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**Main Manuscript for**

Spontaneous Activity is Necessary for the Synchronous Growth of the Calyx of Held and Intrinsic Matching of Postsynaptic Excitability in the Medial Nucleus of the Trapezoid Body

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**Classification:** Biological Sciences (Neuroscience; Developmental Biology)

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Main Text

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**Abstract**

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During neural circuit formation nascent synaptic contacts are exuberant in nature ensuring proper neural connectivity and undergo a period of pruning of superfluous inputs followed by maturation and strengthening of the remaining contacts. In sensory systems, patterned spontaneous activity (SA) originates in the peripheral sense organ and propagates through the central nervous system, prior to the onset of external stimulation, playing an important role in shaping the synaptic architecture. The calyx of Held (CH) is the primary terminus of globular bushy cells (GBCs), whose cell bodies are located in the ventral cochlear nucleus (VCN) and innervates principal neurons (PNs) in the medial nucleus of the trapezoid body (MNTB). Previous manipulations to eliminate SA at the developing CH have involved genetic strategies that also affect cochlear function and may induce homeostatic compensatory mechanisms in GBCs. To overcome this confounding factor, direct manipulation of synaptic transmission through viral vector mediated, rapid-onset expression of tetanus neurotoxin (TeNT) targeting GBCs was employed to silence activity at the CH:MNTB synaptic connection. The effects of *in vivo* synaptic silencing at the CH:MNTB connection delay the maturation of the MNTB PNs intrinsic physiological properties. Following TeNT expression PNs remain in an immature hyperexcitable state, with a tonic firing pattern, high input resistance, and depolarized resting membrane potential. Morphological analysis of transduced calyxes shows impaired growth with reduced volume and increased thickness. This study highlights an important role for SA triggering rapid growth of the CH and the synchronous maturation of the MNTB PN physiological properties.

**Significance Statement**

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The formation of neural circuits can occur independent of neural activity, but the maturation and refinement of nascent connections is an activity-dependent mechanism. The effects of removing cochlear SA on formation of the calyx of Held (CH) located in the auditory brainstem, have proven inconclusive, perhaps due to homeostatic compensation by central neurons. We utilized a viral vector approach to selectively block synaptic transmission at the CH resulting in altered growth of the nerve terminal and delayed maturation of biophysical properties of its postsynaptic neuron. This study reveals specific roles for SA in triggering synchronous maturational events in synaptic partners.

**Main Text**

**Introduction**

Intrinsic patterned spontaneous activity (SA) occurs in several brain regions during development and in sensory systems originates in the peripheral sense organ (Blankenship & Feller, 2010; Galli & Maffei, 1988; Kros et al., 1998; Lippe, 1994; Meister et al., 1991; Tritsch et al., 2007). Interestingly, SA in these sensory systems occurs prior to the onset of external stimulation (in mice, ear canals and eyes open after P10; Findlater et al., 1993; Mikaelian et al., 1965; Poole, 1999), highlighting the importance of stimulus-independent activity during neural circuit formation. SA regulates the patterning of central circuits across sensory systems ensuring precise topographical connectivity (Kersbergen and Bergles, 2024; Kirby et al., 2013). In the developing murine auditory system, intrinsic spontaneous bursting activity originates in inner hair cells in the cochlea, beginning at birth, and propagates throughout the ascending auditory pathway (Babola et al., 2020; Babola et al., 2018; Lippe, 1994; Sonntag et al., 2009; Tritsch, Rodriguez-Contreras, et al., 2010; Tritsch et al., 2007). The onset of cochlea-driven SA occurs synchronously with the rapid formation and early maturation of the calyx of Held (CH) (Tritsch, Rodriguez-Contreras, et al., 2010; Sierksma et al., 2017), the largest nerve terminal in the mammalian brain, and its postsynaptic target, the principal neuron (PN) in the medial nucleus of the trapezoid body (MNTB) in the auditory brainstem (Hoffpauir et al., 2006; Hoffpauir et al., 2010; Holcomb et al., 2013; Kandler & Friauf, 1993; Rusu & Borst, 2011). The CH:PN neural circuit maturation initiates more synchronously (at postnatal day (P)2) and over a shorter time (2-4 days) than other model systems, such as climbing fiber innervation of Purkinje cells in cerebellum (Hashimoto et al., 2009; Ichikawa et al., 2011; Watanabe & Kano, 2011), retinal ganglion cell innervation of dorsolateral lateral geniculate nucleus (Ackman et al., 2012; Blankenship & Feller, 2010; Siegel et al., 2012), or the neuromuscular junction (Balice-Gordon & Lichtman, 1993; Sanes & Lichtman, 1999; Tapia et al. 2012). Therefore, it offers an excellent model to explore the hypothesis that SA changes drive early maturational events, which have been shown to be coordinated between pre- and postsynaptic partners (Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017). Temporal profiles of gene expression from microdissected whole-MNTB tissue show multiple genes with increasing and decreasing profiles between P0-6, including those for biochemical signaling pathways, ion channels, and mediation of whole tissue organization such as the onset of myelination (Heller er al., 2024; Kolson et al., 2016).

Previous studies manipulating intrinsic spontaneous activity in the cochlea have resulted in minimal deficits to synaptic transmission at the CH:MNTB synaptic connection (Erazo-Fischer et al., 2007; Oleskevich et al., 2004; Youssoufian et al., 2005), abnormal morphology of the CH (Clause et al., 2014; Lessle et al., 2024; Youssoufian et al., 2005), or MNTB PN intrinsic physiological properties (Noh et al., 2010). In vesicular glutamate transporter 3 (Vglut3) knockout mice blocking synaptic transmission from inner hair cells in the cochlea, spiral ganglion neurons are hyperexcitable (Babola et al., 2018) suggesting possible homeostatic compensatory mechanisms in the cochlear nucleus. We thus directly silenced synaptic transmission in GBCs through viral vector mediated, rapid-onset expression of tetanus neurotoxin (TeNT) (Kim et al., 2009; Sando et al., 2017; Schiavo et al., 1992). Following unilateral viral vector injections into the VCN at P0, mCherry fluorescence (co-expressed with TeNT) was detectable within 48 hours in CHs innervating the contralateral MNTB. Compared to non-transduced ipsilateral MNTB control recordings, whole cell patch-clamp recordings from transduced MNTB PNs showed a decrease in the frequency and amplitude and increase in the decay rate of spontaneous excitatory postsynaptic currents. The effects of *in vivo* synaptic silencing at the CH:MNTB connection delay the maturation of the MNTB PNs intrinsic physiological properties. Following TeNT expression PNs remain in an immature hyperexcitable state, with a tonic firing pattern, high input resistance, and low threshold current. Morphological analysis of transduced calyxes shows impaired growth with reduced volume and increased thickness. This study highlights an important role for SA in triggering the synchronous maturation of the MNTB PN physiological properties and modulating rapid growth of the CH and increase in PN cell body volume.

**Results**

**Viral Vector Expression of Tent Is Rapid Following Injection at P0 and Precedes Initial Growth of the Calyx of Held at P3**

To directly test the role of SA in maturation of the calyx of Held to principal neuron neural connection (CH:PN) and their functional properties, we utilized a viral vector approach to transduce the presynaptic neuron with the light chain subunit of tetanus neurotoxin (TeNT) (Fig. 1A and B). The light chain of TeNT mediates the proteolytic cleavage of the synaptic vesicle fusion protein synaptobrevin-2, also known as vesicle-associated membrane protein 2 (VAMP2) (Fig. 1A; Breidenbach & Brunger, 2005; Lacy et al., 1998; Schiavo et al., 1992), preventing opening of a fusion pore and subsequent neurotransmitter release (Hernandez et al., 2012; Jahn & Fasshauer, 2012; Zhao et al., 2016). A helper-dependent adenoviral (HdAd) construct, which co-expresses the TeNT light chain driven by the overexpression pUNISHER cassette, allowing for rapid and high levels of expression (Montesinos et al., 2011), along with a fluorescent reporter molecule (mCherry) independently driven by the 470-bp human synapsin promoter, was injected at P0 into the ventral cochlear nucleus at high titer (2.03 x 108 viral particles/mL) to express TeNT light chain in globular bushy cells (GBC). Transduction of GBCs was detectable within 48 hours (Supplemental Fig. 1A) and labeled their cell bodies and axonal projections along the ventral brainstem surface which crossed the midline to form synapses onto PNs of the contralateral medial nucleus of the trapezoid body (Fig. 1B, C). Thus, TeNT expression at the CH:MNTB synaptic connection occurred prior to or coincident with the onset of cochlea induced bursting activity and initial growth of the CH (Sierksma et al., 2017; Tritsch, Rodriguez-Contreras, et al., 2010; Tritsch et al., 2007). Unilateral injections allowed for an in-slice control comparing transduced and non-transduced calyces in the contralateral and ipsilateral MNTB (to the injection site), respectively (Fig. 1C-C’’).

**Evoked Synaptic Transmission is Abolished at the Calyx of Held Following *In Vivo* Viral Vector Expression of TeNT**

The CH is a good model for studying the biophysical and molecular mechanisms of synaptic transmission due to the giant size of the terminal and accessibility to patch-clamp recordings (Borst et al., 1995; Forsythe, 1994). To confirm the efficacy of TeNT mediated synaptic silencing at the CH:MNTB connection, we performed simultaneous paired pre- and postsynaptic whole-cell recordings (Fig. 1D and E). Many transduced CHs formed large terminals by P6-8, recognizable by mCherry fluorescence, which were targeted for paired whole-cell recordings. Alexa fluorophores of two different wavelengths (488 and 647; 50 mM) were incorporated in the patch pipettes for visual confirmation of simultaneous pre- (mCherry and Alexa 488 co-labeling) and postsynaptic (Alexa 647) recordings (Fig. D). Presynaptic action potentials were evoked by a short (1-3 ms), 500 pA depolarizing current injection via the recording pipette and postsynaptic recordings in either voltage or current-clamp configuration recorded the evoked EPSC or MNTB PN action potential, respectively (Fig. 1E). In all paired recordings from the cMNTB (n = 3) a depolarizing current injection in transduced calyces (TeNT expression) failed to drive an evoked EPSC or spike in the postsynaptic MNTB PN (Fig. 1E, right panel). To ensure paired recordings were performed on healthy cMNTB PNs in acute brainstem slices, sEPSCs and the firing pattern from depolarizing current injections were recorded (Supplement) with consistent results to other experimental recordings (Supplement). For comparison paired recordings were performed on P6-P8 CH:MNTB PN synaptic pairs in non-injected mice along with neurons from the iMNTB following viral injection. All paired recordings from these control conditions (non-injected, n = 3; iMNTB, n = 3) displayed the characteristic evoked response (EPSC in voltage clamp and action potential in current clamp) in the PN following a presynaptic spike in the CH.

**Spontaneous Neurotransmission is Reduced at the Calyx of Held Following *In Vivo* Viral Vector Expression of TeNT**

Prior to the growth phase of the CH beginning at P2-P3, MNTB PNs are hyperexcitable, whereby spontaneous vesicle release is sufficient to trigger an action potential (Hoffpauir et al., 2010; Rusu & Borst, 2011). Previous reports utilizing TeNT to block synaptic neurotransmission have shown a reduction of ≈70-90% in spontaneous EPSCs (sEPSC; Kerschensteiner et al., 2009; Lessle et al., 2024; Sando et al., 2017), indicating that not all SA is TeNT-sensitive. We therefore recorded sEPSCs from PNs in both cMNTB (transduced) and iMNTB (non-transduced) following unilateral TeNT injection, and from non-injected control animals at P4, P6, P9, and P14 in order to assay its effect on spontaneous neurotransmission across the first two postnatal weeks. We hypothesized that TeNT expression may prevent or delay CH growth, but we could reliably identify transduced CHs at P6 and P8 and target their postsynaptic PNs for recordings. At P4, the presence of large endogenously labeled fluorescent calyces was less frequent (4/20), so many recordings were made from PNs without an identifiable CH input to achieve a comparable sample size for statistical analysis.

Exemplary voltage-clamp traces at P6 show the significant reduction in the frequency and slower time course of sEPSCs following TeNT expression compared to PNs recorded from the ipsilateral MNTB (iMNTB) and non-injected mice (Fig. 2A). Consistent with the mechanism of action for TeNT preventing synaptic vesicle fusion (Breidenbach & Brunger, 2005; Lacy et al., 1998; Schiavo et al., 1992), PNs in the cMNTB of injected animals had a significant reduction in the frequency of sEPSCs (≈0.5-1Hz at physiological temperature) across neonatal ages (Fig. 2B). By P14, after opening of the ear canals, differences were less significant due to a set of neurons with high sEPSC frequency in both control groups. TeNT expression nearly or completely eliminated spontaneous release in about 50% of cells, and in the remainder the frequency of quantal events was reduced but not abolished. These persistent sEPSCs may arise from non-calyceal inputs, which have slower kinetics compared to calyceal synaptic transmission, consistent with the slower decay time constant in cMNTB PNs (Banks & Smith, 1992; Hamann et al., 2003). Alternatively, persistent sEPSCs from cMNTB PNs may be associated with the non-canonical fusion protein vesicle-associated membrane protein 7 (VAMP7), also known as TeNT-insensitive VAMP, that mediates spontaneous neurotransmission (Bal et al., 2013; Coco et al., 1999; Hua et al., 2011).

Transduced (cMNTB) PNs showed consistently slower decay times from P4-9, with average values > 1ms . At P14 values for both control and experimental groups decreased substantially, but average experimental values were still longer than both control groups (Fig. 2C and Table 3). Kinetics for sEPSC rise times were generally slower in the experimental group from P4-P9, but due to the small values on average and variance within groups, significance was less consistent. At P14 values decreased for all groups, and this trend was not statistically significant. The range of amplitudes was variable following TeNT expression across development, resulting in inconsistent trends across age (experimental group on average smaller (P4), similar (P6 and P14), or larger (P9) (Fig. 2D)). Both control groups (iMNTB of injected animals and non-injected animals) showed no difference across metrics and age, with the exception of the frequency and rise time at P9 (Fig. 2B, D), confirming the utility of our unilateral viral injection approach with an in-slice control (Fig. 1A-C). Overall, viral vector mediatedTeNT expression significantly reduced the frequency of spontaneous vesicle release during the critical growth period of the CH (Hoffpauir et al., 2010; Holcomb et al., 2013; Rodriguez-Contreras et al., 2008).

**Maturation of MNTB Principal Neuron Physiological Properties is Delayed Following *In Vivo* Silencing of Neurotransmission at the calyx of Held**

PNs in general become less excitable between P0 and P6 as input resistance declines from ~1 GΩ to ~250 MΩ (Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017), action potential threshold current increases from ~50 pA to >200 pA, and the resting membrane potential (RMP) hyperpolarizes from -64 to -72 mV. We performed whole-cell current-clamp recordings from PNs in the iMNTB (control) and cMNTB (transduced CHs) following viral vector injections to investigate the role of spontaneous activity (SA) in these maturational profiles (Fig. 3A). Recordings were made from animals after the growth phase of the CH (P4), after most PNs are mono-innervated (≈75% at P6), and bracketing the onset of hearing (P9 and P14) (Holcomb et al., 2013; Mikaelian et al., 1965; Rodriguez-Contreras et al., 2008). Following TeNT expression, cMNTB PNs displayed larger input resistance at P4, more consistent with values at P2 (Hoffpauir et al. 2010; Rusu & Borst, 2011), and did not become similar to iMNTB PNs until P9, although values at P9 and P14 were larger on average but not statistically different (Fig. 3A, B). Membrane time constant in cMNTB PNs followed a similar developmental trajectory, but remained significantly larger at P9 and P14 (Fig. 3A, B). RMP remained on average larger across these ages, but due to large variance in these values was not consistently statistically significant (Fig. 3A, C). Overall, these data reveal a delayed developmental trajectory that converges toward control values, and consistent with previous reports (Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017), at P9 and P14. During hyperpolarizing current injections MNTB PNs exhibit a prominent depolarizing sag due to the activation of hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels (Banks et al.,1993; Koch et al., 2004). To standardize this metric, accounting for differences in input resistance, we measured the depolarization sag following hyperpolarization to -90 mV, which was sufficient to generate absolute sag values ≥ 2mV in most cells (Fig. 3A, D). The values for depolarization sag were greater at P4, P9, and P14, though not statistically significant, indicating an increased hyperpolarized activated conductance (Ih) in cMNTB PNs. At P6 cMNTB PNs displayed a reduced depolarization sag, which could account for the hyperpolarized RMP (Fig. 3C) as an increase in HCN channel expression leads to a concomitant depolarizing shift in the RMP (Rothman & Manis, 2003). Overall, these data show a role for intrinsic spontaneous activity regulating the maturation of MNTB PNs biophysical properties, whereby neurons innervated by transduced CHs remain in an immature hyperexcitable state persisting into the second postnatal week.

**Conversion from Tonic to Phasic Spike Pattern is Delayed Following *In Vivo* Silencing of Neurotransmission at the calyx of Held**

At P3 MNTB PNs begin to transition from a tonic to phasic firing pattern (Hoffpauir et al., 2010; Sierksma et al., 2017) in response to depolarizing current injections, whereas in the mature brain most PNs fire a single AP. Exemplary traces from P6 iMNTB and cMNTB PNs that show the responses to hyperpolarizing and depolarizing current injections display the characteristic phasic firing pattern (iMNTB) found in normally developing animals, and persistent tonic firing (cMNTB) following TeNT expression (Fig. 4A, B). For a more quantitative classification of responses, we plotted the number of evoked action potentials (AP) as the amplitude of a 300 ms current step was increased. Since many cells entered into depolarization block of APs at large depolarizing currents and thus had non-monotonic profiles, for clarity we plotted values up to the peak response (Fig. 4C). These profiles revealed populations of cells with low current thresholds and high maximum rates, cells with low current thresholds and low maximum rates, and cells with high current thresholds and low or high maximum rates. All cMNTB PNs, except two cells, at P4-14 displayed a characteristic tonic firing pattern at current injections ≤ 200 pA, so we chose the number of spikes at 200 pA to classify MNTB PNs as phasic (≤ 3 APs) or tonic (≥4 APs) (Fig. 4B, C insets). Almost all PNs in the cMNTB exhibited a tonic firing pattern across neonatal ages, and thus did not acquire the phasic phenotype through this age range. PNs in the iMNTB began to acquire the characteristic phasic firing pattern from P4-P6 and maintained it at P9 (Fig. 4B). At P14, we recorded a divided population of phasic and tonic cells (Fig. 4B), suggesting reorganization of neural activity after opening of the ear canal at P10-12 (Mikaelian et al., 1965).

**Action Potential Current Thresholds Increase and Kinetics are Slightly Slower Following *In Vivo* Silencing at the CH:MNTB Connection**

We next wondered if the functional delays described in the previous section affected AP characteristics such as PN current and voltage thresholds, and action potential waveform kinetics (Fig. 5 and Table 3.5). These parameters were quantified from the first suprathreshold spikes elicited from depolarizing current injection and their phase-plane plots as illustrated by exemplary waveforms at P6 (Fig. 5A). Phase-plane plots illustrate the effects of TeNT expression on AP properties plotting the time derivative of the voltage (dV/dT) versus the membrane potential showing a depolarizing shift in the spike threshold (inflection point), increased peak amplitude in cMNTB PNs, and reduced repolarization rate, though results were not significant across development (Fig. 5A and 5C-G). The threshold current required to elicit a spike in cMNTB PNs was ≈50-70 pA at P4 and P6, more consistent with P2 in control animals (Hoffpauir et al. 2010), and just exceeded 100 pA at P9, more consistent with P3-P4 in control animals (Hoffpauir et al. 2010) and iMNTB from these data (Fig. 5B). Values were on average lower at P14, but not statistically significant. Voltage thresholds were more depolarized on average by several mV across ages, but due to the variance in values (range 10-20 mV across conditions and ages) were only significantly different at P9 (Fig. 5C). At P4-9, consistently slower depolarization and repolarization rates, although not significant individually, and similar amplitudes, resulted in slightly broader half-width (significant at P4; Fig. 5D-G and Table 3.5).

**Principal Component Analysis Shows the Diverging PN Physiological Maturational Profiles Following *In Vivo* Silencing at the CH:MNTB Connection**

Following TeNT expression apparent differential expression of ion channels is evident by the increased excitability, persistent tonic firing pattern, and slower AP kinetics and spike broadening (Figs. 3, 4, and 5). To better understand how TeNT expression contributes to the delay in maturation of PN physiological properties we performed a principal component analysis (PCA) of the electrophysiological parameters comparing iMNTB and cMNTB PNs across development (Fig. 6). We performed an initial PCA on P9 iMNTB PNs, which represent an end-point of maturation with most physiological parameters plateauing at this age (Hoffpauir et al., 2010; Rusu & Borst, 2011), analyzing 10 electrophysiological parameters (Fig. 6A). The first two principal components accounted for 67.9% of the variance with AP waveform parameters (amplitude, half-width, and maximum depolarization and repolarization rate) and intrinsic properties (RMP, threshold current, input resistance, and membrane time constant) contributing to the variability in the first and second principal components, respectively (Fig. 6A, B). Following PCA of P9 iMNTB PNs a K-means clustering algorithm was applied identifying four clusters of cells with similar electrophysiological properties. The significant distinction between each cluster of PNs is likely associated with the large variability of each individual electrophysiological parameter (Fig. 3, 5, and 6A). The three tonic cells in the P9 iMNTB group are generally more excitable with a depolarized RMP and high input resistance and have larger and faster rising APs likely reflecting increased Na+ conductance, consistent with previous reports characterizing firing pattern in auditory neurons (Bondy et al., 2021; Hoffpauir et al., 2010; Sierksma et al., 2017). To compare the physiological maturation stages of MNTB PNs across development we projected P4 and P6 iMNTB neurons onto the 2D PCA space of P9 iMNTB neurons (Fig. 6C). We chose a projection approach onto lower dimensional space instead of grouping all PNs as traditional PCA standardizes electrophysiological parameters for individual neurons based on the mean and standard deviation for all age groups. Given that during development the physiological properties of MNTB PNs continue to mature after the growth phase of the CH (Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017) we set P9 iMNTB as the maturation end-point and standardized electrophysiological parameters for the other age groups individually, P4 and P6 iMNTB, to the P9 iMNTB dataset (Fig. 6C). From this projection approach the maturational trajectory of MNTB PNs is evident with a majority of the distinction between age groups attributed to the AP waveform kinetics along the first PC axis. A K-means clustering algorithm was applied to the projection data where optimal clustering was achieved for three clusters, identifying age groups (Cluster 1: P4; Cluster 2: P6; Cluster 3: P9) with minimal overlap. This clustering methodology allows for an unbiased approach to identify maturational groups and reduce inherent noise associated with variability in the electrophysiological properties of PNs during normal development (Hoffpauir et al., 2010; Rusu & Borst, 2011). Violin plots of the 10 electrophysiological parameters analyzed in the PCA show the maturational changes of iMNTB PNs physiological properties, with the most significant changes from P4-P9 attributed to the AP properties (Fig. 6D). To better understand the effects of TeNT expression on the maturation of PN physiological properties we performed a combined projection of all age groups and experimental conditions (iMNTB: open symbols and cMNTB: closed symbols) (Fig. 6E). Comparison of P9 iMNTB and cMNTB PNs shows a continuum in 2D PCA space. Interestingly, a majority of P4 and P6 cMNTB PNs occupy distinct spatial domains and when comparing the electrophysiological parameters these cells had larger input resistance, slower membrane time constant, and a depolarized RMP, consistent with immature hyperexcitable PNs (Hoffpauir et al., 2010; Rusu & Borst, 2011) and a delay in maturation. Comparison of the individual physiological properties for P9 iMNTB and cMNTB PNs (Fig. 3, 5) and the projection approach onto P9 iMNTB PCA space (Fig. 6E) shows a convergence of maturation profiles, likely reflecting homeostatic compensation from chronic blockade of activity. However, from the PCA the first two principal components account for only 67.9% of the total variance. Utilizing the same projection approach, for P9 cMNTB mapped onto P9 iMNTB PCA space, a 3D scatter plot with the first three PCs shows a clear sepeartion between the two groups along the PC3 axis (plane at PC3 = 1) (Fig. 6F). From the contribution plot the variability associated with PC3 is mostly attributed to the passive membrane properties (Input resistance, membrane time constant, and RMP) (Fig. 6F). Finally, to better understand the maturation profiles of all age groups following TeNT expression we utilized the same projection approach, mapping all groups onto P9 iMNTB PCA space, taking into account the total variance for the first three PCs (Fig. 6G). A 3D plot of all age groups represented as ellipsoids, volume based on one standard deviation from the centroid, shows the maturational trajectory for iMNTB (control) and cMNTB (TeNT) PNs. By projecting each ellipsoid onto the PC1/PC3 plane a parallel maturational trajectory is evident suggesting divergence from normal development, where blocking SA at the CH:MNTB synaptic connection resulting in PNs with distinct electrophysiological profiles (Fig. 6G).

Computational Modeling Simulating the Effects of TeNT Expression

One of the more striking findings abolishing synaptic transmission at the CH:MNTB connection is the delayed transition from a tonic to phasic firing pattern (Fig. 3.6A), where under normal development PNs transition to phasic firing beginning at P3 (Hoffpauir et al., 2010). This transition to a phasic firing pattern is due in part to expression of a low threshold potassium conductance (KV1) that activates close to the RMP preventing repetitive firing (Brew & Forsythe, 1995; Dodson et al., 2002; Hoffpauir et al., 2010). To better understand the altered biophysical properties of cMNTB PNs following TeNT expression, we generated a single compartment model incorporating various ion channel conductance and activation parameters utilizing Hodgkin-Huxley equations (Hodgkin & Huxley, 1952). The single compartment was a soma of 20 mm diameter, which was implemented based on previous MNTB PN models (Macica et al., 2003; Sierksma et al., 2017; Wang et al., 1998) and contained five conductances: a sodium conductance (), leak conductance (), low threshold potassium conductance (), high threshold potassium conductance (), and hyperpolarization activated conductance () (Fig. 3.8A). All cMNTB PNs across development (P4, P6, and P9) fired trains of APs in response to depolarizing current injections (Fig. 3.6) in contrast to characteristic control neurons firing one or two APs phasically at stimulus onset (Hoffpauir et al., 2010). To compare the effects of TeNT expression we setup our model utilizing ion channel gating kinetics (Macica et al., 2003; Sierksma et al., 2017; Wang et al., 1998) and conductance parameters (, , , , and ), tuned from *in vivo* recordings (Sierksma et al., 2017), based on previous models of MNTB PNs. We simulated a P6 MNTB PN under normal conditions (non-transduced by TeNT) before and after removal of the KV1 conductance (Fig. 3.8B, C). The Hodgkin-Huxley model of a P6 PN with normal KV1 conductance displayed the characteristic single AP phenotype (Dodson et al., 2002; Hoffpauir et al., 2010) in response to a 200-pA, 300 ms depolarizing current injection. Removing the KV1 conductance from the model resulted in a train of APs (32 spikes) in response to the same depolarizing current injection, consistent with electrophysiological recordings in the cMNTB (TeNT expression; 30.3 ± 15.8 spikes, n = 18; 200-pA current injection). Our model recapitulated the effects of TeNT delaying the transition from a tonic to phasic firing pattern in response to depolarizing current injections in MNTB PNs, suggesting differential expression of the low voltage activated potassium channel (KV1). The effects of TeNT expression also resulted in increased excitability, in part due to higher input resistance, compared to iMNTB PNs (Fig. 3.6C) and consistent with more immature PNs (P2; Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017). To further simulate the effects of TeNT expression we tuned the conductance parameters (, , , , and ) to fit our experimental recordings for non-injected mice and following viral vector injections (Fig. 3.8D and E). Experimental current-clamp recordings from P2 and P6 non-injected PNs show the characteristic transition from tonic to phasic firing and decreased input resistance around the RMP, that was accurately simulated from our model (Fig. 3.8D and F). The decrease in excitability of MNTB PNs from P2-P6 is largely due to a depolarizing shift in RMP, increase in KV1 expression, and reduced input resistance (Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017), consistent with experimental recordings showing a delayed development of these parameters following TeNT expression (Fig. 3.6). TeNT expression resulted in increased excitability of P6 cMNTB PNs (Fig. 3.6) with intrinsic properties closer in value to immature P2 neurons (Hoffpauir et al., 2010; Rusu & Borst, 2011; Sierksma et al., 2017). To model the effects of TeNT expression we tuned the conductance parameters for our model of a P6 cMNTB PN by focusing on ion channels that regulated the RMP (i.e., leak, low threshold potassium, and HCN channels), tonic firing (low threshold potassium), and after-hyperpolarization induced action potentials (HCN) to fit our experimental findings (Fig. 3.6 and 3.8D-E). Our model simulating the effects of TeNT for a P6 cMNTB PN had a higher input resistance consistent with P2 non-injected neurons and displayed repetitive firing to depolarizing current injections with the appearance of after-hyperpolarization induced rebound APs (Fig. 3.8D and F) that accurately recapitulates the effects of pharmacological block of KV1 channels with dendrotoxin (Dodson et al., 2002; Hoffpauir et al., 2010). Overall, the results of our experimental recordings and computational modeling show that blocking synaptic transmission at the CH:MNTB connection results in delayed decrease in intrinsic excitability, likely due to reduced low threshold potassium channel expression and increased HCN channel expression.

Impaired Growth of the CH Following *In Vivo* Silencing at the CH:MNTB Connection

Previous studies have shown that synaptogenesis initiates in the absence of spontaneous and evoked synaptic transmission (Varoqueaux et al., 2002; Verhage et al., 2000) but ultimately neurons and synapses are removed. We noted, surprisingly, easy visual identification of transduced calyces during electrophysiological recordings which reveal that the CH initially grows (Fig. 3.4A and 3.5A). To assess the effects of TeNT expression on CH structural development we utilized traditional immunohistochemistry (IHC) approaches along with confocal microscopy on brainstem tissue sections at P6 and P9 (Fig. 3.9). For IHC we used antibodies against vesicular glutamate transporter 1 and 2 (Vglut1/2) to label the CH, dsRed to amplify the signal from transduced calyces (mCherry co-expressed with TeNT), and microtubule associated protein 2 (Map2) to label MNTB PNs. Lower magnification images of the iMNTB and cMNTB following viral vector injections expressing TeNT qualitatively shows expression of mCherry restricted to the calyces in the cMNTB (Fig. 3.9 A and B). Significant co-labeling between mCherry and Vglut1/2 staining in the cMNTB is observed resulting from high titer viral injections and labeling of nearly all GBCs in the cochlear nucleus, consistent with the afferent projection pattern to the cMNTB (Friauf & Ostwald 1988; Kandler & Friauf, 1993; Kuwabara et al., 1991; Morest, 1968; Smith et al., 1991; Spirou et al., 1990; Fig. 3.9B). In the iMNTB, mCherry expression is localized to the trapezoid body fascicles (ventral acoustic stria) passing through the MNTB *en route* to the cMNTB (Friauf & Ostwald, 1988; Kuwabara et al., 1991; Spirou et al., 1990), other SOC nuclei, and ascending auditory projections (Fig. 3.9A; Cant, 2019). High-resolution confocal image stacks visually illustrate the impaired growth of transduced CHs with reduced volume and increased thickness, likely from accumulation of synaptic vesicles in the terminal (Kim et al., 2009; Sando et al., 2017), compared to non-transduced calyces in the iMNTB (Fig. 3.9C and D). For quantitative assessment of the morphological differences between iMNTB and cMNTB calyces we randomly selected a subset of terminals from each image volume for segmentation and morphological quantification (Fig. 3.9C’ and D’). Comparison of calyces from the cMNTB and iMNTB show reduced volume and increased thickness following *in vivo* synaptic silencing (Fig. 3.9E and F).

**Discussion**

From Results: The disparities in intrinsic excitability beginning at P9 suggest possible homeostatic plasticity occurring prior to opening of the ear canal, where chronic inhibition can result in differential gene expression of ion channels to maintain homeostasis of neuronal circuits (Davis, 2013; Desai et al., 1999; Li et al., 2020; Schaukowitch et al., 2017).

in response to a presynaptic spike ensuring temporal fidelity in transmission of synaptic information at high frequencies (reviewed by Borst & Soria van Hoeve, 2012).

Summary of Observations

We investigated the development of sEPSCs during the first two postnatal weeks in MNTB PNs, systematically characterizing the size and time course prior to the growth of the CH (P0-2), during the growth phase (P2-4), after most cells are mono-innervated (P6) and bracketing the opening of the ear canal (P9 and P14) (Kandler & Friauf, 1993; Hoffpauir et al., 2006; Holcomb et al., 2013; Mikaelian et al., 1965). The frequency of sEPSCs was similar across neonatal ages (P0-P9) with a significant increase after the opening of the ear canal at P14, consistent with fenestration of the CH and increase in the number of active zones (Dondzillo et al., 2010; Ford et al., 2009; Satzler et al., 2002; Soria van Hoeve & Borst, 2010; Taschenberger et al., 2002). Beginning at P4 the time course of sEPSCs in MNTB PNs accelerates, correlating with the AMPA receptor isoform switch to the fast-gating flop GluA4 variant resulting in the developmental speeding of AMPAR desensitization from P7-P14 and faster EPSC kinetics (Joshi et al., 2004; Koike-Tani et al., 2005; Yang et al., 2011). Consistent with previous reports the amplitude and frequency of sEPSCS was unchanged after application of TTX at all neonatal ages, indicating quantal events at the CH are not driven by presynaptic activity (Kushmerick et al., 2006; Rusu & Borst, 2011; Sahara & Takahashi, 2001). Similarly, the size and time course of sEPSCs in MNTB PNs was consistent after application of the non-selective VGCC blocker Cd2+ across neonatal ages, when spontaneous release has been shown to be regulated by VGCC in inhibitory, but not excitatory synapses (Tsintsadze et al., 2017; Vyleta & Smith, 2011; Williams et al., 2012). To investigate the role of spontaneous activity in the synchronous maturation of the CH:MNTB synaptic connection we utilized a viral vector approach expressing TeNT to directly silence activity during the critical period of neural circuit formation (P2-P4; Hoffpauir et al., 2010; Holcomb et al., 2013; Rusu & Borst, 2011). The efficacy of TeNT mediated synaptic silencing was experimentally determined through whole-cell voltage-clamp recordings in MNTB PNs showing a significant reduction in the frequency of sEPSCs. The recorded quantal events could arise from non-calyceal inputs (Banks & Smith, 1992; Hamann et al., 2003). Paired simultaneous pre- and postsynaptic recordings of the CH and associated MNTB PN showed abolished evoked neurotransmission in transduced terminals (TeNT expression) confirming our viral vector approach to silence activity at the CH:MNTB connection. We found that blocking spontaneous activity prior to the onset of cochlea induced bursting activity in the MNTB (Babola et al., 2021; Tritsch, Rodriguez-Contreras, et al., 2010; Tritsch et al., 2007) resulted in PNs remaining in an immature hyperexcitable state with delayed maturation continuing into the second postnatal week. Computational modeling of the effects of TeNT showed delayed expression of KV1 and likely increased HCN channel expression regulating the firing pattern. Structural assessment of the effects of TeNT expression on the morphology of the CH showed impaired growth with reduced volume and increased thickness compared to ipsilateral control terminals. Pharmacological manipulation with the L-type VGCCs antagonist isradipine showed expression of L-type Ca2+ channels in MNTB PNs and spike broadening suggesting a contribution of these channels in shaping the AP waveform. This study highlights the importance of intrinsic spontaneous activity in the coincident maturation of the CH growth and postsynaptic MNTB PN physiological properties.

Characterization of Spontaneous Excitatory Postsynaptic Currents in MNTB Principal Neurons

The Ca2+ dependence of spontaneous activity represents a divergence from the other modes of neurotransmission, synchronous and asynchronous vesicle release, with spontaneous vesicle fusion occurring either with or without localized increase in Ca2+ concentration through VGCCs (Emptage et al., 2001; Ermolyuk et al., 2013; Helmchen et al., 1997; Tsintsadze et al., 2017; Williams et al., 2012). The Ca2+ sources regulating spontaneous release include basal concentrations (Babai et al., 2014; Kochubey & Schneggenburger, 2011; Lou et al., 2005; Sun et al., 2007), stochastic opening of VGCCs (Ermolyuk et al., 2013; Tsintsadze et al., 2017; Williams et al., 2012), and release from internal stores (Bal et al., 2013; Emptage et al., 2001; Llano et al., 2000). Within the literature there are conflicting views on the role of VGCCs regulating release at inhibitory and excitatory synapses (Dai et al., 2015; Ermolyuk et al., 2013; Tsintsadze et al., 2017; Vyleta & Smith, 2001; Williams et al., 2012). Recent evidence, recording from P10-P12 MNTB PNs which receive excitatory glutamatergic input from the CH and GABAergic and glycinergic inhibitory inputs (Awatramani et al., 2005), showed that the non-selective VGCC blocker Cd2+ affected spontaneous release only from inhibitory synapses (Tsintsadze et al., 2017). Consistent with these results we have shown a consistent mechanism throughout postnatal development (P0-P14), whereby VGCCs do not trigger spontaneous vesicle release from excitatory synapses in MNTB PNs (Fig. 3.1).

Unlike evoked synaptic transmission, spontaneous vesicle release is independent of presynaptic activity with spontaneous postsynaptic currents or miniature events typically recorded in the presence of the voltage gated sodium channel blocker tetrodotoxin (TTX) (Kaeser & Regehr, 2014). Previous studies recording sEPSCs from MNTB PNs have shown no change in the amplitude or frequency of individual events following application of TTX (Ishikawa et al., 2002; Kushmerick et al., 2006; Rusu & Borst, 2011; Sahara & Takahashi, 2001; Yamashita et al., 2003). These studies were in rat and predominantly from older postnatal ages. Consistent with these earlier studies we have shown that the size and time course of sEPSCs are indistinguishable from mEPSCs (recorded in the presence of TTX) across neonatal ages (Fig. 3.1). The histogram distributions of size and kinetics for all individual quantal events showed a large variance for all neonatal ages (Fig. 3.2B). This variance may be due to variable concentration of neurotransmitter in individual synaptic vesicles, the variable volume of synaptic vesicles, or rapid changes in the availability of postsynaptic receptors associated with AMPA receptor desensitization or saturation (Bekkers & Clements, 1999; Bekkers et al., 1990; Koike-Tani et al., 2005; Meyer et al., 2001; Partin et al., 1994). Developmental changes in the amplitude of sEPSCs were similar across neonatal ages, with the exception of P0 and P9, consistent with previous reports (Fig. 3.2B and C; Chuhma & Ohmori, 1998; Iwasaki & Takahashi, 2001; Rusu & Borst, 2011; Taschenberger & von Gersdorff, 2000). The reduced amplitude of sEPSCs at P0 might be attributed to reduced release probability and size of the readily releasable pool or low expression of AMPA receptors associated with an immature synapse (Chuhma and Ohmori, 1998; Del Castillo & Katz, 1954; Futai et al., 2001; Iwasaki & Takahashi, 2001; Joshi & Wang, 2002). The disparity at P9 could be attributable to structural development of the CH with fenestration occurring after opening of the ear canal, where homeostatic regulation of the expression patterns and redistribution of active zones and postsynaptic receptors may occur in preparation for this morphological change of the CH (Dondzillo et al., 2010; Ford et al., 2009; Kandler & Friauf, 1993; Soria van Hoeve & Borst, 2010). These morphological changes in the calyx also facilitate glutamate clearance, where astrocytic processes occupy the new PN territory mediating rapid clearance of glutamate from the synaptic cleft and may contribute to the reduced sEPSC amplitude at P9 (Ford et al., 2009; Holcomb et al., 2013; Holcomb, in preparation; Palmer et al., 2003; Renden et al., 2005).

Afferent Projections from the Cochlear Nucleus and Potential Off-Target Viral Labeling

Unlike the MNTB, which is comprised of a mostly homogeneous neuronal population, the PN (Altieri et al., 2015; Banks & Smith, 1992; Brandebura et al., 2018; Holcomb et al., 2013), the cochlear nucleus is comprised of a diverse neuronal population characterized from morphological, electrophysiological, and most recently RNA-sequencing techniques (Jing et al., 2023; Manis et al., 2019; Oertel et al., 2009). In order to abolish neurotransmission at the CH:MNTB synapse we utilized a stereotaxic viral vector approach to express TeNT in the cochlear nucleus, targeting the GBCs in the VCN specifically. Due to some intermixing of cell types and the volume of the injection, additional cell types were transduced (Fig. 3.3). Recombinant adenovirus has the ability to undergo retrograde transport, though at low efficiency (Ghadge et al., 1995; Kuo et al., 1995) and AAV can transduce postsynaptic neurons through anterograde trans-synaptic transport with a high initial virus titer (>1013 genome copies/mL) (Zhao et al., 2017; Zingg et al., 2017). In the case of retrograde labeling, we never observed mCherry labeling (co-expressed with TeNT) in the auditory nerve fibers (data not shown), which are the afferent projections of spiral ganglion neurons to the cochlear nucleus (Fekete et al., 1984; Liberman, 1980; Lorente de No, 1933). Immunohistochemical labeling and fluorescent imaging of the auditory brainstem shows prominent labeling of the ventral acoustic stria and contralateral MNTB, consistent with GBC projections terminating on PNs (Fig. 3.3C-C’’; Friauf & Ostwald, 1988; Kandler & Friauf, 1993; Kuwabara et al., 1991; Morest, 1968; Smith et al., 1991; Spirou et al., 1990). In addition to this targeted TeNT expression in GBCs, fluorescent labeling was observed in the ipsilateral, to the injection site, lateral superior olive (LSO) and contralateral LSO and VCN (Fig. 3.3C). The primary projection neurons in the VCN are the spherical and globular bushy cells (SBCs) and planar multipolar cells, also referred to as T-stellate cells (Lauer et al., 2013; Oertel et al., 1990; Xie & Manis, 2017). SBCs project to the ipsilateral LSO (Cant & Casseday, 1986). T-stellate cells send axonal projections to the trapezoid body (ventral acoustic stria) terminating in the ipsilateral LSO and lateral and ventral nucleus of the trapezoid body (VNTB) and contralateral superior paraolivary nucleus and VNTB (Doucet & Ryugo, 2006; Oertel et al., 1990). In addition to the projection patterns of planar multipolar cells, radiate multipolar cells (D-stellate) project to the opposite cochlear nucleus via the commissural pathway and may have anterogradely transported virus to postsynaptic cell bodies, consistent with labeling in the non-injected, contralateral VCN (Fig. 3.3C; Brown et al., 2013; Doucet & Ryugo, 2006; Needham & Paolini, 2003; Schofield & Cant, 1996; Smith et al., 2005). A notable absence of labeling was shown in the MNTB ipsilateral to the injection site, indicating that GBCs in the non-injected cochlear nucleus were not transduced. Further confirmation of this conclusion came from physiological data indicating comparable sEPSCs size and kinetics and PN intrinsic properties to non-injected control mice and previous reports (Fig. 3.3C-C’’ and Fig. B.3; Hoffpauir et al., 2010; Rusu & Borst, 2011).

Efficacy of *In Vivo* Silencing of Spontaneous Activity at the CH:MNTB Synaptic Connection

Our approach to abolish synaptic transmission at the CH:MNTB synapse utilized the clostridial neurotoxin (TeNT), with transduced calyces expressing the light chain of the toxin that cleaves the vesicle fusion protein synaptobrevin 2 (Breidenbach & Brunger, 2005; Schiavo et al., 1992). Previous reports utilizing TeNT or genetic knockout of synaptobrevin-2 have shown a complete block of evoked release and additionally abolished a large portion (≈70-90%) of spontaneous release (Kerschensteiner et al., 2009; Kim et al., 2009; Sakaba et al., 2005; Sando et al., 2017; Schoch et al., 2001) consistent with our observations (Fig. 3.4 and 3.5). Whole-cell patch-clamp recordings from MNTB PNs following viral vector mediated TeNT expression at the CH resulted in a reduction in the frequency of sEPSCs (≈60-80%) across neonatal ages compared to non-injected controls (Fig. 3.4B and C and Fig. B.3). The persistent quantal events in PNs following TeNT could be attributed to non-transduced GBCs, excitatory non-calyceal inputs (Forsythe & Barnes-Davies, 1993; Hamann et al., 2003), or the presence at the active zone of other, TeNT-insensitive, vesicular SNARE proteins (Kavalali, 2015; Takamori et al., 2006). In addition to the large excitatory somatic CH nerve terminal, MNTB PNs receive smaller non-calyceal inputs with slower kinetics and a proportionally larger NMDA receptor-mediated response (Hamann et al., 2003). Though synaptobrevin-2 (VAMP2) regulates a substantial proportion of spontaneous release (Schoch et al., 2001), other non-canonical SNARE proteins, VAMP7 (also known as TeNT-insensitive VAMP) and Vps10p-tail-interactor-1a (Vti1a), regulate this mode of neurotransmission (Bal et al., 2013; Crawford et al., 2017; Hua et al., 2011; Ramirez et al., 2012). In addition to the different SNARE proteins regulating spontaneous neurotransmission, distinct vesicle pools (Melom et al., 2013; Peled et al., 2014) and postsynaptic receptors (Atasoy et al., 2008; Sara et al., 2011; Zenisek, 2008) have been shown to preferentially regulate evoked and spontaneous release. Further investigation is needed to identify the synaptic origin and molecular mechanism associated with the persistent sEPSCs in MNTB PNs following TeNT expression.

Formation of the CH

Structural assessment of the effects of TeNT expression at the CH showed impaired morphology with reduced volume, increased thickness, and persistent collateral branching, yet a recognizable CH managed to grow (Fig. 3.9). Previous manipulations to signaling pathways have resulted in impaired CH size, morphology, and increased polyinnervation (Kronander et al., 2019; Lilley et al., 2014; Xiao et al., 2013), but similar to this study yield an incomplete phenotype. These cumulative studies suggest that the mechanisms regulating formation of the CH constitute a robust process. Extracellular signaling cues also regulate synapse remolding during developmental climbing fiber (CF) innervation of Purkinje cells (PCs) in the cerebellum (Kano & Watanabe, 2019). Knockdown of the secreted semaphorin Sema3A in PCs resulted in a reduction in the number of CFs poly-innervating each PC, whereas Sema7a (membrane-anchored molecule) knockdown resulted in an increase in the number of CFs (Uesaka et al., 2014). Conditional brain-derived neurotrophic factor (BDNF) knockout mice targeting PCs impaired CF synapse elimination (Choo et al., 2017), where retrograde BDNF signaling to tropomyosin receptor kinase B (TrkB) receptor expressed in the CF facilitates elimination in the developing cerebellum (Bosman et al., 2006; Johnson et al., 2007). Anterograde BDNF to TrkB signaling from the CH to MNTB PN inhibits synaptic transmission by slowing the kinetics of VGCC currents and impairing endocytic vesicle recycling (Baydyuk et al., 2015; Wu et al., 2020). In the MNTB, genetic deletion of BDNF resulted in loss of physiological and structural tonotopic gradients in an activity dependent mechanism with disrupted sound evoked cFos expression (Wollet & Kim, 2022). These results are consistent with previous reports showing loss of ion channel gradients along the tonotopic axis *dn/dn* deafness mouse model (Leao et al., 2006). Accumulating evidence has shown that neural activity along with genetic and extracellular signaling cues play a synergistic role in the refinement of synaptic connections during development (Hrvatin et al., 2018; Kano & Watanabe, 2019; Kitazawa et al., 2021; Mikuni et al., 2013; Stroud et al., 2020; Yap & Greenberg, 2018).

Activity Dependent Neural Circuit Maturation and Gene Expression

The traditional model for neural circuit formation follows an early phase of synaptogenesis and coarse wiring controlled by genetic and molecular cues, whereby nascent synapse formation can occur after abolishing neurotransmission (Varoqueaux et al., 2002; Verhage et al., 2000). However, refinement of the connectome architecture is controlled by neural activity (reviewed by Blankenship & Feller, 2010). During development, patterned bursting activity from the cochlea propagates to the MNTB with about 60% of P2 PNs displaying burst firing from *in vivo* recordings (Babola et al., 2020; Babola et al., 2018; Babola et al., 2021; Tritsch, Rodriguez-Contreras, et al., 2010), coincident with the growth phase of the CH and synchronous maturation of the PN biophysical properties (Hoffpauir et al., 2010; Holcomb et al., 2013; Rusu & Borst, 2011). During early postnatal development, prior to the growth phase of the CH, MNTB PNs are hyperexcitable primarily associated with a depolarized resting membrane potential and high input resistance, whereby spontaneous vesicle release was sufficient to trigger an action potential (Hoffpauir et al., 2010; Rusu & Borst, 2011). Our study abolishing synaptic transmission at the CH:MNTB connection showed that without neural activity PNs remained in this immature hyperexcitable state for a longer developmental period. These data are consistent with previous reports showing activity-dependent regulation of homeostatic excitability (Walmsley et al., 2006) and consistent with *in vitro* (Hoffpauir et al., 2010; Rusu & Borst, 2011) and *in vivo* (Sierksma et al., 2017)recordings correlating excitability with input size, where immature PNs with high intrinsic excitability have small inputs and the decrease in excitability of PNs corresponds to the growth phase of the CH from P2-P4 (Holcomb et al., 2013). The intrinsic properties of P6 PNs following TeNT expression corresponded to P2 neurons during normal development (Hoffpauir et al., 2010; Rusu & Borst, 2011), consistent with smaller inputs where the CH grows but is severely impaired structurally (Holcomb et al., 2013; Fig. 3.9). We recapitulated these effects utilizing a compartmental neuron model showing delayed KV1 expression and conductance parameters of a simulated P6 cMNTB PN (TeNT expression) more consistent with immature neurons (P2; Fig. 3.10D-F).

In conventional excitation-transcriptional coupling Ca2+ entry through L-type VGCCs regulates activity-dependent gene expression (Deisseroth et al., 1996; Wheeler et al., 2012). Previous investigation of somatic L-type VGCCs in P10-P14 MNTB PNs resulted in discrepancies between species with expression in mice, but not rats, though both studies did not look at younger neonatal ages (Barnes-Davies et al., 2001; Leao et al., 2004). Genetic knockout of the L-type VGCCs (Cav1.2 and Cav1.3) resulted in decreased volume and 1/3 fewer neurons in the MNTB (Ebbers et al., 2015; Hirtz et al., 2011; Satheesh et al., 2012). These studies utilized either a global (Hirtz et al., 2011) or conditional gene knockout (Ebbers et al., 2015; Satheesh et al., 2012) using the Krox20 promoter (Egr2 Cre-driver line) which is active in progenitor cells in r3 and r5, so recombination will occur in MNTB PNs (Voiculescu et al., 2000). The drastic decrease in MNTB volume from genetic deletion of L-type Ca2+ channels in PNs is likely linked to impaired activity-dependent gene expression mechanisms during development (Tong et al., 2010; Yap and Greenberg, 2018). Our findings showed functional evidence for the presence of L-type VGCCs in PNs with reduced AP frequency and spike broadening following pharmacological manipulation with the dihydropyridine isradipine (Fig. 3.10). Comparison of the AP kinetics and spike rate following application of isradipine showed variable results across development and is likely associated with the reduced sensitivity of CaV1.3 compared to CaV1.2 to dihydropyridines, with both expressed in the MNTB (Brandebura et al., 2022), and slow development of inhibition resulting in little effect on L-type currents induced by action potential-like stimuli (Berjukow & Hering, 2001; Helton et al., 2005; Lipscombe et al., 2004). Previous studies using organotypic cultures of the auditory brainstem have shown an upregulation of phosphorylated cyclic adenosine monophosphate (cAMP)-responsive element binding protein (pCREB), which regulates immediate early gene expression (Yap and Greenberg, 2018), in PNs following chronic depolarization with high K+ culture medium resulting in increased KV3 currents (Tong et al., 2010). Future studies are needed to investigate the link between L-type VGCC regulating activity-dependent gene expression and ion channel expression during early postnatal development in the MNTB.

**Materials and Methods**

Animals

All procedures involving animals were approved by the University of South Florida Institutional Animal Care and Use Committees. FVB mice (NCI; Frederick, MD and Jackson Laboratory; Bar Harbor, ME; RRID:IMSR\_ARC:FVB) of either sex were used in all experiments, unless otherwise specified.

DNA Construct and Recombinant Viral Vector Production

A recombinant helper-dependent adenovirus (HdAd28E4) plasmid was optimized for rapid, high-level expression of the 50 kDa tetanus neurotoxin light chain (TeTxLC), a metalloprotease that cleaves the synaptic vesicle fusion protein synaptobrevin-2 (Breidenbach & Brunger, 2005; Lacy et al., 1998; Schiavo et al., 1992), independently of the fluorescent reporter mCherry. The dual expression plasmid utilizes the pUNISHER cassette (Montesinos et al., 2011) to drive expression of TeTxLC along with a separate neuron specific mCherry expression cassette driven by the 470 bp hSyn promoter. Production of the HdAd was carried out as previously described (Montesinos et al., 2016). For stereotaxic viral injections the amount of the virus injected did not exceed a total of 2.03x108 viral particles/μL. Preparation of the viral injection solution followed the protocol previously described (Keine et al., 2023), with the virus stock solution diluted in storage buffer (250 mM Sucrose, 10 mM HEPES, 1 mM MgCl2 dissolved in nanopure H2O) and 20% mannitol solution.

Stereotaxic Viral Injection

Viral vector injections were performed on newborn (postnatal day (P)0) pups. Anesthesia was induced by deep hypothermia. Individual fingers of nitrile gloves were cut-off and neonatal pups were placed inside positioned with the head facing up and immersed in ice cold water (2-3°C) for 5 minutes. The depth of anesthesia was verified by toe pinch and a lack of ensuing reflex response. Pups were positioned on a chilled, clay-filled aluminum block (custom built) in the prone position and held in place with additional clay. The aluminum block was positioned on a Kopf stereotaxic frame (model 940 digital; David Kopf Instruments, Tujunga, CA). A small incision was made in the skin at the injection site using a sterile 26G needle. Micropipette glass capillary needles (Blaubland; IntraMARK) were pulled using a Narishige puller (model PC 10, RRID:SCR\_022057), clipped to an approximate tip diameter of 20-50 µm, and loaded with the virus solution (HdAd28E4 Cpun TeTxLC syn mCherry) using capillary action. For P0 mouse pups lambda and bregma (where coronal and sagittal sutures intersect) is identified visually by evident suture lines in the skull. An injection glass needle is positioned directly above lambda and slowly moved to bregma assuring proper anterior/posterior stereotaxic alignment of the mouse pup. From bregma (location considered 0.0 mm) the coordinates for the ventral cochlear nuclei are as follows: anterior-posterior: -5.1 mm, medial-lateral: -1.4 mm, and dorsal-ventral: -2.9-3.2 mm (assuming a 3.5mm distance from lambda to bregma) (Fig. 3.3A). The virus solution (up to 1 μL, but typically 200-500 nL) was then slowly infused at a rate of 0.1 μL/min. After injection of virus, the injection glass capillary needle was left in place for 1 minute prior to slow withdrawal. The animal was placed under a heat lamp (≈35°C) to recover and restore physiological temperature. Pups were rolled in bedding material to ensure that they were accepted by their mother upon return to the cage.

Slice Preparation for Electrophysiology Experiments

Acute brainstem slices were prepared from neonatal mice as previously described (Hoffpauir et al., 2006; Hoffpauir et al., 2010). FVB mouse pups (P4-9) were decapitated, and the brain was immediately dissected in ice-cold, low Ca2+ artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 3 MgCl2, 0.1 CaCl2, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myo-inositol, and 2 sodium pyruvate, pH 7.3. Coronal 250-300 m brainstem slices containing the MNTB were cut using a vibratome (VT1200S, Leica), stored at 37°C for 1 hr and kept at room temperature (RT) in normal ACSF containing the following (in mM): 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myo-inositol, and 2 sodium pyruvate, pH 7.3 until experimentation. All solutions were constantly bubbled with 95% O2/5% CO2.

Electrophysiology

All electrophysiology recordings were performed at near physiological temperature (33-35°C). Solution temperature was regulated using a dual-channel temperature controller with both in-line and chamber heaters (TC-344B, Warner Instruments). ACSF was constantly bubbled with 95% O2/5% CO2 and perfused over the slice at a rate of ≈2 mL/min using a peristaltic pump (P720, Instech). Neurons were visualized and targeted for whole-cell recordings using a two-photon microscope (HyperScope, Scientifica) equipped with Dodt Gradient Contrast (DGC) optics and an EM-CCD camera (C9100, Hamamatsu). All recordings were made from the medial 1/3, high frequency region of the MNTB to minimize the effects of differential ion channel expression and maturational gradients along the tonotopic axis (Leao et al., 2006; Weatherstone et al., 2017; Wollet & Kim, 2022). Patch-pipettes were pulled to a tip resistance of 2-4 MΩ for postsynaptic recordings (PNs) and 5-7 MΩ for presynaptic recordings (CH) using a micropipette puller (P-1000, Sutter Instrument Co.). The internal recording solution contained the following (in mM): 114 potassium gluconate, 26 KCl, 2 MgCl2, 0.1 CaCl2, 1.1 EGTA-Na4, 10 Hepes, 5 sodium phosphocreatine, and 4 ATP-Mg, pH 7.3, In some recordings the intracellular solution included a 10,000 MW dextran-conjugated fluorophore (Alexa Fluor 488 or 647; 50 mM) for visual identification. Whole-cell voltage and current-clamp recordings were made using a patch-clamp amplifier (model EPC 10 USB, HEKA Electronik, RRID:SCR\_018399) and data was acquired at a sampling rate of 20 kHz and 50 kHZ for current-clamp and voltage-clamp recordings, respectively. For voltage-clamp protocols series resistance during pre- and postsynaptic recordings was in the range of 8-25 MΩ and 4-10 MΩ, respectively and compensated 50-90% with a lag of 10 ms. For current-clamp recordings, pipette capacitance neutralization and bridge balance were adjusted and monitored during all recording sessions. Potentials were corrected online for a -13 mV liquid junction potential, calculated at 35°C using pCLAMP 9.2 software (RRID:SCR\_011323; Hoffpauir et al., 2010). For all recordings the glycine receptor antagonist Strychnine (2 mM) (European Pharmacopoeia) and GABAA receptor antagonist SR 95531 hydrobromide (gabazine; 10 mM) (Alomone Labs, RRID:SCR\_013570) were added to the extracellular solution to block inhibitory glycinergic and GABAergic currents, respectively (Awatramani et al., 2005). In some recordings the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 10 mM) (Tocris Bioscience, RRID:SCR\_003689) was bath applied to record MNTB PN intrinsic physiological properties. For recordings of spontaneous miniature excitatory postsynaptic currents (mEPSCs) the voltage-gated sodium channel blocker tetrodotoxin (TTX 1 mM) (HelloBio, RRID:SCR\_021047) was bath applied where indicated below, though mEPSCs kinetics were unaffected by TTX (Kushmerick et al., 2006; Rusu & Borst, 2011; Fig. 3.1).

Electrophysiology Data Analysis

Data were analyzed offline with custom routines using Igor Pro (Wavemetrics, RRID:SCR\_000325). Individual mEPSC events were detected utilizing a sliding template algorithm (Clements & Bekkers, 1997; Jonas et al., 1993) with custom code implemented in Igor Pro. The mEPSC template parameters included the peak amplitude, 20-80% rise time, and decay time constant (τ) and were defined based on average values recorded from MNTB PNs at physiological temperature. The detection threshold for individual events was set at 1 to 2 standard deviations above the baseline noise. Quantification of the decay time constant (τ) was determined by fitting a single exponential function to individual events. The goodness of fit was determined for representative cells across developmental ages (Fig. S3.1). The input resistance of MNTB PNs was calculated around the resting membrane potential by plotting the voltage-current relationship from the steady state and fitting a regression line to three points. Measurement of action potential (AP) waveform kinetics were made at rheobase, determined from 200 ms current steps at 5 pA increments. AP amplitudes were measured and reported from the baseline and voltage threshold. The voltage threshold was determined as the membrane potential at which the dV/dt exceeds 10 V/s (P2) and 20 V/s (P4-P14). The AP half-width was determined using the voltage threshold to peak amplitude measurement.

Computational Neuron Modeling

The MNTB PN model consists of a single compartment (soma) implemented using the NEURON simulation software (RRID:SCR\_005393; Hines & Carnevale, 2001). The Hodgkin-Huxley type model was based on previous numerical simulations of MNTB PNs (Macica et al., 2003; Sierksma et al., 2017; Wang et al., 1998). The PN model consisted of a single electrical compartment with a membrane capacitance (Cm) connected in parallel with a sodium current (*INa*), leak current (*ILeak*), high-threshold potassium current (*IKHT*), low-threshold potassium current (*IKLT*), and hyperpolarization-activated cation current (*Ih*). The changes to the membrane potential (*V*) was described by the following differential equation:

The leakage current was modeled by the following equation:

where is the maximum steady-state conductance and is the reversal potential for the leak current. The voltage dependence of the sodium current was modeled by the following equation:

where is the maximum steady-state conductance, is the reversal potential for the sodium current, and *m* and *n* are gating variables that model the open probability of the channel. The gating variables (*m* and *n*) are dimensionless parameters that model activation (*m*) and inactivation (*n*) of the sodium channel. For the MNTB PN model the outward potassium current was simulated utilizing a high-threshold Kv3.1-like current and a low-threshold Kv1.1 and Kv1.2-like current. The voltage dependence of the *IKHT* and *IKLT* currents were modelled by the following equations:

where and is the maximum steady-state conductance for the low and high-threshold potassium current, respectively. The gating variables model the activation and inactivation (*n* and *j*) of the high-threshold potassium channel and activation (*o*) of the low-threshold potassium channel. The voltage dependence of the hyperpolarization-activated current was modeled by the following equation:

where is the maximum steady-state conductance for the *Ih* current. The gating variable *u* models the activation of the hyperpolarization-activated cyclic nucleotide–gated channel. The time-dependence for the gating variable was governed by the following differential equation:

where *x* corresponds to the gating variables for each ion channel modeled (*m, h, n, j, o,* and *u*) and for a given voltage *x* approaches a steady-state value with a time constant . The steady-state value for each ion channel open probability was calculated from the following equation:

where and are the activation and inactivation rate constants, respectively for each ion channel (*x = m, h, n, j, o,* and *u*). For each ion channel the voltage dependency of the rate constants was described from the following equations:

where , , and are constants that determine the rates and voltage dependence (*V*) of current activation. The parameters for voltage activation rate constants for each ion channel are listed in Table 3.1. The reversal potential for each ion channel modeled was: *ELeak* = -73 mV, *ENa* = 50 mV, *EKHT* = -80 mV, *EKLT* = -80 mV, and *Eh* = -45 mV.

Immunofluorescence Imaging

FVB mice at P2, P4, P6, and P9 were anesthetized using hypothermia/cryoanesthesia (P2) or with Avertin (P4 and older; 250 mg/kg intraperitoneal injection) and perfused transcardially with 10 mM phosphate-buffered saline (PBS), pH 7.4, followed by a solution of 4% paraformaldehyde (PFA) in PBS. Each brain was removed from the skull and post-fixed overnight at 4°C in 4% PFA in PBS. The brains were transferred to cryoprotectant (30% sucrose in PBS) at 4°C for 1-2 days prior to freezing and sectioning. Coronal sections of the brainstem were cut at ~50-60 m thickness using a freezing microtome (model HM 450, Microm, Waltham, MA). Epitope retrieval was performed in 10mM citric acid, pH 6.0, at 95°C for 30 minutes before sections were moved into blocking solution (3% donkey serum in PBS containing 0.1% Triton X-100) for 1 hour. The sections were then incubated overnight at RT on an orbital shaker with primary antibodies diluted in 3% donkey serum in PBS. Primary antibodies included: dsRed (1:500, Cat# 632496: Takara Bio, RRID:AB\_10013483) to amplify endogenous mCherry signal, microtubule associated protein 2 (Map2, 1:2500, catalog # CPCA-Map2; EnCor Biotechnology) to label MNTB PNs, vesicular glutamate transporter 1 (Vglut1, 1:2500, Cat# AB5905; EMD Millipore) and Vglut2 (1:2500, Cat# AB2251-I) to label the CH. After primary antibody incubation slices were washed three times (5 min each) with PBS followed by incubation with the appropriate secondary antibodies (Molecular Probes, Grand Island, NY; Jackson Immunoresearch Laboratories, West Grove, PA) diluted in blocking solution at 1:500 for 2 h at RT. Slices were washed three times (5 min each) with PBS before being imaged on an inverted confocal microscope equipped with a motorized stage (SP8 LIGHTNING Confocal Microscope, RRID:SCR\_018169) and a Leica 63X HC PL APO/1.4 NA oil immersion objective. Z-stacks were collected at 0.3-μm steps. Scanning parameters (e.g. laser power and gain) were optimized based on fluorescence signal intensity from the MNTB contralateral to the injection site (transduced, TeNT expression) and the same parameters were utilized for image acquisition of the ipsilateral MNTB (non-transduced, control). Images were postprocessed with Lightning deconvolution using Leica imaging software (Leica Application Suite X, RRID:SCR\_013673) for increased contrast and resolution.

Segmentation and 3D Reconstruction of Calyces from Confocal Image Stacks

Confocal image volumes were imported into the 3D virtual reality software syGlass (www.syglass.io, IstoVisio, Inc; RRID:SCR\_017961) for manual segmentation and reconstruction of calyces. Three animals from at least two different litters were processed for IHC staining and imaging. For each image stack all calyces that were fully contained within the volume were identified and assigned a number. We then randomly selected, using a random number generator script (Python Programming Language, RRID:SCR\_008394), a subset of 5-10 calyces for segmentation. A region of interest (ROI) was drawn around each CH. The 3D “painting tool” in syGlass was utilized to manually segment each structure and export surface meshes for quantitative analysis of the volume, surface area, and apposed surface area between each CH and associated MNTB PN. Following segmentation, the resulting objects were imported into 3D visualization software (Blender v2.79, RRID:SCR\_008606) for rendering for illustrations presented in the figures.

Statistics

All statistical analyses were performed in GraphPad Prism (GraphPad Software, San Diego, CA, United States). The normality of datasets was analyzed using the Kolmogorov-Smirnov test, with parametric or non-parametric statistical tests carried out accordingly. To compare the two groups, an unpaired t-test with Welch’s correction (parametric) or Mann-Whitney U test (non-parametric) was carried out. To compare three or more groups, a non-parametric Kruskal-Wallis test with an uncorrected Dunn’s test for multiple comparisons was carried out. All values indicated in the results are presented as the mean ± standard deviation. Significance levels reported in the results and figures is indicated according to the following convention: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, and not significant (ns).

**Acknowledgments**

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Paste your main manuscript references here. They should be listed in order of citation.

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**Figures and Tables**

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**Figure 1.** Experimental protocol and efficacy of unilateral viral vector injections into the VCN expressing TeNT, blocking synaptic activity. (A), Schematic diagram showing the injection coordinates corresponding to a P0 mouse pup and the mechanism of action for TeNT light chain cleaving the synaptic vesicle fusion protein Synaptobrevin-2. (B), (Top) Timeline for viral vector injection and experimental data collection (red arrows). (Bottom) Schematic diagram of the auditory brainstem circuit. Globular bushy cells (GBCs) in the ventral cochlear nucleus (VCN) project contralaterally synapsing on principal neurons in the medial nucleus of the trapezoid body (MNTB) forming the large calyx of Held (CH) nerve terminal. The HdAd construct expresses tetanus neurotoxin (TeNT) light chain driven by the pUNISHER overexpression cassette and mCherry is driven by a 470 bp human synapsin (hsyn) promoter. (C), High titer viral vector injection into the VCN at P0 showing the expression pattern of the fluorescent protein mCherry, co-expressed with TeNT, at P6. Arrow indicates injection site. Dashed line indicates the midline. The ipsilateral and contralateral MNTB (iMNTB and cMNTB, respectively), to the injection site is outlined in a dashed line. The pipette indicates the location in the MNTB for whole-cell electrophysiological recordings targeting cells in the medial 1/3 region. Arrowhead indicates labeling in the non-injected cochlear nucleus. Scale bar: 500 mm. (C’ and C’’), High-magnification images showing fluorescent labeling of calyces in the cMNTB and not in the iMNTB, to the injection site. Scale bar: 200 mm. (D), Fluorescent images labeling the CH and MNTB PN following simultaneous whole-cell paired recording. The presynaptic CH was labeled endogenously (mCherry co-expressed with TeNT) and with Alexa 488 conjugated dextran following patching. The postsynaptic MNTB PN was labeled with Alexa 647 conjugated dextran following patching. The merged image shows co-labeling with endogenous mCherry and Alexa 488 confirming presynaptic recording from a transduced (TeNT expression) CH. Additionally the merged image shows confirmation of a whole-cell patch-clamp recording from an MNTB PN with a transduced CH. Dashed lines outline the pre- and postsynaptic patch pipettes. Scale bar: 10 mm. (E), Paired pre- and postsynaptic simultaneously patching the CH and associated MNTB PN. Evoked responses in PNs, following a 3 ms, 500 pA depolarizing current injection via the presynaptic recording pipette, in voltage (EPSC) and current-clamp (action potential) configuration. Paired recordings were performed from CH:MNTB PN synaptic pairs in a non-injected (control) and in the iMNTB (non-transduced control) and cMNTB (TeNT expression) following viral injection. The experimental recordings in the cMNTB corresponds to the fluorescent images in panel (D) targeting a P6 PN in the contralateral (to the injection site) MNTB. Abbreviations: 7N, seventh nerve; DCN, dorsal cochlear nucleus; LSO, lateral superior olive.

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**Figure 2.** Effects of *in vivo* silencing of presynaptic activity on spontaneous EPSCs in MNTB PNs. (A), Exemplary voltage-clamp traces of P6 PN sEPSCs recorded from a non-injected control mouse and from the ipsilateral (non-transduced, control) and contralateral (transduced, TeNT expression), to the injection site, MNTB. Averaged sEPSC waveforms from each individual cell are shown at the bottom. Cells were clamped at a holding potential of -73 mV. Recordings were made after bath application of the inhibitory synaptic blockers Gabazine (GABAA receptor antagonist, 10 mM) and strychnine (glycine receptor antagonist, 2 mM). Inset shows the grand averaged sEPSC waveforms for the representative traces, with expanded time scale and color-coordinated, showing slower kinetics of cMNTB PN sEPSC. (B-E), Plots showing the average amplitude, decay time constant (τ), rise time, and frequency of sEPSCs for each MNTB PN across age. Non-injected control recordings from MNTB PNs (P4, n = 10 cells; P6, n =15 cells; P9, n = 13 cells; P14, n = 12 cells) were compared to PNs in the iMNTB (Control, P4, n = 20 cells; P6, n =20 cells; P9, n = 19 cells; P14, n = 13 cells) and cMNTB (TeNT, P4, n = 20 cells; P6, n =21 cells; P9, n = 17 cells; P14, n = 7 cells) following unilateral viral injection, across neonatal ages. Horizontal gray lines represent the mean with standard deviation error bars. Statistical significance within each age group was assessed by performing a Kruskal-Wallis ANOVA followed by an uncorrected Dunn’s test for multiple comparisons between ages with significance levels indicated according to the convention: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001.

A graph of a slope resistance

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**Figure 3.** Effects of *in vivo* silencing of presynaptic activity on MNTB PN intrinsic physiological properties. (A-D), Plots showing the resting membrane potential (RMP), slope resistance, membrane time constant, and sag ratio for iMNTB (Control, P4, n = 20 cells; P6, n =20 cells; P9, n = 19 cells; P14, n = 13 cells) and cMNTB (TeNT, P4, n = 20 cells; P6, n =21 cells; P9, n = 17 cells; P14, n = 7 cells) PNs across neonatal ages. Horizontal gray lines represent the mean with standard deviation error bars. Statistical significance was assessed by performing a Mann-Whitney U test with significance levels indicated according to the convention: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, and not significant (ns).

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**Figure 4.** Effects of *in vivo* silencing of presynaptic activity on the firing pattern of MNTB PNs. (A), Exemplary current-clamp traces of P6 PNs recorded from the ipsilateral (non-transduced, control) and contralateral (transduced, TeNT expression), to the injection site, MNTB. Data was recorded at resting membrane potential and shown as a −100 pA current step with successive steps at 20 pA increments. (B), MNTB PN expressing TeNT show a delayed transition from a tonic to phasic firing pattern. At P4 both control and TeNT expressing cells displayed a tonic firing pattern. Beginning at P6 the percentage of phasic cells in control conditions increased and was consistent at P9. All recordings from PN expressing TeNT displayed a phasic firing pattern. Numbers inside the bars indicate sample size. (C), Plots showing the relationship between the number of action potentials elicited following 300 ms depolarizing current injection, comparing PNs in the ipsilateral and contralateral MNTB following viral injection. Each line represents the number of spikes/current injection step for an individual neuron. For clarity, cells that went into depolarization block had plots clipped at the peak spikes/current injection.

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**Figure 5.** Effects of *in vivo* silencing of presynaptic activity on MNTB PN action potential kinetics. (A), (Top) Exemplary action potential (AP) waveforms recorded from P6 PNs in the ipsilateral (non-transduced, control) and contralateral (transduced, TeNT expression), to the injection site, MNTB. AP waveform kinetics were analyzed at rheobase. APs were aligned at the inflection point (voltage threshold) showing an increased amplitude and broadening of the waveform kinetics. (Bottom) Phase-plane plots corresponding to the representative AP waveforms are shown plotting the dV/dt against the membrane potential showing a depolarizing shift in voltage threshold, increased peak amplitude, and reduced repolarization rate following TeNT expression. The voltage threshold and peak amplitude are denoted by the arrow and arrowhead, respectively (B-G), Plots showing the AP threshold current, amplitude, half-width, voltage threshold, and max depolarization and repolarization rate for iMNTB (Control, P4, n = 20 cells; P6, n =20 cells; P9, n = 19 cells; P14, n = 13 cells) and cMNTB (TeNT, P4, n = 21 cells; P6, n =21 cells; P9, n = 17 cells; P14, n = 7 cells) PNs across neonatal ages. Horizontal gray lines represent the mean with standard deviation error bars. Statistical significance was assessed by performing a Mann-Whitney U test with significance levels indicated according to the convention: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, and not significant (ns).

**Figure X**. Effects of *in vivo* silencing of presynaptic activity on CH morphology. (A-B), Confocal images from the ipsilateral and contralateral MNTB (iMNTB and cMNTB, respectively), to the injection site, following unilateral viral vector injection into the ventral cochlear nucleus expressing TeNT. mCherry staining (red, co-expressed with TeNT) labels the globular bushy cell axons passing and CH in the cMNTB. Vglut1/2 staining (magenta) labels the CH with more punctate labeling in the cMNTB. Scale bar: 10 mm. (C-D), 63X confocal images from the iMNTB (non-transduced) and cMNTB (transduced, TeNT expression), respectively. Transduced CHs were smaller in volume, had less extension over the somatic surface, and were thicker. Map2 staining (green) labels the PN with smaller and irregular shaped cells in the cMNTB . Scale bar: 10 mm. (C’), 3D reconstruction of the non-transduced CH in panel (c). (D’), 3D reconstruction of the transduced CH in panel (D). (E-F), Plots of the volume and thickness of non-transduced (control) and transduced (TeNT expression) from reconstructed calyces. Error bars represent the mean ± standard deviation. Statistical significance was assessed by performing a Mann-Whitney U test with significance levels indicated according to the convention: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, and not significant (ns).

A collage of images of a human brain

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**Supplemental Figure 1.** Expression pattern following unilateral viral vector injection into the ventral cochlear nucleus (VCN) across development. (A-D), Injection of the dual expression HdAd vector (mCherry reporter molecule coexpressed with TeNT light chain) into the VCN was performed at P0 followed by transcardial perfusion with 4% PFA at P2, P4, P9, and P14. Endogenous mCherry labeling was amplified with primary antibodies against dsRed following standard immunohistochemistry procedures. Arrows indicate the viral injection site. Vertical dashed line indicates the midline. The ipsilateral and contralateral MNTB (iMNTB and cMNTB, respectively), to the injection site is outlined in a dashed line. Labeling in the injected cochlear nucleus is confined to the neuronal cell bodies in VCN, with afferent projection fibers to the dorsal cochlear nucleus (DCN) labeled. Scale bar: 500 mm. (A’-D’ and A’’-D’’), High-magnification images showing fluorescent labeling of calyces in the cMNTB and not in the iMNTB, to the injection site. Labeling in the iMNTB is confined to the trapezoid fibers projecting to the cMNTB. Scale bar: 200 mm. Abbreviations: LSO, lateral superior olive.

Table 1.Parameters for the kinetics of each ion channel modeled

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ***NaV***  ***Activation*** | ***NaV***  ***Inactivation*** | ***KHTV***  ***Activation*** | ***KHTV***  ***Inactivation*** | ***KLTV***  ***Activation*** | ***HCN***  ***Activation*** | ***KA***  ***Activation\**** | ***KA Inactivation\**** | ***KA Inactivation\**** |
|  | ***m*** | ***h*** | ***n*** | ***p*** | ***o*** | ***u*** | ***a*** | ***b*** | ***c*** |
| ***Cα (ms-1)*** | 76.4 | 0.000533 | 0.2719 | 0.00713 | 6.947 | 9.12 x 10-8 | 0.0892 | 0.0405 | 0.016 |
| ***kα (mV-1)*** | 0.037 | -0.0909 | 0.04 | -0.1942 | 0.03512 | -0.01 |  |  |  |
| ***Cβ (ms-1)*** | 6.930852 | 0.787 | 0.1974 | 0.0935 | 0.2248 | 2.1 x 10-3 | 0.3691 | 0.0103 | 0.0041 |
| ***kβ (mV-1)*** | -0.043 | 0.0691 | 0 | 0.0058 | -0.0319 | 0 |  |  |  |

*Parameters for the voltage dependence and kinetics were based on previous models of PNs (Macica et al., 2003; Sierksma et al., 2017).*

Table 2.Parameters for MNTB principal neuron sEPSCs following TeNT expression

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **MNTB PN Age** | **Amplitude (pA)** | **Decay Time Constant, τ (ms)** | **Risetime 20-80% (ms)** | **Frequency (Hz)** |
|  |  |  |  |  |
| P4 Non-injected/Control  (n = 12) |  |  |  |  |
| P4 iMNTB/Control  (n = 12) | 61.84 ± 7.72 | 0.77 ± 0.16 | 0.30 ± 0.09 | 2.08 ± 1.62 |
| P4 cMNTB/TeNT  (n = 13) | 50.31 ± 11.10 | 1.17 ± 0.26 | 0.37 ± 0.11 | 0.55 ± 0.79 |
|  |  |  |  |  |
| P6 iMNTB/Control  (n = 19) | 62.44 ± 14.63 | 0.72 ± 0.18 | 0.23 ± 0.06 | 2.60 ± 1.94 |
| P6 cMNTB/TeNT  (n = 18) | 60.42 ± 15.76 | 1.09 ± 0.27 | 0.36 ± 0.11 | 0.65 ± 0.41 |
|  |  |  |  |  |
| P9 iMNTB/Control  (n = 12) | 44.97 ± 6.56 | 0.54 ± 0.17 | 0.28 ± 0.09 | 1.17 ± 1.69 |
| P9 cMNTB/TeNT  (n = 9) | 62.78 ± 20.29 | 1.02 ± 0.16 | 0.29 ± 0.06 | 1.01 ± 0.54 |
| P14 Non-injected/Control  (n = 9) |  |  |  |  |
| P14 iMNTB/Control  (n = 9) |  |  |  |  |
| P14 cMNTB/TeNT  (n = 9) |  |  |  |  |
|  |  |  |  |  |

*Data presented as means ± standard deviation based on the average values for individual cells. n: number of cells*

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