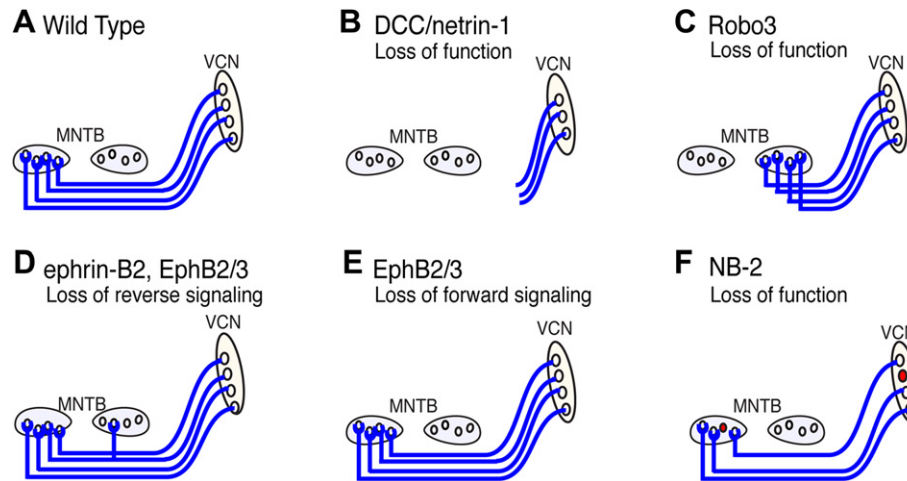
Fig. 1. Summary of mutations affecting VCN–MNTB axon targeting. A, In wild type mice, VCN axons project to the contralateral MNTB only. B, VCN axons never reach the midline in *DCC* and *netrin-1* deficient mice (Howell et al., 2007). *DCC* deficient mutant mice also have less VCN neurons than heterozygous and wild type littermates (Howell et al., 2007). C, VCN axons abnormally project to the ipsilateral, not contralateral, MNTB in *Krox20::cre;Robo3* mutant mice (Renier et al., 2010). D, Mutations in Eph proteins that reduce reverse signaling through ephrin-B ligands include deletions of the intracellular domain of ephrin-B2 and null mutations in EphB2 and EphB3. These mutations result in a significant number of aberrant ipsilateral VCN–MNTB projections (Hsieh et al., 2010). E, When only the intracellular domain is deleted in EphB2, forward signaling is reduced while reverse signaling through ephrin-B2 is intact. These mice have normal, strictly contralateral VCN–MNTB projections (Hsieh et al., 2010), suggesting that reverse, but not forward, EphB/ephrin-B signaling regulates the precision of this pathway. F, In *NB-2* knockout mice, a percentage of MNTB principal neurons lack calyces of Held. The number of apoptotic neurons (red) in MNTB and VCN, is greater in these knockout mice compared to wild type mice (Toyoshima et al., 2009). Adapted, with permission, from Hsieh et al. (2010).

of peaks I and II. However, mutants show an increase in the latency of peak III and the ipsilateral peak IV is absent. These peaks are thought to represent activity in the SOC and lateral lemniscus, respectively (Wada and Starr, 1989). Also, the latency of the contralateral peak IV is greater in these *Robo3* mutant mice. In the mouse spinal cord, an isoform of Robo3 in pre-crossing commissural axons blocks the repulsive activity of Slit/Robo1 and -2, allowing netrin-1-mediated attraction towards the midline (Chen et al., 2008; Sabatier et al., 2004). Similarly, it may be that Robo3 in VCN axons modulates Slit/Robo repulsion before crossing the midline and that without Robo3 the axons are prematurely repelled from the midline, which results in innervation of the ipsilateral MNTB. This possibility is further supported by the evidence that netrin-1 alone is not sufficient to attract VCN axons to the midline (Poe and Brunso-Bechtold, 1998).

Signaling through the Eph proteins, including the Eph receptors and their ephrin ligands, also plays a role in the establishment of auditory brainstem circuit formation (Cramer, 2005). Signaling through Eph proteins is bidirectional: forward signaling refers to signaling in the Eph receptor-expressing cell and reverse signaling refers to signaling in the ephrin-expressing cell. During early postnatal development, the receptor EphB2 is expressed in VCN axons, while one of its ligands, ephrin-B2, is expressed in MNTB (Hsieh et al., 2010). Mice lacking EphB2 and EphB3 have an increased number of aberrant VCN projections to the ipsilateral MNTB compared to wild type mice. However, transgenic mice that express an EphB2 fusion protein that allows EphB2-induced reverse signaling, but not EphB2 forward signaling, have normal VCN–MNTB projections. Additionally, mice that have impaired ephrin-B2 reverse signaling, but intact ephrin-B2 forward signaling, show an increased number of aberrant calyces in the ipsilateral MNTB. Together, the data suggest that EphB2- and EphB3-induced reverse signaling through ephrin-B2, but not EphB2 and EphB3 forward signaling, inhibits the formation of ipsilateral VCN–MNTB projections (Hsieh et al., 2010; Fig. 1D and E).

Further characterization of the development of the ipsilateral projections in mutant mice revealed that they form concurrently with their contralateral counterparts and have a normal morphology, suggesting that ephrin-B2 reverse signaling normally impairs calyx formation in the ipsilateral MNTB, but not contralateral MNTB.

EphB2, EphB3, and ephrin-B2 do not appear to control VCN axon growth to the midline, midline crossing, innervation of the contralateral MNTB, or calyx of Held formation, since all mutants still have a robust contralateral VCN–MNTB pathway (Hsieh et al., 2010). Some of the VCN axons that project to the ipsilateral MNTB continue to project horizontally to the midline, while fewer project to the ipsilateral MNTB only and do not emerge from branches that project to the midline. A remaining question is whether individual ipsilateral-projecting axons also have calyces in the contralateral MNTB. Additionally, the cell type (globular versus spherical bushy cell) from which these axons arise is not known. It is likely that ipsilateral calyces arise from branches of mistargeted globular bushy cells, as other cell types in VCN do not normally terminate in calyceal endings.

VCN axons course through the ipsilateral MNTB during embryonic development. It is unclear why these axons fail to branch and terminate in the ipsilateral MNTB, whereas they do so in the contralateral MNTB. The extended time period between outgrowth of VCN axons and formation of connections in the contralateral MNTB suggests that the molecular environment encountered by VCN axonal growth cones may be substantially different on the ipsilateral side compared to the contralateral side. One possibility is that signals at the midline change the responsiveness of crossed VCN axons such that synapse formation is permitted after these interactions (Brittis et al., 2002). Alternatively, it is possible that Eph family proteins are selectively targeted to distinct regions of VCN axon branches (McLaughlin et al., 2003). An important consideration is that in this large family of proteins, relative concentrations of ligands and receptors determine net attraction or repulsion (Brown et al., 2000; Hansen et al., 2004; Reber et al., 2004). Further studies examining the spatiotemporal expression of other Eph proteins during embryonic and postnatal development and projection patterns in other Eph mutant mice are thus needed to better understand the contribution of this family of proteins.

In addition to target selection, Eph proteins may also be required for the formation of topography in the VCN axonal projections to MNTB. *EphA4* mutant mice have normal, contralateral MNTB innervation, unlike the *EphB2* and *EphB3* double mutant and *ephrin-B2* mutant mice (Hsieh et al., 2007). However, the tonotopic terminations of VCN axons along the mediolateral axis of the

contralateral MNTB are degraded in *EphA4* mutant mice (Hsieh and Cramer, 2006b). Moreover, after exposure to 40-kHz pure tones, *EphA4* mutant mice have altered patterns of activity in MNTB compared to wild type controls, as assessed with immunoreactivity for c-fos, an immediate early gene (Miko et al., 2007). The iso-frequency band of c-fos-positive cells in mice lacking EphA4 is narrower and more laterally positioned. These experiments provide additional evidence to support the hypothesis that Eph protein signaling ensures tonotopic innervation of auditory brainstem nuclei (Huffman and Cramer, 2007). Thus, different Eph proteins may play distinct roles in various stages of auditory brainstem circuit assembly.

The function of Eph proteins in VCN–MNTB development may involve neuron–glial interactions, as demonstrated in other brain areas (Klein, 2009; Murai et al., 2003; Petros et al., 2006). For example, EphB1 in retinal axons binds to eprhin-B2 in radial glia during ipsilateral targeting at the optic chiasm (Petros et al., 2009; Williams et al., 2003). EphB/ephrin-B complexes formed between neurons are endocytosed to permit repulsion; a similar mechanism has been shown to transfer full length EphB2 from neurons to glial cells (Lauterbach and Klein, 2006; Zimmer et al., 2003). The role of Eph signaling in neuron–glial interactions in the development of auditory pathways is incompletely understood. Glial cells in MNTB receive excitatory inputs from the calyx of Held (Muller et al., 2009) and communicate with MNTB neurons (Reyes-Haro et al., 2010). The presence of glia in MNTB results in morphological changes in the calyx after the first few postnatal weeks (Ford et al., 2009). Further studies are needed to determine whether interactions between glial cells and VCN axon terminals are mediated by Eph signaling, and whether these neuron–glial interactions are important for the development of the VCN–MNTB projection at early postnatal ages.

4. Formation of calyceal contacts

The earliest synaptic contacts between VCN axons and MNTB neurons are made by E17 (Hoffpauir et al., 2010). An immature, protocalyx of Held becomes recognizable at postnatal day 2 (P2). Unlike the mature morphology, the protocalyx has a closed, spoon-like appearance, but like the mature connectivity, there is only one protocalyx per MNTB neuron (Hoffpauir et al., 2006; Kandler and Friauf, 1993). However, axon collaterals emanating from the calyx also innervate adjacent MNTB neurons during early postnatal development, a phenomenon that is thought to ensure that there is only one calyx per MNTB principal neuron once the circuitry is mature (Rodriguez-Contreras et al., 2008).

Interestingly, about 10% of MNTB neurons in mice lacking NB-2, a cell recognition molecule of the contactin family, lack a calyx of Held during early postnatal development at P6 (Toyoshima et al., 2009; Fig. 1F). Additionally, a significant portion of MTNB principal neurons undergoes apoptosis in the *NB-2* knockout mice. This does not occur in wild type littermates and is likely the result of insufficient calyx formation or maturation (Toyoshima et al., 2009). This finding is consistent with deafferentation-induced cell death in VCN, where neurons are dependent on cochlear input during early postnatal development (Hashisaki and Rubel, 1989; Moore, 1990; Mostafapour et al., 2000; Trune, 1982). However, studies testing the dependence of MNTB neurons on afferent innervation have yet to be completed. *In vivo* imaging of calyx formation and maturation (Rodriguez-Contreras et al., 2008) in *NB-2* knockout mice may provide further insight into the role of NB-2 in these processes. It is possible that the collaterals of the calyx of Held are less dynamic in the *NB-2* knockout mice, which may decrease flexibility during this period. An alternative possibility is that the postsynaptic MNTB neurons are not capable of maintaining

calyceal inputs without NB-2. The NB-2 mutation also results in abnormal ABRs; interpeak latencies between peaks II and III and between peaks III and IV were greater compared to wild type controls, but peripheral thresholds and peak amplitudes did not seem to be affected by the mutation. Since the vast majority (about 90%) of MNTB principal neurons do receive their normal calyceal contacts in *NB-2* knockout mice, additional proteins that regulate the formation and maturation of the calyx of Held still need to be identified.

The results of these studies together suggest that multiple signaling families ensure the appropriate architecture and distinct aspects of the VCN–MNTB projection. For example, NB-2 normally *promotes* calyx of Held formation and/or maintenance in the *contralateral* MNTB, while ephrin-B reverse signaling normally *inhibits* calyx formation in the *ipsilateral* MNTB. Additionally, increasing the number of aberrant ipsilateral calyces does not affect the number of MNTB principal neurons in *ephrin-B2* transgenic mice, while decreasing the number of contralateral calyces results in less MNTB neurons in *NB-2* knockout mice. This comparison highlights the importance of different families of molecules acting in a coordinated fashion during the formation of the VCN–MNTB pathway.

5. Morphological maturation of the calyx of Held

During the second and third postnatal weeks, shortly after hearing onset, the calyx of Held develops its characteristic, highly-fenestrated appearance (Kandler and Friauf, 1993; Kil et al., 1995). Fenestration of the calyx, the process by which the presynaptic membrane is reduced to many digit-like processes, occurs along the tonotopic axis of MNTB, beginning with the high-frequency (medial) region of the nucleus (Ford et al., 2009). This gradient of development is activity-dependent, in that it is not present in animals that undergo cochlear ablation surgery or in animals that receive ototoxic drugs. However, the fenestration process itself does not require external sound stimuli. This change in morphology reflects the interdigitation of glial processes that contain glutamate receptors and transporters to occupy the open space within the calyx. These glial cells may facilitate rapid clearance of glutamate from the synaptic cleft, increasing the temporal precision of synaptic transmission (Ford et al., 2009).

6. Maturation of VCN–MNTB synaptic transmission

6.1. Presynaptic waveform and synaptic delay

Calyces of Held and MNTB neurons mature physiologically during postnatal life and these changes parallel the changes in calyceal morphology (von Gersdorff and Borst, 2002). The wave-form of the presynaptic action potential in the calyx of Held becomes quicker and shorter during this time (Taschenberger and von Gersdorff, 2000). Changes in the presynaptic waveform are due to developmental changes in voltage-dependent Na^+ and K^+ channels and their respective currents. Between the first and second postnatal weeks, the kinetics of Na^+ current inactivation and recovery from inactivation shorten, which may be due to an increase in the expression of the α–subunit $Na_V1.6$ in the unmyeli-nated region of the VCN axon heminode (Leão et al., 2005). The $Na_V1.6$ α–subunit has faster kinetics and is more resistant to cumulative inactivation than $Na_V1.2$, the other Na^+ channel α–subunit expressed in axons and nodes of Ranvier in the central nervous system (Leão et al., 2005; Zhou and Goldin, 2004). Like-wise, K^+ currents, which contribute to the stabilization and repo-larization of the calyx, become larger and their activation kinetics become faster. These changes may be due to changes in Kv1- and

Kv-3-mediated K^+ currents (Nakamura and Takahashi, 2007). The synaptic delay also decreases from P7 to P14 (Taschenberger and von Gersdorff, 2000).

6.2. Ca^{2+} channels

Shortening of the presynaptic action potential during development reduces the number of recruited voltage-gated calcium channels (VGCCs) and the duration of Ca^{2+} influx, thereby allowing better control of quantal output and more efficient glutamate release (Yang and Wang, 2006). Concomitantly, VGCCs become more tightly coupled to synaptic vesicles (Fedchyshyn and Wang, 2005) so that these briefer Ca^{2+} currents become more effective in causing neurotransmitter release (Kochubey et al., 2009). This coupling of Ca^{2+} influx and glutamate release is regulated by the filamentous protein septin 5 (Yang et al., 2010). In immature calyces, septin 5 localizes to the release face of the terminal where it may create a spatial barrier between synaptic vesicles and the active zone. In mature calyces it is removed from the active zones of the calyx, allowing synaptic vesicles to be more closely associated with this region of the axon terminal. In support of these observations, the calyces of young *septin 5* knockout mice exhibit functional properties similar to calyces of mature, wild type mice. Additionally, developmental changes in the expression of Ca^{2+}-sensing proteins, like the synaptotagmins, may also play a role in these processes (Xiao et al., 2010). For example, virus-mediated overexpression of a mutated synaptotagmin in VCN during early postnatal development disrupts the position of synaptic vesicles in the calyx terminal in relation to calcium channels, thereby reducing the effectiveness of Ca^{2+} influx in causing synaptic vesicle release (Young and Neher, 2009). These developmental modifications correspond with a decline in the mean probability of synaptic vesicle release (Taschenberger et al., 2002).

In immature calyces of Held Ca^{2+} can enter VCN neurons through N-, P/Q-, and R-type Ca^{2+} channels, but later Ca^{2+} influx is mostly through P/Q-type channels only (Iwasaki and Takahashi, 1998; Wu et al., 1999). These changes in Ca^{2+} channel subtype coincide with a steady increase in the amplitude of Ca^{2+} currents from P5-P11 because Ca^{2+} influx through N- and R-type channels is less effective at triggering synaptic vesicle release due to their more distant localization to release sites (Chuhma and Ohmori, 1998). Changes in Ca^{2+} concentration are important because intracellular Ca^{2+} regulates K^+ channel expression in MNTB neurons (Tong et al., 2010) and most likely in VCN axons as well. Ca^{2+} influx and release from intracellular stores results in increased phosphorylation of cAMP-response element binding protein, which modulates K^+ channel expression (Tong et al., 2010), thereby controlling intrinsic neuronal excitability (Johnston et al., 2010).

6.3. Vesicular glutamate transporters and vesicular endocytosis

The expression of vesicular glutamate transporter VGLUT1 increases substantially in the calyx of Held between P5 and P29, while the level of VGLUT2, which co-localizes to the same VGLUT1-positive calyces, remains constantly high throughout development and adulthood (Billups, 2005). Mechanisms of synaptic vesicle endocytosis also change throughout postnatal development. In immature, pre-hearing onset calyces, vesicular endocytosis requires activated calmodulin and calcineurin, but is independent of GTP hydrolysis (Xu et al., 2008; Yamashita et al., 2010). In contrast, this process does not require calmodulin and calcineurin activation in more mature, post-hearing calyces, but has now become dependent on GTP hydrolysis (Yamashita et al., 2010). It is possible that mechanisms of vesicular endocytosis must change because of decreases in residual bulk intracellular Ca^{2+}

concentration and calcineurin expression during postnatal development (Yamashita et al., 2010).

6.4. Presynaptic inhibition

The expression of metabotropic glutamate receptors (mGluRs) in the presynaptic calyx is also developmentally regulated. Calyceal expression of group II (mGluR2/3) and III (mGluR4 and mGluR8) mGluRs decreases between the second and third postnatal weeks (Elezgarai et al., 1999; Renden et al., 2005). Activation of mGluRs controls the release of glutamate from the calyx by decreasing P/Q-type Ca^{2+} channel conductance (Takahashi et al., 1996). Similarly, noradrenaline binds to $\alpha 2$-adrenergic receptors on the calyx, which inhibit presynaptic Ca^{2+} channels, thereby decreasing glutamate release (Leão and Von Gersdorff, 2002). This effect of noradrenaline dramatically decreases during development. Additionally, serotonin (through 5-HT$_{1B}$ receptors) and adenosine (through A$_1$ receptors) can inhibit presynaptic release of glutamate through similar mechanisms during early postnatal development, but not in adulthood (Kimura et al., 2003; Mizutani et al., 2006). Thus, presynaptic inhibition of neurotransmitter release via mGluRs activation, noradrenaline, serotonin and adenosine most likely have developmental roles distinct from their function in mature calyces. For example, mGluR activation in mature calyces does not change the net release of neurotransmitter, but it alters the synaptic state by increasing the vesicle pool size and by decreasing the probability of release (Billups et al., 2005). However, in mature calyces of Held, presynaptic inhibition is mediated by GABA$_B$ receptors via suppression of Ca^{2+} channels through G-protein signaling (Isaacson, 1998; Takahashi et al., 1998).

6.5. Postsynaptic glutamate receptors

The rise time and duration of AMPA receptor (AMPAR)-mediated EPSCs in MNTB neurons shorten during development, but the amplitudes do not change (Taschenberger and von Gersdorff, 2000). The deactivation and desensitization times of AMPARs become faster, which results in a faster decay time. However, the contribution of desensitization to speeding up decay times decreases during development. These changes may be due to an increase in the flop variant of the glutamate receptor subtype GluR4, which has faster desensitization kinetics than its flip counterpart (Koike-Tani et al., 2005). The amplitude of the NMDA-mediated EPSC is decreased significantly upon maturation, but the time course becomes quicker. This change is due to an increase in the expression of the NR2A and NR2C subunits and a decrease in the functional contribution of NR2B subunits (Steinert et al., 2010). Despite the small amplitude of the NMDA EPSC in mature MNTB neurons, it is important for neuronal nitric oxide synthase (nNOS) activation. Nitric oxide influences MNTB excitability by inhibiting Kv3 channels, which increases the duration of action potentials (Steinert et al., 2008).

6.6. Short-term plasticity

Frequency-dependent short-term depression at calyx of Held-MNTB synapses decreases between the first and second postnatal weeks (Taschenberger et al., 2002). This decrease in short-term depression is mostly due to changes in the level of calmodulin activation. In immature calyces, presynaptic Ca^{2+} currents are inactivated by Ca^{2+} and calmodulin after low-frequency stimulation (Xu and Wu, 2005). However, calmodulin-mediated inactivation of Ca^{2+} channels is limited to young calyces of Held, mostly likely due to a developmental decrease in intracellular Ca^{2+} concentration, which may not be sufficient to activate calmodulin

(Nakamura et al., 2008). Despite decreased inactivation of Ca^{2+} channels in mature calyces, other mechanisms, such as reduced amplitudes of presynaptic action potentials, vesicle pool depletion, AMPAR desensitization and possibly AMPAR saturation, mediate short-term depression in older animals (Schneggenburger and Forsythe, 2006; von Gersdorff and Borst, 2002).

6.7. Ultrastructural changes

Myelination of VCN axons in MNTB becomes more prominent by the second postnatal week (Taschenberger et al., 2002). The average number of docked synaptic vesicles per active zone is about halved between the first and second postnatal weeks, but the estimated numbers of docked vesicles per calyx increases by about 25%, most likely due to an increase in the estimated number of active zones per calyx (Taschenberger et al., 2002). Furthermore, there is a change in the arrangement of synaptic vesicles in the presynaptic terminal. At around hearing onset, synaptic vesicles become arranged in donut-like rings around central clusters of interconnected mitochondria, which differs from the homogenous distribution of synaptic vesicles in pre-hearing calyces (Wimmer et al., 2006).

The cell body and nuclei of MNTB principal neuron become rounder and larger during development, corresponding with an increase in the surface area (Taschenberger et al., 2002). The average surface area of individual postsynaptic densities (PSDs) decreases by more than one-third between the first and second postnatal weeks, but the total PSD surface area per calyx increases. This change is most likely due to the breaking up of individual, larger-sized PSDs into many smaller-sized PSDs, which parallels the fenestration of the presynaptic calyceal membrane, and an increase in the size of MNTB neurons (Taschenberger et al., 2002).

7. Role of afferent activity

The majority of developmental processes that result in a mature calyx of Held are independent of the postnatal sensory experience, as they occur before the onset of hearing. Moreover, such processes do not require spontaneous activity in the auditory nerve; calyx of Held development occurs normally in mouse models of congenital deafness. In congenitally deaf mice, synaptic transmission at the calyx is normal during early and late postnatal development and the calyx of Held appears morphologically similar to the calyces of normal-hearing mice (Oleskevich et al., 2004; Youssoufian et al., 2005, 2008). However, spontaneous activity generated in the cochlea before it degenerates may instruct some of these processes in congenitally deaf mice.

The development of membrane properties of MNTB neurons seems to require auditory nerve activity during development. Properties of Na^+ channels and developmental regulation of Na^+ channel subunit expression in MNTB neurons are disrupted in congenitally deaf mice (Leão et al., 2006a). Moreover, MNTB neurons in these transgenic mice are more excitable than the MNTB neurons of wild type mice. This increase in excitability is associated with a reduction of low-voltage activated K^+ conductances (Leão et al., 2004a). Inhibitory transmission to MNTB neurons is also affected by a lack of spontaneous activity; the frequency of glycinergic mIPSCs is greater in congenitally deaf mice, but the amplitude of these currents is less. Additionally, the MNTB neurons of these mice have more gephyrin clusters, which are indicative of inhibitory synaptic sites, than the MNTB neurons of wild type mice (Leão et al., 2004b).

Tonotopic gradients of multiple voltage-gated currents (Brew and Forsythe, 2005; Leão et al., 2006b) and channels (Gazula et al., 2010; Li et al., 2001) exist in the MNTB of normal mice.

Gradients of membrane currents in MNTB produce a source of time delays of action potential initiation across the tonopotopic axis of MNTB (Leão et al., 2006b). These tonotopic gradients of voltage-gated currents in MNTB are dependent on spontaneous auditory nerve activity during development (Leão et al., 2006b). Additionally, the maintenance of the tonotopic gradient of expression of the K^+ channel Kv3.1 appears to be dependent on auditory activity, as it is lost in hearing-impaired mice (von Hehn et al., 2004).

The cellular structure of MNTB neurons is dependent on afferent activity during development and adulthood. Developing mice that are deprived of airborne sound stimuli have smaller MNTB neurons (Webster and Webster, 1977). A reduction in MNTB soma size is also observed after cochlear damage in the adult (Jean-Baptiste and Morest, 1975). This reduction in cell size is most likely the result of presynaptic changes. While VCN axons do not degenerate, VCN axon terminals become fragmented in MNTB and other SOC nuclei (Morest et al., 1997) and the number and size of synaptic vesicles in the calyx of Held are reduced (Jean-Baptiste and Morest, 1975). Changes in MNTB neuron size are evident 48 h, but not 24 h, after cochlear ablation or administration of tetrodotoxin (TTX; a sodium channel blocker) in the auditory nerve. This effect is reversible in TTX-treated animals. MNTB neuron size returns to normal 7 days after TTX administration (Pasic et al., 1994). These experiments suggest that the morphological integrity of MNTB principal neurons relies on activity-dependent release of trophic substances from the calyx of Held.

8. Modification of circuitry following changes in input

The VCN—MNTB pathway can be modified after changes in cochlear input during early postnatal development. Following unilateral cochlea removal, the intact VCN sprouts novel axonal projections, which form calyces within the denervated MNTB (Kitzes et al., 1995; Russell and Moore, 1995). The induced ipsilateral projections form morphologically normal calyces in a tonotopically appropriate region of MNTB (Russell and Moore, 1995). Deletion of the Eph receptor EphA4 increases the amount of deafferentation-induced calyces in MNTB (Hsieh et al., 2007), consistent with the view that Eph proteins limit ipsilateral projections during normal development (Hsieh et al., 2010), although the relative contributions of individual Eph family members to development and lesion-induced changes remains to be determined. The extent of reorganization to MNTB after unilateral deafferentation decreases as the pathway matures, but VCN sprouting to the ipsilateral MNTB can still occur after hearing onset if the cochlear nucleus is removed directly (Hsieh and Cramer, 2006a).

9. Changes to VCN—MNTB pathway with aging

Cell counts have shown substantial decreases in the number of MNTB neurons in aging rats (Casey and Feldman, 1982). In the SOC, this aging effect seems to be specific to MNTB because the numbers of MSO and LSO neurons remains unchanged throughout the aging processes from 3 to 30 months of age (Casey, 1990). It may be that MNTB neurons are more susceptible to death than MSO and LSO neurons because they have only one major excitatory input. Expression levels of Kv3.1 decrease in MNTB with age (Zettel et al., 2007). The number of MNTB neurons expressing calbindin D-28k, a neuron-specific protein involved in regulating levels of intracellular Ca^{2+}, decreases in a strain of mice with age-related hearing loss during adulthood (O'Neill et al., 1997). VCN axonal degeneration and spongiform lesions become prominent in MNTB of old gerbils (Gleich and Strutz, 2002; McGinn and Faddis, 1998). All of these changes may contribute to the diminished sound localization abilities in aged animals (Brown, 1984; Harrison, 1981).

10. Conclusion

During development, VCN axons display extraordinary specificity in target selection, and the calyx of Held undergoes substantial anatomical and physiological modifications. Several of the molecular signaling pathways that lead to its precise development have been identified. However, many questions remain regarding the development of the VCN—MNTB pathway and calyx of Held. Future experiments are needed to elucidate the mechanisms of neuronal migration of VCN and SOC neurons. The roles of other families of axon guidance molecules, such as the semaphorins, and morphogens such as sonic hedgehog, Wnts, and Bone Morphogenic Proteins, remain relatively unexplored in the formation of auditory brainstem nuclei and their circuitry. Moreover, the molecular mechanisms that give rise to the specialized calyceal structure are not known, as no mutations thus far have led to disrupted morphology.

Large-scale genomic screens to asses changes in gene expression in VCN axons and MNTB neurons after unilateral deafferentation may be useful in determining mechanisms of reorganization in the auditory brainstem, especially in regard to critical periods for lesion-induced VCN axon sprouting, and are likely to provide insights into the normal development of these pathways. The distinctive, stereotyped nature of the VCN—MNTB projection provides a relatively simple and quantifiable system in which to study lesion-induced sprouting, which may be of general relevance to issues related to brain recovery and repair.

While the calyx of Held is atypical and specialized in several respects, these specializations make it an excellent system in which to study molecular and cellular mechanisms of development and synaptic maturation. Few areas of the mammalian central nervous system contain projections this clearly identifiable and precise. Collectively, work on this system has elucidated many aspects of the entire process, from the birth of neurons to the synaptic and circuit functions that mediate localization of sound sources. These studies will allow us to determine which aspects of its development are shared with other regions of the nervous system, and which are unique adaptations that permit development and function of auditory processing.

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