VOLTAGE-GATED POTASSIUM CHANNEL CURRENTS OF BINAURAL HEARING NEURONS IN THE AVIAN SOUND LOCALIZATION CIRCUIT

A dissertation submitted to Kent State University in cooperation with Northeast Ohio Medical University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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<tr>
<td>α2-R</td>
<td>alpha2-noradrenergic receptor</td>
</tr>
<tr>
<td>β-R</td>
<td>beta-noradrenergic receptor</td>
</tr>
<tr>
<td>AIS</td>
<td>axon initial segment</td>
</tr>
<tr>
<td>AMPA-R</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AN</td>
<td>auditory nerve</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
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<tr>
<td>EPSP</td>
<td>excitatory postsynaptic potential</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GABA_A-R</td>
<td>gamma-aminobutyric acid-A receptor</td>
</tr>
<tr>
<td>GABA_B-R</td>
<td>gamma-aminobutyric acid-B receptor</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization and cyclic-nucleotide gated channel</td>
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<tr>
<td>HF</td>
<td>high characteristic frequency nucleus laminaris neuron</td>
</tr>
<tr>
<td>HTK</td>
<td>high-threshold voltage-gated K+ channel</td>
</tr>
<tr>
<td>I_h</td>
<td>hyperpolarization-activated current</td>
</tr>
<tr>
<td>I_onset</td>
<td>onset current</td>
</tr>
<tr>
<td>I_SS</td>
<td>steady state current</td>
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<tr>
<td>IEI</td>
<td>interevent interval</td>
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<tr>
<td>ILD</td>
<td>intraural level difference</td>
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<td>IPD</td>
<td>intraural phase difference</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>IPSC</td>
<td>inhibitory postsynaptic current</td>
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<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
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<td>ITD</td>
<td>intraural time difference</td>
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<tr>
<td>$K_V$</td>
<td>voltage-gated K+ channel</td>
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<td>LF</td>
<td>low characteristic frequency nucleus laminaris neuron</td>
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<tr>
<td>LTK</td>
<td>low-threshold voltage-gated K+ channel</td>
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<tr>
<td>MF</td>
<td>middle characteristic frequency nucleus laminaris neuron</td>
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<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
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<tr>
<td>mGluR II</td>
<td>group II metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MSO</td>
<td>medial nucleus of superior olive</td>
</tr>
<tr>
<td>$Na_V$</td>
<td>voltage-gated Na+ channel</td>
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<tr>
<td>NA</td>
<td>nucleus angularis</td>
</tr>
<tr>
<td>NL</td>
<td>nucleus laminaris</td>
</tr>
<tr>
<td>NM</td>
<td>nucleus magnocellularis</td>
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<tr>
<td>PPCR</td>
<td>perforated patch clamp recording</td>
</tr>
<tr>
<td>PSC</td>
<td>postsynaptic current</td>
</tr>
<tr>
<td>PSP</td>
<td>postsynaptic potential</td>
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<tr>
<td>$R_a$</td>
<td>access resistance</td>
</tr>
<tr>
<td>$R_{in}$</td>
<td>input resistance</td>
</tr>
<tr>
<td>$R_S$</td>
<td>series resistance</td>
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<tr>
<td>RMP</td>
<td>resting membrane potential</td>
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<tr>
<td>sIPSC</td>
<td>spontaneous inhibitory postsynaptic current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>sEPSC</td>
<td>spontaneous excitatory postsynaptic current</td>
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<tr>
<td>$V_{1/2}$</td>
<td>half-maximum activation potential</td>
</tr>
<tr>
<td>$V_{\text{command}}, V_C$</td>
<td>voltage command</td>
</tr>
<tr>
<td>$V_{\text{hold}}$</td>
<td>holding voltage, holding potential</td>
</tr>
<tr>
<td>$V_{\text{prepulse}}$</td>
<td>prepulse voltage, prepulse potential</td>
</tr>
<tr>
<td>WCR</td>
<td>whole cell recording</td>
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</tbody>
</table>
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This dissertation is dedicated to my mother who helped guide me throughout my life. Her support allowed me to transcend the unstable lifestyle of the inner city of New York to the calm and fruitful lifestyle of academia. I never knew I could do this but she never let me forget my potential and that I'm able to achieve anything I choose to pursue.

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CHAPTER I

INTRODUCTION

Unlike other sensory epithelia, such as the retina or integument, the cochlea does not process the location of the sensory stimuli. This presents a problem for organisms that use auditory information to locate food sources, predators, and mates. In many species, specialized neuronal circuits compute the location of sound in space using temporal cues, spectral cues, or both to determine the source of a sound in a three-dimensional world. Much research on sound localization has focused on localization in the horizontal plane (azimuth plane) and revealed that two important mechanisms have evolved to encode azimuth sound location: interaural level differences (ILD) and interaural time differences (ITD) (Konishi 2003). Humans (and many terrestrial animals) utilize both ITD and ILD processing to determine the horizontal position of a sound source.

ILD processing utilizes sound-shadows created by sound transfer across the animal’s head (Irvine 1992). A sound wave generated on to the left side of an animal, will be slightly 'louder' in the left ear and as the sound wave is reflected and refracted by the mass of the head, it is slightly dampened when it arrives in the right ear. In ITD-coding, submillisecond differences in the time a sound stimulus reaches one ear versus the other ear are exploited (Joris et al. 1998; Bernstein 2001). In this case, a sound generated to the
left of an animal will arrive at the left ear first, and it will reach the right ear with a short
time delay because of the longer distance the sound wave must travel to the right ear.

**Phylogenetic differences in utilization of ITD and ILD coding**

So what determines whether ILDs or ITDs are used to encode sound location? More than a century ago, Lord Rayleigh developed a profound hypothesis to answer this question. His theory, termed the duplex theory of sound localization (Strutt 1907), stated that ITDs are useful to encode low frequency sounds (up to 3000 Hz) and ILDs are useful for encoding the location of high frequency sounds (above 3000 Hz; Klumpp and Eady 1956; Zwislocki and Feldman 1956; Searle et al. 1976; Carlile 1996).

While the theory was developed to describe sound localization in humans, observations from a variety of animal species revealed that head-size, in addition to frequency range, influence the degree to which ITD and ILD encoding is used (Roth et al. 1980; Wightman and Kistler 1992; Carlile 1996; Blauert 1997; Yin 2002; Konishi 2003; Carr and Köppl 2004; Harper and McAlpine 2004). In mammals, the nucleus where ITD-coding is first coded, the medial superior olive (MSO), shows a general trend such that mammals with larger heads and a bias towards low-frequency perception (<3,000 Hz) tend to have a larger and well-developed MSO (Harrison and Irving 1966; Irving and Harrison 1967; Moore and Moore 1971; Masterson et al. 1975; Schwartz 1992). Animals with smaller heads and a bias towards high-frequency perception use different strategies. For example, mice have a small and poorly-developed MSO and tend to use ILD coding rather than ITD coding for sound localization (Ollo and Schwartz 1979; Ehret and Dreyer
This concept extends to avian species as well. Chickens, which have moderately sized heads and detect low frequency sounds, possibly even infrasound (<100 Hz; Gray and Rubel 1985; Saunders and Salvi 1993; Hill et al. 2014), possess a well-developed ITD-coding nucleus (nucleus laminaris, NL) that enables sound localization in azimuth space (Hyson et al. 1994; Carr and Köppl 2004; Ohmori 2014).

Despite the broad acceptance of the duplex theory, it has shortcomings that become evident when the theory is applied to ecologically complex sound environments (Henning 1974; Leakey et al. 1958; McFadden and Pasanen 1976; Wightman and Kistler 1992). For example, the degree to which ITD cues are utilized is considerably influenced by additional sound localization mechanisms, such as spectral cues (Slattery and Middlebrooks 1994) and even ILD cues (Wightman and Kistler 1997; Macpherson and Middlebrooks 2002). While this and other pitfalls in the duplex theory exist, frequency hearing range and head size are still critical factors in determining how the location of the sound source is encoded (Carlile 1996).

A mechanistic view of ITD coding: coincidence detection

ITD coding is a particularly useful strategy for sound localization in humans because the head size is relatively large and important signals such as speech occur in the low frequency range. As a result, a question auditory neuroscientists and clinicians have asked is, How does ITD coding occur on a cellular level?
The cellular process underlying ITD-coding is termed coincidence detection (Jeffress 1948; Joseph and Hyson 1993; Reyes et al. 1996; Goldberg and Brown 1969; Yin and Chan 1990; Spitzer and Semple 1995; Brand et al. 2002). The process of coincidence detection requires that a neuron produces an action potential (AP) when two or more inputs coincide in timing. For coincidence detection in ITD-coding, the ITD-coding neuron receives inputs from both ears, compares the arrival time of each input, and if the inputs occur within a very small window of time (in humans this window can be as small as 10 µs; Mills 1958), the cell will fire APs.

As a result, coincidence detection is a delicate process that requires many morphological, synaptic, and intrinsic specializations in order for the coincidence detector neurons to ignore asynchronous or poorly timed inputs. In addition, ITD-coding neurons must produce APs in response to coincident inputs with a high degree of temporal precision (e.g., reduced variability in the timing to produce an AP) and often at rates greater than 100 Hz (Trussell 1997, 1999; Joris et al. 1998; Kuba 2007; MacLeod and Carr 2012). To further complicate the issue of sound localization, the accuracy of the localization differs depending on the sound frequency (Carr and Konishi 1990; Yin and Chan 1990), indicating that a neuron that codes a particular sound frequency, which is termed its characteristic frequency (CF), performs coincidence detection differently than neurons with different CFs. The goal of this dissertation was to understand how individual ITD-coding neurons are specialized in their physiology at the cellular level to localize sounds at different frequencies.
Addressing this issue is profoundly complicated in mammals largely because the ITD circuit is particularly complex, requiring a multitude of nuclei, nuclei with heterogeneous cell populations, and furthermore, a striking variation between species exists. First of all, ITD-coding in mammal requires temporally precise bilateral excitation and inhibition (Grothe and Sans 1993; Han and Colburn 1993; Zhou et al. 2005; Couchman et al. 2010; Manis et al. 2012). The use of bilateral excitation and inhibition utilizes at least five different nuclei, each containing multiple cell types (Tabor 1961; Cant 1982; Adams 1986; Cant 1991; Moore 1991; Cant and Hyson 1992; Cant 1992; Schwartz 1992; Spirou et al. 1998). In the first ITD-coding nucleus, the MSO, there can be up to four types of neurons, depending the species studied (Scheibel and Scheibel 1974; Schwartz 1977; Schwartz 1980; Kiss and Majorossy 1983; Schwartz 1992; Kulesza 2007). Additionally, there is substantial variability across mammalian species regarding the ITD circuit, including the size and presence of the MSO, tonotopic projections to the MSO, and cell types within the MSO (Moore and Moore 1971; Masterson et al. 1975; Irvine 1992; Schwartz 1992). Ultimately, the multitude of nuclei and cell types contributing to ITD coding and vast species differences make understanding frequency-dependent differences in sound localization circuit more difficult to address in mammalian circuits.

However, ITD-coding NL neurons in birds receive bilateral excitation and ipsilateral inhibition with each nucleus predominantly composed of homogeneous groups of neurons. Like mammals, the avian ITD-coding NL neurons are arranged by CF, i.e. tonotopically arranged. More importantly, despite the evolutionary distance between
chickens and humans, the cellular mechanisms of ITD-coding are very similar between these classes of animal (Golding 2012; MacLeod and Carr 2012).

Therefore, to understand cellular mechanisms of sound localization at different sound frequencies, the chicken offers an excellent model. The chicken NL contains a well-organized map of frequency along its caudolateral-rostromedial axis and a map of sound location along the lateral-medial axis (Fig 1.1; Rubel and Park 1975; Smith 1981; Lippe and Rubel 1983; Seidl et al. 2010; Wang et al. 2010). For these reasons, I used the chicken ITD-coding nucleus to address the overarching question: how do cellular properties of ITD-coding neurons vary with sound frequency and how do these tonotopic variations improve coincidence detection?

**ITD-coding circuitry in birds**

Acoustic information is first processed in the inner ear by hairs cells on the sensory epithelium, the basilar papilla, conveying information regarding sound amplitude, frequency, and timing to the auditory nerve (AN; Gleich and Manley 2000; Nicolson 2012). Properties of sound are "relayed" from the auditory nerve to the cochlear nucleus magnocellularis (NM) and nucleus angularis (NA) (Carr and Boudreau 1991; Carr and Code 2000; Rubel and Fritzsch 2002).

The temporal features of a sound are extracted and processed in the NM, a homogeneous set of neurons, via large axosomatic inputs called endbulbs. The endbulb is a large axonal terminal of an auditory nerve axon that acts as a highly secure synapse, such that release of glutamate from the AN terminal to the neuronal soma almost always
Figure 1.1 Organization of the nucleus laminaris (NL) in Gallus gallus. The NL of chick contains two axes upon which sound frequency and sound location are coded. Along the lateral to medial axis, sound location is coded and the caudal (caudolateral) to rostral (rostromedial) axis, sound frequency is coded. As all neurons in the NL use coincidence detection to code ITD, this arrangement allows observations of the tonotopic variations to be observed, manipulated, and reported. Image created by Hamlet for the current literature review.
results in the production of an AP in the NM neuron. Thus, the endbulb synapse makes the NM neuron a faithful relay point. However, in addition to relaying information reliably, the endbulb can also improve the temporal fidelity of NM neuron’s output (Fukui et al. 2008), especially when NM response is phase-locked to the sine-wave of the sound stimulus (Carr 1992; Joris and Smith 2008; Manis et al. 2012). Phase locking, the ability to produce an AP at a certain phase of a sound wave, is an important property of many auditory neurons and intimately involved in ITD coding (most especially at low frequencies, as the interaural phase difference in the timing of phase-locked inputs can be compared to determine the location of sound (IPD; ITD = product of IPD and frequency).

Input to the NM from the auditory nerve is tonotopically organized with LF axons terminating in the caudolateral NM, HF axons terminating in the rostromedial NM, and middle CF (MF) axons terminating in the intermediate region (Parks and Jackson 1984; Young and Rubel 1986). This tonotopic organization observed in the NM is preserved in the ascending ITD-coding neurons, the NL, with LF inputs in the caudolateral NL, HF input in the rostromedial NL, and MF inputs in the intermediate region (Rubel and Parks 1975; Smith and Rubel 1979; Smith 1981; Young and Rubel 1986).

The temporal properties of sound encoded by the NM are "relayed" bilaterally to ITD-coding ascending neurons in the NL (Rubel and Parks 1975; Young and Rubel 1983; Wang et al. 2010). ITDs are extracted in the NL by segregated and independent excitatory input from the NM. Excitatory inputs from multiple NM neurons converge onto individual dendrites and to a lesser extent, the soma, of a NL neuron (Parks et al. 1993). Inputs from the ipsilateral and contralateral NM are strongly segregated to the
ventral and dorsal dendrites of NL neurons, respectively (Young and Rubel 1983; Overholt et al. 1992). The independence and segregation of ipsilateral and contralateral inputs in this way strongly improve the ability of these neurons to extract submillisecond differences in the arrival times of input from each NM.

While birds do not utilize bilateral inhibition, inhibitory synaptic input is critical for ITD-coding. In birds, unilateral inhibitory feedback is known to improve ITD coding. Inhibition arises from projections of NL neurons to the superior olivary nuclei (SON), a heterogeneous population of neurons which in turn project inhibitory axons to the ipsilateral NL and contralateral SON (Takahashi and Konishi 1988; Carr et al. 1989; Carr and Boudreau 1993; Lachica et al. 1994; Burger et al. 2005).

One issue which arises when discussing the ITD circuitry is how the coincidence detecting neurons of ITD-coding nuclei receive simultaneous ipsilateral and contralateral input given that the contralateral axons travel longer distances to reach the ipsilateral ITD-coding nucleus. The longer distance would confound the coincidence detection process, as the contralateral inputs would arrive at a later time point than ipsilateral inputs. To account for this difference, Jeffress (1948) proposed that axons from the ipsilateral and contralateral ascending nuclei would traverse a different path of distance depending on ITD, ensuring that the arrival of excitatory ipsilateral and contralateral inputs could occur simultaneously on to a given ITD-coding neuron. Using the Jeffress model, a place code of sound location could be created where the physical location of an ITD-coding neuron would represent the physical location of a sound in space (Fig. 1.2). There is strong evidence for delay lines and the use of a place code in birds (Lippe and
Fig. 1.2 Jeffress hypothesis of sound localization. The Jeffress Model hypothesized that sound location could be extracted when fibers from the first auditory nucleus split, with one set traveling to an ipsilateral sound localizing nucleus and the other to the contralateral sound localizing nucleus. In this model, both the ipsilateral and contralateral axons (tertiary fibers) would travel an equidistant path so that the two inputs coincide at a given coincidence detecting neuron. Sound location could be then represented using a 'place code' strategy, where the physical location of the neuron represents the location of the sound in azimuth space and ultimately represented a map of all sound-locations within the bilateral nucleus. Image adapted from Jeffress (1948).
Numerous morphological and physiological tonotopic variations have been reported in the chicken NL, including differences in cellular morphology (Rubel and Parks 1975; Smith and Rubel 1979), intrinsic physiology (Parameshwaran et al. 2001; Parameshwaran-Iyer et al. 2003; Kuba et al. 2005; Yamada et al. 2005; Hamlet and Lu 2014), synaptic responses (Kuba et al. 2005; Tang et al. 2011; Tang and Lu 2012b; Yamada et al. 2013; Hamlet and Lu 2014) and modulation and plasticity (Tang et al. 2013; Okuda et al. 2013; Lu 2014). The overarching goal of this work was to explore the tonotopic differences in the intrinsic and synaptic physiology of NL neurons. In Chapter II, I will explore the interaction between tonotopic variations of inhibitory inputs and low-threshold voltage-gated K$_V$ (LTK) currents. Chapter III will further explore the tonotopic variations in voltage-gated potassium (K$_V$) channel modulation by a particular subset of tonotopically arranged G-protein-couple receptor (GPCR), group II metabotropic glutamate receptors (mGluR IIs).

**Tonotopic variations in chicken ITD-coding neurons**

The differences in dendritic morphology of NL neurons in the chick are striking, with LF neurons having up to 11-fold longer and 5 fold-thicker dendrites than HF neurons (Smith and Rubel 1979). The role of dendritic computation has been illustrated
in computer models (Agmon-Snir et al. 1998) and highlighted in experiments using the mammalian ITD-coding neurons of the MSO, where active conductances such as $I_h$ (Khurana et al. 2012) and LTK currents (Matthews et al. 2010; Roberts et al. 2013) can strongly influence synaptic integration of the bilateral inputs (Van der Heijden, et al. 2013; Franken, et al. 2015). However, the physiological significance of different dendritic length in avian NL neurons still remains elusive.

In addition to dendritic differences, LF neurons have larger cell bodies than HF neurons. Given that the somas of coincidence detecting neurons act as current sinks to effectively summate bilateral excitatory inputs, the relatively larger dendritic trees in LF NL neurons may require larger somas to summate bilateral excitatory inputs (Agmon-Snir, et al. 1998; Van der Heijden et al. 2013).

**Tonotopy of physiological properties of NL neurons**

Besides the differential morphology of NL neurons in different CF regions, there are a multitude of physiological properties that vary across the tonotopic axis of the chicken NL. In the following section, I will review the known tonotopic variations in intrinsic physiology, synaptic excitation, synaptic inhibition, and GPCR induced modulation in the chicken NL. I will also discuss how these tonotopic variations influence coincidence detection and ITD-coding, and describe the existing gaps in our knowledge that led to the design of my dissertation.
As discussed in the first section, coincidence detector neurons possess a number of neuronal properties that are critical for their function. These properties include specialized voltage-gated sodium (Na\textsubscript{v}) currents that define an ITD-coding neuron's threshold to produce APs when bilateral excitatory inputs coincide, strong hyperpolarization-activated currents (I\textsubscript{h}) and LTK currents at rest which act as a high-pass filter by reducing the membrane time constant, and strong high threshold K\textsubscript{v} (HTK) currents to enable ITD-coding neurons to entrain AP generation during ongoing acoustic stimulation (i.e., produce APs repeatedly to an ongoing acoustic stimulus). These intrinsic properties are tonotopically distributed in the avian NL (more details below), resulting in cellular gradients adapted to ITD coding at different CF.

The axon initial segment (AIS) is a region of the axon where there is a large accumulation of Na\textsubscript{v} channels to initiate an AP (Bender and Trussell 2012; Kole and Stuart 2012). In the NL, the distance of the AIS from the soma and the length of AIS vary along the tonotopic axis. There appears a gradient whereby the AIS is short and located close to the soma of LF neurons and becomes longer and located more distally from the soma in HF neurons (Kuba et al. 2006). This tonotopic variation is shaped during development by acoustic input. The AIS is of equal length and distance from the soma prior to the hearing onset in chickens (embryonic day 12), and shortens during maturity a few days later (embryonic day 14-15) in all CF regions. A more dramatic shortening and retraction from the soma are observed in HF neurons. Interestingly, the shortening of the AIS does not occur if the cochlea is ablated at embryonic day 2, indicating that this change in the AIS structure is to some extent activity-dependant (Kuba et al. 2014). The
function of this variation in AIS is proposed to A) reduce the capacitive and conductive load incurred by LF neurons due to their relatively large membrane area, and B) prevent temporal summation of excitatory inputs in HF neurons, thus allowing HF neurons to be as sensitive as possible to discrete excitatory inputs (Kuba 2007).

One of the most critical features of ITD-coding neurons that enable coincidence detection is the great leakiness of the neuron's membrane. A leaky membrane (observed via input resistance, $R_{in}$, of the membrane while at or near a resting voltage) allows a neuron to act as a high-pass filter, capable of sifting through slow, small, and asynchronous inputs and respond only to coinciding, high frequency, excitatory inputs from each ear (Svirskis et al. 2002, 2003). Two complimentary currents are active at rest which contribute to the low $R_{in}$, and other biophysical properties that indicate the leakiness of a neuron: $I_h$ and LTK currents (Golding 2012). $I_h$ and LTK intimately interact to control the extent of voltage deflections in time encoding neurons, maintaining the neuron at a resting state until coincident inputs arrive (Bal and Oertel 2000; 2001; Hassfurth et al. 2009; Khurana et al. 2011).

In time encoding auditory neurons strong $I_h$ currents improve coincidence detection (Golding et al. 1995; Bal and Oertel 2000; Cao et al. 2007; Khurana et al. 2012), especially in mammalian MSO as well as in avian NL. HCN channels, which generate the $I_h$ current, are present in all regions of the NL. Among the four different types of HCN subunits with different activation kinetics and sensitivities to cyclic nucleotides (Ludwig et al. 1998; Santoro and Tibbs 1999; Santoro et al. 2000; Moosmang et al. 2001), HCN1 and HCN2 are found in the NL (Yamada et al. 2005). In general,
HCN1 channels activate faster and are activated at more depolarized voltages than HCN2 channels, while HCN2 channels tend to have a greater sensitivity to cyclic nucleotides such as cAMP (Santoro and Tibbs 1999). While HCN2 was evenly distributed throughout the NL, HCN1 showed a gradual increase with decreasing CF, with little to no expression in HF neurons and strongest expression in the LF neurons. As a result, $I_h$ currents more strongly regulate the intrinsic properties and synaptic responses of LF neurons (Yamada et al. 2005).

While $I_h$ currents drive the resting voltage of a cell in a positive direction at rest, active LTK currents counterbalance $I_h$ currents by hyperpolarizing the cell (Golding 2012). LTK currents are often active at rest in ITD-coding neurons and improve coincidence detection in multiple ways: set the resting membrane properties, regulate the threshold for APs, act as a high-pass filter for synaptic inputs (i.e.; postsynaptic currents; PSCs), and influence the size and shape of action potentials (Svirskis et al. 2002; 2003; Scott et al. 2005; Kuba et al. 2005; Matthews et al. 2010; Roberts et al. 2013; Hamlet et al. 2014). LTK currents in the NL vary across the tonotopic axis such that MF and HF neurons have larger and stronger LTK currents (Kuba et al. 2005; Hamlet et al. 2014). The variation of LTK in the NL likely relates to the amount of spontaneous PSCs and the amplitude of PSCs a neuron receives, allowing MF and HF neurons to better filter the asynchronous and irrelevant inputs and only produce APs when large bilateral inputs coincide over a relatively narrow time window (Svirskis et al. 2002, 2003; Kuba et al. 2005; Slee et al. 2010; Hamlet et al. 2014).
In addition to LTK currents, ITD-coding neurons also possess tonotopically arranged HTK currents (Parameshwaran et al. 2001; Parameshwaran-Iyer et al. 2003). HTK currents activate at the peak of an action potential and repolarize the neuronal membrane to its resting state (Rudy et al. 1999; Rudy and McBain 2001; Gutman et al. 2005; Gutman et al. 2005). In this way, neurons with stronger HTK currents repolarize quicker; thus ultimately allowing the neuron to produce more APs in a given period of time (Gan and Kaczmarek 1998; Johnston et al. 2010; Golding 2012, MacLeod and Carr 2012). In the NL, K$_V$3.1 subunit, the predominant component of HTK channels, is expressed more strongly in MF and HF neurons than in LF neurons (Parameshwaran et al. 2001). Thus the tonotopic organization of HTK currents presumably mirrors that of the LTK currents, with stronger HTK and LTK currents in HF neurons than in LF neurons.

**Tonotopy of synaptic physiology in the NL: excitatory inputs**

Coincidence detecting neurons require short duration excitatory PSCs (EPSCs), and therefore fast deflections in the membrane voltage or postsynaptic potential (PSP). The speed of an excitatory PSP (EPSP) is determined by a multitude of factors, including the passive membrane properties of the neurons (e.g., membrane time constant; Geiger et al. 1997), the subunit composition of the postsynaptic receptor channel (Levin et al. 1997), the rate of desensitization (Raman and Trussell 1995a), channel opening duration (Raman et al. 1994; Raman and Trussell 1995b), the clearance of the neurotransmitter (Otis et al. 1996), and the strength of LTK (Ströhmann et al. 1995; Reyes et al. 1996).
While these factors apply to all neurons, the convergence of these variables in coincidence-detecting neurons creates extremely fast EPSPs (Trussell 1999; Parks 2000; Wang et al. 2010).

The size and strength of individual stimulus evoked EPSCs (eEPSC) and EPSP (eEPSP) are dramatically different across the tonotopic axis, with eEPSCs and eEPSPs being slower (longer duration) in LF neurons than MF and HF neurons (Kuba et al. 2002; 2005). Additionally, in vitro (Kuba et al. 2005; Sanchez et al. 2010) and in vivo (Nishino et al. 2008) studies have shown that LF neurons have fewer spontaneous EPSCs (sEPSC) and EPSP (sEPSP). Despite the tonotopic variation of excitatory inputs, NL neurons at different frequency regions can perform as equally accurate and reliable ITD coding (Kuba et al. 2005; Kuba 2007).

Several mechanisms have been proposed to explain how tonotopic variations of excitatory input allow accurate and reliable coding of sound localization. Reyes et al. (1996) showed that certain postsynaptic properties of the coincidence detecting neuron are required given the number and size of PSCs from the NM to NL. Specifically, activation of strong outward currents is needed to counteract numerous spontaneous and stimulus-evoked PSCs to minimize temporal summation of the PSCs. In addition to the properties of postsynaptic membrane, in vitro experiments revealed fast phasic synaptic inhibition in LF neurons (see below for discussion on tonotopic variation in synaptic inhibition, Yamada et al. 2013; Hamlet et al. 2014), which may provide timely precise inhibition in ITD coding-circuits, as found in the mammalian MSO. In fact, in vivo experiments have shown that sound-intensity dependent inhibition from the SON can
improve ITD coding at low frequencies (Nishino et al. 2008). Moreover, the role of
dendrites, which likely improve coincidence detection (Agmon-Snir et al. 1998) and tend
to be the longest and thickest in LF neurons (Smith and Rubel 1979), may also be
underappreciated in slice physiology. Recent work by Slee et al. (2010) proposed that the
totality of postsynaptic membrane properties, inhibitory inputs, and dendritic filtering
work together with tonotopic variations of EPSCs to produce differential band-pass
filtering to accurately encode ITD at different sound frequencies.

*Tonotopy of synaptic physiology in the NL: inhibitory inputs*

In the NL an unusually high intracellular Cl\(^-\) concentration that persists into
maturation generates an inward GABAergic current, which causes a depolarizing
response upon activation of GABA\(_A\) channels. Therefore, as opposed to the classical
hyperpolarizing effects of GABAergic and/or glycinenergic inhibition (as in the mammalian
ITD circuit), in the NM and NL, GABAergic inhibition generates a slow and sustained
depolarizing inhibitory PSC (IPSC, Hyson et al. 1995; Yang et al. 1999; Lu and Trussell
2000; Kuo et al. 2009; Tang et al. 2009). In the auditory brainstem of chick, slow and
sustained depolarizing inhibition can appear contradictory to the notion of inhibitory
action, as it is capable of producing excitation when over-activated (Tang et al. 2009;
Hamlet et al. 2014). However, in spite of the positive deflection of the membrane voltage
following an IPSC, the depolarizing inhibition reduces the membrane R\(_{in}\), in part by
activation of LTK channels (Funabiki et al. 1998; Howard et al. 2007), and in part by
forming accommodation of spike threshold, through Na\(_V\) channel inactivation (Monsivais
and Rubel 2001), preventing its excitatory action. Depolarizing IPSCs have been shown to enhance phase-locked input from the NM to the NL, and in turn sharpens ITD coding in the NL (Monsivais et al. 2000; Fukui et al. 2010).

Although slow and sustained depolarizing inhibition is a property of NL neurons, recent work has shown that depolarizing inhibition is not always slow and sustained in the NL. Slow and asynchronous inhibition appears to be a property of MF and HF NL neurons while LF neurons tend to receive a fast and phasic inhibition (Tang and Lu 2012b; Yamada et al. 2013; Hamlet et al. 2014). Interestingly, a tonic inhibition, mediated by GABA$_A$ receptors containing the δ subunit, is present in MF and HF neurons but not LF neurons (Tang et al. 2011). In the MF and HF neurons, δ subunits endow GABA$_A$ receptors with the ability to produce a sustained but small inward current which improves coincidence detection through a continuous form of shunting inhibition (Tang et al. 2011). In LF neurons, local GABAergic neurons provide fast phasic IPSCs to NL neurons. Fast and phasic inhibition is at least partly due to the strong expression of GABA$_A$ α1 subunit (Yamada et al. 2013).

One aim of this dissertation was to assess the role of LTK currents in shaping inhibitory synaptic inputs in different CF regions. Previous work in the auditory brainstem on the role of LTK currents has shown that K$_V$1 channels on the presynaptic terminal have slightly different subunit composition, which regulates neurotransmitter release and thus postsynaptic responses (Dodson and Forsythe 2004; Trussell and Roberts 2008). Specifically, presynaptic LTK channels reduce the number of APs that invade the presynaptic terminal, improving the synchrony of transmitter release. The LTK channels
also decrease the width of single APs, reducing the amount of neurotransmitter released during each AP (Geiger et al. 2000; Dodson et al. 2002; 2003; Shu et al. 2007). As discussed in Chapter II, stronger presynaptic LTK currents appear to be present on the inhibitory terminals innervating LF neurons compared to MF and HF neurons, revealing differential interactions between LTK channels and synaptic inhibition along the NL frequency axis (Hamlet et al. 2014).

**Tonotopy of modulation in the NL**

Until now, the discussion of morphological and physiological specialization has implied that these are static, unchanging properties. It is important to recognize that modulation of these properties is a critical property of neuronal signaling. Modulation allows for changes in neuronal function via alterations in morphological and physiological properties that can occur on timescales ranging from seconds to months. While a variety of integral membrane proteins, such as GPCRs and tyrosine-kinases, modulate cellular function, GPCRs are the most ubiquitous sources of modulation in the central nervous system (McCudden et al. 2005; Luttrell 2006), so understanding GPCR function is critical to understanding cellular communication.

While not much is known about modulatory mechanisms in the NL, much less is known regarding the tonotopic arrangement of modulation. Some recent work in the NL has shown that modulatory receptors are also tonotopically arranged (Yamada et al. 2005; Tang et al. 2013; Okuda et al. 2013), such as noradrenergic receptors (Yamada et al. 2005), GABA<sub>B</sub> receptors, and metabotropic glutamate receptors (mGluRs) (Tang et al.
2009; Tang and Lu 2012a; Okuka et al. 2013). These GPCRs influence or modulate ITD-coding in a coding frequency dependent manner. For example, activation of noradrenergic receptors has been shown to alter the activation kinetics of $I_h$ currents more dramatically in HF neurons compared to MF and LF neurons (Yamada et al. 2005). As described earlier, the predominant expression of HCN2 subunits in HF neurons likely makes HF neurons more sensitive to noradrenaline than MF and LF neurons. This is due to the fact that HCN2 subunits have greater sensitivity to cAMP (Kusch et al. 2012), a downstream signaling molecule of several adrenergic receptors including α2-R, and most β-Rs (Xiao 2001; Zhang and Linden 2004; Qin et al. 2008). The shift in $I_h$ current activation made HF neurons more selective for a particular ITD, which ultimately enhances sound localization when noradrenaline is present (Yamada et al. 2005). The behavioral outcome of altered sound localization when noradrenaline is present may be to heighten attention to sound-source during periods of arousal.

Certain types of mGluRs are also tonotopically expressed in NL (Okuda et al. 2013). Of the three broad groups of mGluRs (Nicoletti et al. 2011; Niswender and Conn 2010), group II mGluRs (mGluR II) seem to have more dense expression and predominant function in LF neurons than MF and HF neurons. Okuda et al. (2013) have shown that presynaptic mGluR II help improve the accuracy of ITD coding in LF neurons by reducing the amount of synaptic depression that occurs during relatively long periods of stimulation. While the presynaptic function of mGluR II in the NL may improve ITD-coding preferentially in LF neurons, no work has addressed whether postsynaptic mGluR II can alter the intrinsic properties of NL neurons. In chapter III, the postsynaptic
functions of mGluR II are addressed. In line with Okuda et al. (2013), mGluR II are most active in LF neurons of the NL. Activation of mGluR II increased HTK currents, allowing LF neurons to produce APs more reliably for high rates of stimulation.

**Summary of the specific aims of the dissertation**

Given the tonotopic organization of the auditory system, sound localization circuitry is expected to be specialized at different sound frequencies. This leads to the question of how frequency differences in sound localizing neurons are expressed on a cellular level. This dissertation addresses this issue by investigating the tonotopic arrangement of Kv currents and their interaction with synaptic inhibition in the NL, and how Kv currents are modulated by excitatory neurotransmission. We hypothesized that there are robust tonotopic variations in Kv currents, and these variations differentially regulate synaptic activity and are subject to tonotopically-arranged modulation. The specific aims are:

**Specific Aim 1: To determine the role of LTK currents in modulating the inhibitory synaptic inputs to the NL.**

When the inhibitory neurotransmitter GABA activates receptors in NL neurons, the membrane potential becomes more positive (depolarizes). The depolarization can activate LTK currents, which ultimately prevent or shunt further membrane depolarization. Our results confirmed prior research showing that LTK currents were the strongest in the MF and HF neurons. Furthermore, results indicated that presynaptic
GABAergic terminals also possess tonotopically organized LTK, with stronger presynaptic LTK currents in LF neurons than in MF and HF neurons. These experiments clearly show that LTK currents are active both presynaptically and postsynaptically to regulate neurotransmission and the excitability of NL neurons. Most importantly, these experiments suggest that ITD-coding in LF neurons may utilize mechanisms more similar to mammalian ITD-coding neurons than in MF and HF NL neurons.

**Specific Aim 2: To determine the role of group II metabotropic glutamate receptors in modulating postsynaptic Kv currents in the NL.**

Glutamate is the major neurotransmitter that directly causes excitation in NL neurons; however glutamate can also bind to modulatory mGluRs. Recent research has shown that a subset of mGluRs, group II mGluRs (mGluR II) act as autoreceptors on NL terminals to reduce synaptic depression in LF neurons. Although mGluR II are expressed in postsynaptic NL neurons, the postsynaptic effects of mGluR II on intrinsic properties of NL neurons are unknown, preventing a comprehensive understanding of mGluRs in auditory processing in coincidence detector neurons. We hypothesized that mGluR II exert a postsynaptic effect on intrinsic neuronal properties in the NL. Utilizing both an mGluR II agonist and endogenously released glutamate, I established that mGluR IIs selectively enhance HTK currents. Further work with pharmacological agents revealed a tonotopic arrangement of mGluR II modulation, the mechanism of mGluR II modulation, and the function of this modulation on AP production in NL neurons. The results show
that mGluR II provides a dynamic feedforward mechanism to improve the ability of NL neurons to fire APs during periods of high activity.
Neurons rely on a variety of intrinsic and synaptic neuronal properties to ensure precise coding of temporal information from sensory inputs. An extensive body of research has demonstrated the prominent roles of synaptic inhibition in auditory neurons that encode the location of sound in azimuth space using interaural time difference (ITD) as a cue (e.g. Grothe and Sanes 1993; 1994; Funabiki et al. 1998; Brand et al. 2002; Grothe 2003; Zhou et al. 2005; Pecka et al. 2008; Fukui et al. 2010). These neurons encode sound location by producing maximal spiking activity when bilateral excitatory inputs from the two cochleae converge, a process termed coincidence detection (Jeffress 1948; Konishi 2003; MacLeod and Carr 2012). Synaptic inhibition can improve coincidence detection via shunting the impact of depolarizing postsynaptic currents on the membrane potential and thus sharpening the time window for spike generation.

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1 The information in this chapter was published in: Hamlet WR, Liu YW, Tang ZQ, Lu
(Brückner and Hyson 1998; Funabiki et al. 1998; Ingham and McAlpine 2005; Howard and Rubel 2010; Tang et al. 2011; Roberts et al. 2013).

In most mature neurons, synaptic inhibition mediated by ionotropic GABA_\text{A} and glycine receptors produces conventional hyperpolarizing inhibitory postsynaptic currents (IPSCs). In contrast, GABAergic and glycineergic IPSCs in chicken auditory brainstem neurons are depolarizing, caused by a depolarized reversal potential for Cl^- (between -45 and -35 mV) that appears to be maintained in mature animals (Hyson et al. 1995; Lu and Trussell 2001; Monsivais and Rubel 2001; Kuo et al. 2009; Tang et al. 2009). Of particular interest, sound-localizing neurons in the nucleus laminaris (NL) of the chick receive depolarizing inhibitory inputs originating primarily from ipsilateral superior olivary nucleus (SON) neurons and sparsely from local GABAergic neurons (von Bartheld et al. 1989; Code and Churchill 1991; Lachica et al. 1994; Burger et al. 2005; Tang et al. 2011; Yamada et al. 2013). There exists a tonotopic distribution of GABAergic inhibition along the frequency axis of NL. Neurons coding low frequency (LF) sound receive small and fast phasic inhibition with minimal tonic inhibition, whereas neurons coding middle and high frequency (MF and HF, respectively) sound receive large and slow phasic inhibition with prominent tonic inhibition (Tang et al. 2011; Tang and Lu 2012; Yamada et al. 2013). The depolarizing inhibition improves coincidence detection via both a shunting effect of the GABAergic conductance and activation of a low threshold voltage-gated potassium (LTK) conductance (Funabiki et al. 1998; Monsivais et al. 2000; Howard et al. 2007; Tang et al. 2011).
LTK currents are prominent in auditory brainstem neurons involved in sound localization circuitry (Manis and Marx 1991; Forsythe and Barnes-Davies 1993; Reyes et al. 1994; Brew and Forsythe 1995; Rathouz and Trussell 1998; Golding et al. 1999; Brew et al. 2003; Barnes-Davies et al. 2004). In these neurons, LTK currents minimize the impact of small and slow excitatory postsynaptic currents (EPSCs) on membrane potential, regulate the threshold for action potential generation, and suppress hyperexcitability at presynaptic terminals (Dodson et al. 2002; 2003; Svirskis et al. 2002; Scott et al. 2005; Gittelman and Temple 2006; Mathews et al. 2010). Ion channels containing subunits from Kv1, Kv4, and Kv7 subfamilies underlie the LTK currents (Coetzee et al. 1999; Johnston et al. 2010). In the tonotopically organized NL (Rubel and Parks 1975), Kv1.1 and Kv1.2 subunits show stronger anatomical expression in MF and HF neurons compared to LF neurons, and physiological data also suggests stronger LTK channel activity at rest in MF and HF neurons (Kuba et al. 2005). Therefore, LTK channels are poised to strongly affect synaptic integration of depolarizing synaptic inputs, particularly in MF and HF neurons. Given the unusual depolarizing nature of the inhibitory input to NL neurons and the robust presence of LTK channels in these neurons, it is important to determine how LTK currents interact with synaptic inhibition at subthreshold and suprathreshold membrane potentials, and how this interaction differs across the tonotopic axis.

Materials and Methods

Slice preparation and in vitro whole-cell recordings
Brainstem slices (250–300 μm in thickness) were prepared from chick embryos E17–E18 as described previously (Tang et al. 2009). An ice-cold artificial CSF (ACSF) used for dissecting and slicing the brain tissue contained the following (in mM): 250 glycerol, 3 KCl, 1.2KH₂PO₄, 20 NaHCO₃, 3 HEPES, 1.2 CaCl₂, 5 MgCl₂, and 10 dextrose (pH 7.4 when gassed with 95% O₂ and 5% CO₂). The procedures have been approved by the Institutional Animal Care and Use Committee at Northeast Ohio Medical University, and are in accordance with National Institutes of Health policies on animal use. Slices were incubated at 34–36°C for approximately 1 h in normal ACSF containing the following (in mM): 130 NaCl, 26 NaHCO₃, 3 KCl, 3 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, and 10 dextrose, pH 7.4. For recording, slices were transferred to a 0.5 ml chamber mounted on an upright Olympus BX51 microscope (Japan) with a 40x water-immersion objective. The chamber was continuously superfused with ACSF (1–2 ml/min) by gravity. Recordings were performed at 34–36°C, except Kᵥ current recordings which were performed at 22-24°C (room temperature). Patch pipettes were drawn on an Electrode Puller PP-830 (Narishige) to 1–2 μm tip diameter using borosilicate glass micropipettes (inner diameter of 0.86 mm; outer diameter of 1.60 mm) (VWR Scientific). The electrodes had resistances between 3 and 5 MΩ when filled with a solution containing the following (in mM): 105 K-gluconate, 35 KCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 4 ATP-Mg, and 0.3 GTP-Na, with pH of 7.2 (adjusted with KOH) and osmolarity between 280 and 290 mOsm/L. The Cl⁻ concentration (37 mM) in the internal solution approximated the physiological Cl⁻ concentration in NL neurons (Tang et al., 2009). Placement of recording electrodes was controlled by a micromanipulator NMN-25.
The liquid junction potential was 10 mV, and data were corrected accordingly. Voltage- and current clamp experiments were performed with an AxoPatch 200B and an AxoClamp 2B amplifier, respectively (Molecular Devices). Voltage-clamp recordings were obtained at a holding potential of -60 mV. Data were low-pass filtered at 2–10 kHz and digitized with a Data Acquisition Interface ITC-18 (InstruTECH) at 20 kHz. Recording protocols were written and run using the acquisition and analysis software AxoGraph X (AxoGraph Scientific). In \( K_V \) current recordings, \( R_S \) compensation was done at approximately 75%. In current clamp experiments, bridge balance was used to compensate for the voltage drop across the electrode resistance.

All chemicals were purchased from Sigma-Aldrich except: (1S,2S)-2-[[3-(1H-Benzimidazol-2yl)propyl]methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-(1-methylethyl)-2-naphthalenyl methoxyacetoacetate dihydrochloride (Mibefrindil), 4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD-7288), which were obtained from Tocris, and 6-imino-3-(4-methoxyphenyl)-1(6\( H \))-pyridazine butanoic acid (SR95531), and 6,7-Dinitroquinoxaline-2,3-dione (DNQX) which were obtained from Abcam.

**Synaptic stimulation and recordings of synaptic responses**

Extracellular stimulation was performed using concentric bipolar electrodes with a tip core diameter of 127 \( \mu \)m (World Precision Instruments). The stimulating electrodes were placed using a Micromanipulator NMN-25 (Narishige) at the lateral fiber bundle,
which carries both excitatory and inhibitory fibers. All synaptic recordings were conducted in the presence of the AMPA receptor blocker DNQX (20 μM) to completely block EPSCs. Square electric pulses (0.2 ms duration) were delivered through a Stimulator A320RC (World Precision Instruments). Optimal stimulation parameters were selected for each cell to give postsynaptic potentials of maximal amplitude. In experiments designed to examine the effect of LTK currents on subthreshold responses, QX-314 (5 mM) was included in the pipette solution to block Na\textsubscript{v} channels. A comparison of K\textsubscript{v} recordings with and without QX-314 in the pipette solution showed little to no difference in LTK current size, although QX-314 can block other K\textsuperscript{+} conductances (Andrade, 1991; Alreja and Aghajanian, 1994).

**Identification of tonotopic characteristic frequency (CF) region**

It is not possible to define the characteristic frequency of NL neurons in an *in vitro* slice preparation. Therefore, to categorize neurons into LF, MF, and HF regions, we used an approach modified from Kuba et al. (2005), by using the rostral-caudal and medial-lateral position as an indicator of CF. Generally 5 slices of brainstem tissue containing relevant nuclei were collected, and the most caudal one was slice #1 and the most rostral one slice #5. Neurons in the lateral NL of slices 2 and 3 were considered LF neurons. MF neurons were considered to be present in the medial NL of slice 2 and 3 and the lateral portion of slice 4. HF neurons were found in the medial portion of slice 4 and in entire slice 5 (Fig. 1). The images were taken with a Provis AX70 (Olympus) microscope using Spot software (Diagnostic Instruments), from freshly sliced tissue.
Image contrast and colorization was adjusted using Creative Suites v2.0 (Adobe). Because boundaries between the regions are subjective, we record neurons clearly present in one of the three CF regions.

**Data analysis**

The resting membrane potential was read from the amplifiers immediately after the whole-cell configuration was established. The input resistance was calculated from the voltage responses to a somatic current injection (50 pA). Current density was calculated by normalizing raw current to each cell's membrane capacitance, recorded during $C_m$ compensation prior to recording. For $K_V$ current recordings, leak subtraction was performed using a linear fitting to $V_{command}$ -100 to -70 mV. The amount of inactivated current was calculated by subtracting the maximum current ($I_{max}$) evoked during the $V_{command}$ from the minimum current ($I_{min}$) evoked during the $V_{command}$. Current activation was measured by a single exponential function, $f(t) = A*\exp(-t/\tau)$, in which $t$ stands for time and $\tau$ for time constant. Spontaneous synaptic events were detected by a template using a function for product of exponentials, $f(t) = [1 - \exp(-t/rise time)]*\exp(-t/decay \tau)$. Due to differences in synaptic event size and shape within the NL, the values of these parameters for the template were determined based on the average of real events from individual cells. The detection threshold was three to four times the noise standard deviation, which allowed the detection of most of the events with the least number of false positives. Graphs were made in Igor (Wavemetrics). Means and SEMs are reported. Prior to hypothesis testing, normality and outliers were observed within
Figure 2.1 Determination of characteristic frequency (CF) regions of nucleus laminaris (NL) in coronal brainstem slices. Usually a total of 5 slices (300 µm in thickness) from each animal were collected and numbered 1 through 5, in a caudal to rostral direction. Slice 1 is characterized by the nucleus magnocellularis (NM, green) without NL. The NM is present in all but the slice 5. The NL appears in slice 2 and contains mostly low frequency (LF, blue) neurons, with some middle frequency (MF, red) neurons present at the most medial end. MF neurons are most prevalent in slice 3, with LF neurons at the lateral end of the nucleus. In slice 4, HF (yellow) neurons are located in the medial portion of the NL while MF neurons are found laterally. In slice 5 only HF neurons are present. The colored boundaries between CF regions are approximate.
each set of data to determine the appropriate statistical test. Outliers (> 2x larger than the interquartile range) were dropped from the dataset. Wilks-Shapiro test was used to confirm whether sample distributions were approximately normally distributed. Violations of normality were present when \( p < 0.05 \). When significant violations of normality were present or where datasets contained \( n_i \leq 7 \), nonparametric inferential statistical tests were used. Statistical differences were determined by Analysis of Variance (ANOVA) and Kruskall-Wallis analysis of variance for parametric and nonparametric samples, respectively. When significant group differences were found, a Tukey’s HSD post hoc test or Mann-Whitney U-test was conducted. Paired-samples t-tests were conducted for repeated-measures sample comparisons. When necessary, alpha-levels were corrected using the Holm-Bonferroni method.

**Results**

NL neurons were categorized into 3 groups based on CF region as function of position; caudolateral, caudomedial/rostrolateral, and rostromedial neurons corresponded to LF, MF, and HF groups, respectively (Fig. 2.1). A total of 162 neurons were recorded, and 50, 57, and 55 cells were from LF, MF, and HF regions, respectively.

**Characterization of LTK currents along the frequency axis of NL**

In order to assess the interaction between LTK currents and synaptic inhibition, a detailed analysis of LTK currents along the frequency axis of NL was conducted. LTK currents were isolated in the presence of blockers for Na\(_V\) channels (TTX, 1 µM), low
threshold CaV channels (Mibefradil, 10 µM), HCN channels (ZD7288, 80 µM), AMPA receptors (DNQX, 20 µM), and GABA_A receptors (Gabazine, 20 µM). LTK currents showed a striking tonotopic variation in the amplitude of the onset current (I_{Onset}, measured at 4-9 ms after the onset of the V_{command}) and steady state current (I_{SS}, measured at 94-99 ms) (Fig. 2.2). Because K_V currents activated at -40 mV represent primarily the LTK component, with little contamination of high threshold K_v (HTK) (Brew and Forsythe 1995; Wang et al. 1998), we analyzed and compared LTK current amplitude at -40 mV. I_{Onset} was significantly smaller in LF (n = 15, 0.4 ± 0.2 nA) compared to MF (n = 19, 1.3 ± 0.2 nA) and HF (n = 16, 1.1 ± 0.2 nA) neurons, and no difference was observed between MF and HF neurons (p = 0.002, Fig. 2.2D). I_{SS} at -40 mV was also significantly smaller in LF (0.6 ± 0.2 nA) neurons than in MF (1.4 ± 0.2 nA) neurons but not HF (1.3 ± 0.2 nA) neurons (p = 0.021, Fig. 2.2H). Due to differences in membrane area across the tonotopic axis (gradual reduction in area from LF to MF and HF regions of the NL), current density (defined as the ratio of current amplitude over cell capacitance) was compared. Both I_{Onset} and I_{SS} densities at -40 mV were significantly smaller in LF (7.6 ± 4.1 and 10.0 ± 4.5 pA/pF) neurons compared to MF (26.1 ± 3.8 and 27.1 ± 4.0 pA/pF) and HF (34.0 ± 4.0 and 38.4 ± 4.4 pA/pF) neurons (I_{Onset}: p < 0.0001; I_{SS}: p < 0.0001, Fig. 2.2E & 2.2I). These data confirm anatomical data that there is a robust tonotopic variation in LTK channels in NL neurons such that MF and HF neurons have substantially higher LTK current amplitude than LF neurons (Lu et al., 2004; Kuba et al, 2005).
Figure 2.2 MF and HF NL neurons have larger LTK currents than LF neurons. (A) Sample protocol ($V_{\text{hold}} = -60 \text{ mV}$; $V_{\text{command}} (V_c) = -90$ to $-30 \text{ mV}$, 100 ms duration), and representative LTK current recordings from LF (blue, n = 15), MF (red, n = 19) and HF (yellow, n = 16) NL neurons. (B-C) Onset current ($I_{\text{onset}}$) amplitude and current density in MF and HF neurons are higher than those in LF neurons. (D) At $-40 \text{ mV}$, LF (0.4 ± 0.2 nA) neurons have significantly smaller $I_{\text{onset}}$ than MF (1.3 ± 0.2 nA) and HF (1.1 ± 0.1 nA) neurons. MF and HF neurons do not significantly differ. (E) $I_{\text{onset}}$ density at $-40 \text{ mV}$ shows similar tonotopic variations to $I_{\text{onset}}$ amplitude (LF: 7.6 ± 2.1 pA/pF; MF: 26.1 ± 4.1 pA/pF; and HF: 34.0 ± 4.9 pA/pF). (F-G) Steady state current ($I_{\text{SS}}$) amplitude and current density reveal similar tonotopic variations to $I_{\text{onset}}$, with higher current amplitude and density in MF and HF neurons compared to LF neurons. (H) At $-40 \text{ mV}$, LF (0.6 ± 0.2 nA) neurons have significantly smaller $I_{\text{SS}}$ than MF (1.4 ± 0.2 nA) and HF (1.3 ± 0.1 nA) neurons. (I) $I_{\text{SS}}$ density at $-40 \text{ mV}$ shows similar tonotopic variations to $I_{\text{SS}}$ amplitude (LF: 10.0 ± 3.9 pA/pF; MF: 27.1 ± 4.1 pA/pF; and HF: 38.4 ± 4.8 pA/pF). Mean ± SEM are shown in this and subsequent figures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA, Tukey's post hoc analysis). Cells were held at -60 mV for voltage clamp experiments.
Coincident detecting neurons in the auditory brainstem have fast membrane time constants, partly due to a strong active LTK conductance at rest (Kuba et al., 2005; Scott et al., 2005; Matthews et al., 2010). Previous work has suggested that in the NL, MF neurons possess more LTK current at rest than LF and HF neurons (Kuba et al., 2005), however little is known about the amount of active LTK conductances at rest along the tonotopic axis. Therefore, we assessed how much LTK current was available at rest (about -60 mV for NL neurons; Reyes et al., 1994; Funabiki et al., 1998; Gao and Lu, 2008) by introducing a -90 mV pre-pulse (1000 ms duration) prior to the voltage commands (100 ms duration) (Fig. 2.3). At -60 mV, neither $I_{\text{onset}}$ nor $I_{\text{ss}}$ amplitude was significantly different across the tonotopic axis (Fig. 2.3D, 2.3H). However, the onset and steady state current density of MF neurons were significantly higher than that of LF and HF neurons (Fig. 2.3E, 2.3I). Specifically, a significant difference in $I_{\text{onset}}$ density between MF ($n = 7$, $8.9 \pm 1.9 \text{ pA/pF}$) and HF ($n = 10$, $2.1 \pm 1.7 \text{ pA/pF}$) emerged ($p = 0.038$) (Fig. 2.3E), and $I_{\text{ss}}$ density of MF neurons ($10.9 \pm 2.3 \text{ pA/pF}$) was higher compared to LF ($n = 10$, $3.5 \pm 2.3 \text{ pA/pF}$) neurons ($p = 0.041$, Fig. 2.3I). To some extent, LTK current was active at rest in all CF regions, with MF neurons having the largest conductance.

LTK current kinetics may also vary across the tonotopic axis, which may influence how LTK currents interact with inhibitory synaptic input. Therefore we assessed LTK kinetics across the tonotopic axis by examining LTK activation and steady state inactivation. For activation kinetics, we analyzed the fast membrane time constants
Figure 2.3 MF neurons have higher LTK conductance active at rest. (A) Sample protocol ($V_{\text{hold}} = -60 \text{ mV}$, $V_{\text{prepulse}} = -90 \text{ mV}$, 1000 ms duration; $V_c = -110$ to -30 mV, 100 ms duration), and representative LTK current recordings from LF (blue, n = 8), MF (red, n = 7) and HF (yellow, n = 10) NL neurons. (B-E) At -60 mV, MF (0.5 ± 0.1 nA) neurons appear to have higher $I_{\text{Onset}}$ amplitude than LF (0.3 ± 0.1 nA) and HF (0.2 ± 0.1 nA) neurons. The $I_{\text{Onset}}$ density at -60 mV in MF (8.9 ± 1.9 pA/pF) neurons is significantly higher than in HF (2.1 ± 1.7 pA/pF) neurons, while LF neurons have intermediate current density (4.3 ± 1.9 pA/pF). (F-G) $I_{\text{SS}}$ amplitude and current density reveal similar tonotopic variations to $I_{\text{Onset}}$. (H) $I_{\text{SS}}$ amplitude at -60 mV in MF (0.6 ± 0.1 nA) neurons seems to be higher than LF (0.2 ± 0.1 nA) and HF (0.3 ± 0.1 nA) neurons. (I) $I_{\text{SS}}$ density at -60 mV in MF (10.9 ± 2.3 pA/pF) neurons is significantly higher than in LF (3.5 ± 2.3 pA/pF) neurons. HF neurons have intermediate current density (5.4 ± 2.0 pA/pF).
(tau < 10 ms) because they are relevant to the peak amplitude and rise time of the depolarizing synaptic conductances. To do this, we used an exponential function, \( f(t) = A \times \exp\left(-t/\text{decay } \tau\right) \). A fast tau was found in most MF (14 / 19) and HF (13 / 16) neurons but in less than half LF (5 / 14 cells) neurons (Fig. 2.4A). The absence of fast tau in many LF neurons may suggest that LF neurons lack prominent fast activating LTK currents. At -45 mV, MF (1.6 ± 0.3 ms) neurons had significantly faster tau than LF (2.3 ± 0.3 ms) and HF (3.1 ± 0.5 ms) neurons (Fig. 2.4B, p = 0.018). LTK inactivation was analyzed from K\(_V\) currents obtained at two voltages: -45 mV and -30 mV (Fig. 2.4C). While at -45 mV virtually only LTK currents were activated, the current evoked at -30 mV may be contaminated by small amounts of HTK currents (Brew and Forsythe 1995; Wang et al. 1998). In many recordings, a fast transient inward current was evoked at -45 and -30 mV, likely caused by a low threshold Ca\(_V\) current incompletely blocked by Mibefradil (Blackmer et al. 2009). Because the fast inward current corrupted accurate measurement of \( I_{\text{Onset}} \), we analyzed \( I_{\text{SS}} \) LTK inactivation. \( I_{\text{SS}} \) showed relatively little inactivation and similar kinetics across the tonotopic axis. Nonetheless, HF neurons had slightly less LTK channel inactivation (15-25% inactivated) compared to LF (20-30%) and MF (20-30%) neurons (Fig. 2.4D-F). MF (n = 8, 0.45 ± 0.8 nA) had a larger amount of current inactivation compared to LF (n = 11, 0.14 ± 0.6 nA) and HF (n = 11, 0.19 ± 0.6 nA) neurons at -45 mV (p = 0.008, Fig. 2.4G). No tonotopic differences in the amount of current inactivation were observed at -30 mV (Fig. 2.4H). Taken together, these data suggest that LTK inactivation kinetics are relatively similar across the tonotopic axis, and the LTK currents are more readily activated in MF neurons.
Figure 2.4 LTK currents in MF neurons are of faster activation and stronger inactivation than in LF and HF neurons. (A) To obtain the time constant (tau) for LTK current activation, a single or double exponential fitting (black dashed line) was performed to the LTK currents recorded at $V_c$ from -45 to -30 mV ($V_{\text{hold}} = -60$ mV). (B) At -45 mV, MF (1.6 ± 0.3 ms) neurons have significantly faster tau than LF (2.3 ± 0.3 ms) and HF (3.1 ± 0.5 ms) neurons. (C) Sample protocol used to study LTK inactivation at -45 mV and -30 mV following increasing voltage steps (-120 mV to -45 mV, 100 ms duration, 5 mV increment). The open circle indicates where $I_{SS}$ was measured. The traces with darker colors were obtained at -30 mV and those with lighter colors at -45 mV. Inset shows the $I_{\text{max}}$ and $I_{\text{min}}$ used to calculate the amount of inactivated current. (D) Normalized $I_{SS}$ inactivation at -45 (n = 11, $V_{1/2} = -83.9$ mV; k = 19.6) and -30 mV (n = 13, $V_{1/2} = -71.9$ mV; k = 7.7) in LF neurons. (E) Normalized $I_{SS}$ inactivation at -45 (n = 8, $V_{1/2} = -83.1$ mV; k = 14.4) and -30 mV (n = 12, $V_{1/2} = -82.3$ mV; k = 13.8) in MF neurons. (F) Normalized $I_{SS}$ inactivation at -45 (n = 11, $V_{1/2} = -85.0$ mV; k = 13.6) and -30 mV (n = 12, $V_{1/2} = -82.5$ mV; k = 8.6) in HF neurons. (G, H) The raw amount of inactivated current was calculated by subtracting the maximum current from the minimum $I_{SS}$. MF (0.45 ± 0.8 nA) neurons had significantly more inactivated LTK current at -45 mV than LF (0.14 ± 0.6 nA) and HF (0.19 ± 0.6 nA) neurons. No differences between CF regions were observed in the amount of inactivated LTK current at -30 mV.
One caveat to the LTK currents reported here is that the recordings were made at room temperature to achieve a better voltage clamp (Rathouz and Trussell 1998). LTK currents at physiological temperature would be expected to be slightly larger in peak amplitude and show faster activation kinetics (Cao and Oertel 2005). Another caveat is the use of embryonic neurons in this study. Although Kv currents are relatively mature by E18 (Gao and Lu 2008), there is evidence that membrane conductances including LTK currents increase after E21 (Kuba et al. 2002). However, the effects of DTX on the firing properties of NL neurons are similar between early chick hatchlings and late embryos (Kuba et al. 2002). Despite these caveats, our data confirm and elaborate on previous research, indicating that LTK current size and kinetics vary across the tonotopic axis of NL.

**Characterization of IPSPs along the frequency axis of NL**

The driving question behind this study is how LTK currents differentially modulate depolarizing inhibitory postsynaptic potentials (IPSPs) in NL neurons. Prior work from our lab has demonstrated a robust tonotopic difference in synaptic inhibition in the NL. LF neurons show less frequent spontaneous inhibitory postsynaptic currents (sIPSCs) than MF and HF neurons, and IPSCs of LF neurons are smaller and faster (Tang et al. 2011; Tang and Lu 2012). We sought to confirm these tonotopic variations in current clamp recordings (Fig. 2.5). Evoked IPSPs were isolated by stimulating the fiber bundle lateral to the NL in the presence of AMPA receptor blocker DNQX (20 µM). To prevent action potentials from occurring on the top of IPSP, QX-314 (5 mM) was
included in the intracellular recording solution. A comparison of LTK currents recorded with and without QX-314 in the pipette solution revealed little to no difference in LTK currents (data not shown). No tonotopic differences in IPSP peak amplitude and 10-90% rise time were observed (Fig. 2.5B-C). However, IPSP half width was significantly different across the tonotopic axis (Fig. 2.5D, p = 0.035). The half width of IPSPs was significantly larger in HF (n = 13, 97.3 ± 32.8 ms) neurons compared to LF (n = 14, 39.0 ± 9.6 ms) neurons, while MF (n = 14, 61.3 ± 13.0 ms) neurons had an intermediate value. Analysis of spontaneous IPSPs (sIPSPs) revealed that while there was no tonotopic variation in the sIPSP kinetics (half width and rise time), the frequency and peak amplitude of sIPSPs were different across the tonotopic axis (Fig. 2.5E-I). The inter-event interval (IEI) of sIPSPs was significantly smaller in MF (n = 14, 120.0 ± 11.0 ms) and HF (n = 13, 120.4 ± 21.4 ms) neurons compared to LF (n = 12, 333.3 ± 58.9 ms) neurons (Fig. 2.5F, p = 0.001). Peak amplitude was also significantly different across the tonotopic axis (Fig. 2.5G, p = 0.003). LF neurons had smaller sIPSP amplitude (1.6 ± 0.2 mV) compared to MF (3.3 ± 0.3 mV) and HF (2.5 ± 0.2 mV). The data on sIPSP frequency and amplitude are consistent with our previous voltage clamp studies on sIPSC parameters (Tang et al. 2011; Tang and Lu 2012). The lack of differences in sIPSP kinetics across CF regions may result from differential influences of LTK currents and other intrinsic conductances on the depolarizing inhibition.
**Figure 2.5** MF and HF neurons tend to have wider IPSPs and larger sIPSPs than LF neurons. (A) Sample evoked inhibitory postsynaptic potentials (IPSP) recordings, with the thick trace being the average. A square current pulse (0.2 ms) was delivered to a mixed excitatory and inhibitory fiber tract, and IPSPs were isolated in the presence of DNQX (20 µM). QX-314 (5 mM) was present in the intracellular solution to prevent action potentials in the recorded neurons. LF (blue, n = 14) neurons tend to have more narrow IPSPs than MF (red, n = 14) and HF (yellow, n = 13) neurons. (B) Peak IPSP amplitude is not significantly different in LF (7.1 ± 0.8 mV), MF (7.8 ± 1.4 mV), and HF (7.7 ± 1.1 mV) neurons. (C) 10-90% rise time of IPSP is not significantly in LF (9.5 ± 1.9 ms), MF (10.4 ± 3.0 ms), and HF (17.1 ± 3.8 ms) neurons. (D) The half width of IPSPs is significantly larger in HF (97.3 ± 32.8 ms) neurons compared to LF (39.0 ± 9.6 ms) neurons (p = 0.035). MF (61.3 ± 13.0 ms) neurons do not differ from LF or HF neurons. (E) Sample spontaneous IPSP (sIPSP) recordings from LF (blue, n = 11), MF (red, n = 14), and HF (yellow, n = 13) neurons, with a sample single sIPSP shown as an inset. (F) MF (120.0 ± 11.0 ms) and HF (120.4 ± 21.4 ms) neurons have significantly shorter inter-event intervals (IEI) than LF (315.2 ± 58.8 ms) neurons. (G) LF neurons (1.6 ± 0.2 mV) have smaller sIPSP peak amplitude than MF (3.3 ± 0.3 mV) and HF (2.5 ± 0.2 mV) neurons. (H-I) sIPSP half width and 10-90% rise time are not different between CF regions.
Interactions between LTK currents and IPSPs along the frequency axis of NL

To assess the role of LTK currents in regulating subthreshold changes in membrane potentials caused by activation of inhibitory inputs to NL neurons, we studied the effects of DTX-I (0.08 μM), a selective blocker for Kv1.1-subunit containing channels (Harvey 2001), on IPSPs (Fig. 2.6) and sIPSPs (Fig. 7). DTX significantly increased the input resistance (R\text{in}) in MF (n = 12, control: 51.6 ± 6.5 MΩ, DTX-I: 101.2 ± 16.9 MΩ, p = 0.008) and HF (n = 11, control: 68.6 ± 6.8 MΩ, DTX: 108.1 ± 12.0 MΩ, p = 0.001) neurons, but not in LF (n = 12, control: 88.4 ± 8.2 MΩ, DTX: 126.6 ± 26.5 MΩ, p = 0.054) neurons (Fig. 2.6B). R\text{in} was increased significantly more in MF (99.8 ± 25.6 %) compared to LF (38.3 ± 13.2 %) neurons (p = 0.028) (Fig. 2.6C), agreeing with Kuba et al. (2005). DTX significantly increased the peak IPSP amplitude in all CF regions: LF (control: 6.7 ± 0.8 mV, DTX-I: 14.5 ± 2.1 mV, p = 0.004), MF (control: 7.8 ± 1.4 mV, DTX-I: 11.7 ± 2.1 mV, p = 0.030), and HF (control: 6.8 ± 1.1 mV, DTX-I: 12.5 ± 2.1 mV, p = 0.002, Fig. 2.6D). DTX also increased the half width of IPSPs all CF regions: LF (control: 50.9 ± 9.6 ms, DTX-I: 97.6 ± 14.8 ms, p = 0.002), MF (control: 66.2 ± 13.0 ms, DTX-I: 179.3 ± 59.2 ms, p = 0.040) and HF (control: 79.4 ± 32.8 ms, DTX-I: 197.4 ± 73.6 ms, p = 0.028) neurons (Fig. 2.6F). No tonotopic differences in the percent effect of DTX on IPSP peak amplitude or half width were detected (Fig. 6E, G). The analysis of coefficient of variation (CV) of synaptic responses can be used as one indicator of whether changes in IPSP size and shape is due to pre- or postsynaptic mechanism (Scheuss et al. 2002; Clements 2003). No significant differences in 1/CV^2 were found.
Figure 2.6 LTK currents regulate evoked IPSP size in the NL. (A) Sample IPSP recordings before (dark traces) and after (color traces) bath application of DXT (0.08 µM), in LF (n = 12), MF (n = 12) and HF (n = 11) NL neurons. QX-314 (5 mM) was present in the intracellular solution. Above and below are raw (grey or light color) and averaged (black or dark color) IPSP recordings. Normalized averaged IPSP recordings before (black) and after (color) DTX-I are shown in the center. (B) DTX-I significantly increased the input resistance ($R_{in}$) in MF (control: 51.6 ± 6.5 MΩ, DTX-I: 101.2 ± 16.9 MΩ), and HF (control: 68.6 ± 6.8 MΩ, DTX-I: 108.1 ± 12.0 MΩ) neurons but not in LF (control: 88.4 ± 8.2 MΩ, DTX-I: 126.6 ± 26.5 MΩ). (C) $R_{in}$ was increased significantly more in MF (99.8 ± 25.6 %) compared to LF (38.3 ± 13.2 %) neurons, while HF (64.2 ± 15.8 %) did not differ from LF or MF neurons. (D) DTX-I significantly increased the peak IPSP amplitude in LF (control: 6.7 ± 0.8 mV, DTX-I: 14.5 ± 2.1 mV), MF (control 7.8 ± 1.4 mV, DTX-I: 11.7 ± 2.1 mV), and HF (control: 6.8 ± 1.1 mV, DTX-I: 12.5 ± 2.1 mV) neurons. (E) No tonotopic differences in the effect of DTX-I on peak IPSP amplitude. (F) DTX-I significantly increased the half width of IPSP in LF (control: 50.9 ± 9.6 ms, DTX-I: 97.6 ± 14.8 ms), MF (control: 66.2 ± 13.0 ms, DTX-I: 179.3 ± 59.2 ms) and HF (control: 79.4 ± 32.8 ms, DTX-I: 197.4 ± 73.6 ms) neurons. (G) No tonotopic differences in the effect of DTX-I on IPSP half width. (H) $1/CV^2$ was not significantly changed in LF, MF, or HF neurons after DTX-I application. (I) Percent change in $1/CV^2$ was not significantly different across the tonotopic axis. (J) Peak IPSP amplitude plotted against the percent change in peak amplitude after DTX reveals that neurons with smaller IPSP peak amplitudes showed a greater change in IPSP peak amplitude after DTX treatment. The change appeared to be larger in LF ($r^2 = 0.215$, $p = 0.076$) neurons than MF ($r^2 = 0.067$, $p = 0.193$) and HF ($r^2 = 0.061$, $p = 0.233$) neurons. (K) IPSP half width plotted against the percent change in half width after DTX reveals that neurons with smaller IPSP half width showed a greater change in IPSP half width after DTX treatment. The change appeared to be larger in LF ($r^2 = 0.347$, $p = 0.022$) neurons than MF ($r^2 = 0.009$, $p = 0.386$) and HF ($r^2 = 0.010$, $p = 0.382$) neurons.
between control and DTX-I conditions, nor among CF regions (Fig. 2.6H-I). However, LF (64.9 ± 26.1) neurons had significantly larger 1/CV² in control conditions than MF (18.0 ± 3.2) and HF (16.1 ± 4.6) neurons (p = 0.041, not shown in figure) and LF neurons tended to show a reduction in 1/CV² after DTX-I application (control: 64.9 ± 26.1; DTX-I: 28.7 ± 13.5; p = 0.069). LF neurons also showed a correlation between control IPSP peak amplitude and half width with their respective percent changes (amplitude: r² = 0.215, p = 0.076; half width: r² = 0.347, p = 0.022) after DTX application (Fig. 2.6J-K). These data demonstrate that LTK currents regulate the size and shape of IPSPs in NL neurons across the entire frequency axis and suggest the possibility of a presynaptic component to this effect in LF neurons.

These findings were somewhat surprising because MF and HF neurons had stronger LTK current amplitude and density than LF neurons (Figs. 2.2 & 2.3), and therefore MF and HF neurons were expected to show a greater change in IPSP parameters than LF neurons. One potential explanation for this apparent discrepancy is that presynaptic LTK currents regulate the inhibitory inputs to LF neurons to a greater extent than in MF and HF neurons. Analysis of the effects of DTX on sIPSP supported this hypothesis (Fig. 2.7). DTX significantly decreased IEI in LF (n = 11, control: 347.2 ± 62.6 ms, DTX-I: 192.3 ± 30.2 ms, p = 0.011) but not in MF (n = 13, control: 120.0 ± 11.0 ms, DTX-I: 124.7 ± 15.1 ms, p = 0.646) and HF (n = 10, control: 120.4 ± 21.4 ms, DTX-I: 164.5 ± 57.2 ms, p = 0.374) neurons (Fig. 2.7B). The percent decrease in IEI was significantly different in LF (-35.3 ± 10.4 %) neurons compared to MF (8.1 ± 9.8 %) and HF (15.5 ± 14.4 %) neurons (p = 0.017, Fig. 2.7C). There was a significant increase in
sIPSP peak amplitude after DTX treatment in LF (control: 1.5 ± 0.2 mV, DTX: 2.1 ± 0.4 mV, p = 0.010) and HF (control: 2.5 ± 0.2 mV, DTX-I: 3.2 ± 0.4 mV, p = 0.018) neurons but not in MF neurons (Fig. 2.7D). DTX increased in sIPSP half width in HF (control: 21.9 ± 1.7 ms, DTX-I: 28.6 ± 2.8 ms, p = 0.038) but not LF and MF neurons (Fig. 2.7G).

In spite of robust tonotopic differences in postsynaptic LTK currents, there were no significant differences in the effect of DTX on percent changes in peak amplitude or half width of sIPSP among LF, MF and HF neurons (Fig. 2.7E, 2.7G), and LTK currents appear to regulate the sIPSP frequency in LF neurons, suggesting that presynaptic LTK currents regulate inhibitory synapses.

**LTK currents on IPSCs: action loci of DTX**

To better understand the presynaptic versus postsynaptic roles of LTK currents in regulating synaptic inhibition, we studied the effects of DTX on IPSCs recorded under voltage clamp (\(V_h = -60\) mV). Ideally, under voltage clamp, changes in IPSCs observed after DTX application should be attributable to blockade of presynaptic LTK channels because postsynaptic LTK channels are not activated. Evoked IPSCs were subject to LTK modulation (Fig. 2.8). DTX tended to increase the peak amplitude, decay time constant, and amount of charge (Q) in LF neurons (n = 10; peak amplitude: control: -429.8 ± 72.6 pA, DTX: -656.4 ± 112.7 pA, p = 0.031; decay time constant: control 55.4 ± 10.7 ms, DTX: 113.5 ± 23.7 ms, p = 0.071; Q: control: 12.7 ± 2.4 pC, DTX: 30.7 ± 7.3 pC, p = 0.012), and in MF neurons (n = 8; peak amplitude: control: -357.1 ± 83.3 pA, DTX: -662.5 ± 172.7 pA, p = 0.018;
Figure 2.7 LTK currents strongly regulate sIPSP frequency in LF neurons and sIPSP shape in HF neurons. (A) Sample sIPSP recordings before (dark traces) and after (color traces) bath application of DXT (0.08 µM) in LF (n = 11), MF (n = 13) and HF (n = 10) NL neurons. See Figure 6A for legend. (B) Inter-event intervals (IEI) significantly decreased in LF (control: 347.2 ± 62.6 ms, DTX-I: 192.3 ± 30.2) but not MF (control: 120.0 ± 11.0 ms, DTX-I: 124.7 ± 15.1 ms) and HF (control: 120.4 ± 21.4 ms, DTX-I: 164.5 ± 57.2 ms) neurons after DTX-I. (C) The percent decrease in IEI of sIPSPs is significantly greater in LF (-35.3 ± 10.4 %) neurons compared to MF (8.1 ± 9.8 %) and HF (15.5 ± 14.4 %) neurons. (D) There is a significant increase in sIPSP peak amplitude after DTX-I treatment in LF (control: 1.5 ± 0.2 mV, DTX-I: 2.1 ± 0.4 mV) and HF (control: 2.5 ± 0.2 mV, DTX-I: 3.2 ± 0.4 mV) neurons but not in MF (control: 3.0 ± 0.3 mV, DTX-I: 3.1 ± 0.6 mV) neurons. (E) The percent change in sIPSP peak amplitude was not different across the tonotopic axis. (F) DTX-I increased the sIPSP half width in HF (control: 21.9 ± 1.7 ms, DTX-I: 28.6 ± 2.8 ms) but not LF (control: 23.7 ± 2.7 ms, DTX: 26.3 ± 3.0 ms) and MF (control: 22.5 ± 2.1 ms, DTX-I: 25.2 ± 1.5 ms) neurons. (G) Although HF neurons show a substantial increase in sIPSP half width, the percent change in sIPSP half width is not significantly different across the tonotopic axis (p = 0.075).
decay time constant: control: 101.9 ± 25.3 ms, DTX: 170.5 ± 29.3 ms, p = 0.063; Q: control: 31.0 ± 13.0 pC, DTX: 76.4 ± 22.5 pC, p = 0.012) (Fig. 2.8D, 2.8F, 2.8H). HF neurons (n = 9) did not show increases in peak amplitude, decay time constant, or amount of charge, but showed a significant increase in 10-90% rise time (control 5.8 ± 3.3 ms, DTX: 10.5 ± 3.1 ms, p = 0.045) (Fig. 2.8B). The percent change in 10-90% rise time, peak, decay time constant, and amount of charge did not differ across the tonotopic axis (Fig. 2.8C, 2.8E, 2.8G, 2.8I). These data suggest that presynaptic LTK may, to some extent, regulate IPSCs in all CF regions but more prominently in LF neurons.

To further confirm and define the role of presynaptic LTK currents, we studied the effect of DTX on spontaneous IPSCs (sIPSCs) (Fig. 2.9). Cumulative probability of sIPSC IEI revealed that DTX decreased IEI predominantly in LF (n = 12, -53.1 ± 8.3 %) neurons compared to MF (n = 11, -22.7 ± 4.2 %) and HF (n = 13, -19.8 ± 6.4 %) neurons (Fig. 9B-E, p = 0.002). sIPSC peak amplitude also similarly increased after DTX application in LF (46.6 ± 17.7 %), MF (33.7 ± 18.2 %), and HF (26.1 ± 12.2 %) neurons (Fig. 2.9F-I). sIPSC decay time constant was significantly increased in LF (control: 6.0 ± 0.4 ms, DTX: 8.0 ± 0.7 ms, p = 0.022) but not in MF (control: 9.1 ± 1.5 ms, DTX: 9.3 ± 1.2 ms, p = 0.829) or HF (control: 9.7 ± 0.8 ms, DTX: 10.9 ± 1.4 ms, p = 0.193) neurons (Fig. 2.9J). The amount of charge transferred per sIPSC (Q) increased after DTX application in LF (control: 0.5 ± 0.1 pC, DTX: 0.8 ± 0.1 pC, p = 0.011) and HF (control: 0.9 ± 0.1 pC, DTX: 1.2 ± 0.2 pC, p = 0.040), but not in MF (control: 1.1 ± 0.2 pC, DTX: 1.4 ± 0.3 pC, p = 0.078) neurons (Fig. 2.9L). No differences in the percent change in peak amplitude, decay time constant, or charge were observed between LF, MF, and HF
Figure 2.8  DTX-I preferentially regulates IPSC frequency and shape in LF and MF neurons. (A) Sample evoked IPSC recordings before (dark traces) and after (color traces) bath application of DXT (0.08 µM) in LF (n = 10), MF (n = 8) and HF (n = 9) NL neurons. See Figure 6A for legend. (B-C) DTX-I increases the 10-90% rise time in HF (control: 5.8 ± 3.3 ms, DTX-I: 10.5 ± 3.1 ms) neurons, but not in LF (control: 2.6 ± 0.4 ms, DTX-I: 4.8 ± 1.2 ms) and MF (control: 6.1 ± 1.9 ms, DTX-I: 6.6 ± 2.4 ms) neurons, without significant differences in percent changes across tonotopic axis. (D) DTX-I increased the IPSC peak amplitude in LF (control: -429.8 ± 72.6 pA, DTX-I: -656.4 ± 112.7 pA) and MF (control: -357.1 ± 83.3 pA, DTX-I: -662.5 ± 172.7 pA) but not in HF (control: -379.9 ± 66.6 pA, DTX-I: -460.4 ± 113.4 pA) neurons. (E) Percent change in IPSC peak amplitude is not significantly different between LF (58.5 ± 20.6%), MF (103.8 ± 32.5%), and HF (15.2 ± 17.4%) neurons (p = 0.056). (F) DTX-I tends to increase the decay time constant in LF (control: 55.4 ± 10.7 ms, DTX-I: 113.5 ± 23.7 ms, p = 0.071) and MF (control: 101.9 ± 25.3 ms, DTX-I: 170.5 ± 29.3 ms, p = 0.063) neurons. No change in the decay time constant was observed in HF (control: 158.8 ± 27.3 ms, DTX-I: 178.7 ± 25.1 ms) neurons. (G) Percent change in IPSC decay time constant is not significantly different across CF regions. (H) DTX-I increases the amount of charge per IPSC (Q) in LF (control: 12.7 ± 2.4 pC, DTX-I: 30.7 ± 7.3 pC) and MF (control: 31.0 ± 13.0 pC, DTX-I: 76.4 ± 22.5 pC) neurons but not in HF (control: 45.6 ± 10.8 pC, DTX-I: 66.6 ± 20.9 pC) neurons. (I) The percent change in Q is not different between CF regions. (J) No significant differences are observed between CF regions in 1/CV² after DTX-I treatment. (K, L) Control peak amplitude and total area plotted against the percent change in peak amplitude and total area, respectively, after DTX-I reveals that there was little to no correlation between control and percent change after DTX in all CF regions: LF (peak amplitude: r² = 0.076, p =0.413; area: r² = 0.156, p = 0.237), MF (amplitude: r² = 0.140, p = 0.361; area: r² = 0.088, p = 0.475), and HF (amplitude: r² = 0.060, p =0.525; area: r² = 0.190, p = 0.241).
neurons (Fig. 2.9I, 2.9K, 2.9M). These data support the notion that LTK currents have a presynaptic role in modulating IPSC size and shape in NL neurons especially in LF neurons. The discrepancy in the effects of DTX on sIPSCs versus evoked IPSCs in MF neurons might be caused by relatively weak influences of LTK currents on the spontaneous release of GABA in MF neurons.

**LTK currents on excitability of NL neurons**

Finally, we assessed the role of LTK currents in regulating neuronal excitability in NL. Specifically, we tested whether the presence of LTK currents prevented GABA-induced action potentials in NL neurons, as suggested in NM neurons (Monsivais et al. 2000; Howard et al. 2007). We thus investigated such interactions in NL neurons using current clamp recordings (Fig. 2.10). We first confirmed the effects of DTX (0.1 µM) on the intrinsic firing properties of NL neurons in MF and HF regions. Under control conditions, NL cells fired one action potential in response to prolonged suprathreshold current injections followed by a plateau of subthreshold membrane potential (Fig. 2.10A), a characteristic hallmark of time-coding neurons in the central auditory system. DTX produced a small depolarization (2 mV) in RMP, substantially lowered the threshold current, and changed the phasic firing pattern to a tonic mode (Fig. 2.10B, 2.10C), consistent with previous findings in auditory brainstem neurons where Kv1-containing channels are highly expressed (e.g., Brew and Forsythe 1995; Gittelman and Tempel 2006). Furthermore, spontaneous action potentials, which were absent under control conditions, appeared prior to the onset and after the termination of the current injections.
**Figure 2.9** LTK currents strongly regulate the frequency of sIPSC in LF neurons. 

(A) Sample sIPSC recordings before (dark traces) and after (color traces) bath application of DXT (0.08 µM) in LF (blue, n = 12), MF (red, n = 11) and HF (yellow, n = 13) NL neurons. See Figure 6A for legend. 

(B-D) Cumulative fraction of IEI in LF (B), MF (C), and HF (D) neurons before (black) and after (color) DTX-I. 

(E) Percent change in IEI is significantly larger in LF (-53.1 ± 8.3%) neurons compared to MF (-22.7 ± 4.2%) and HF (-19.8 ± 6.4%) neurons. No differences were observed between MF and HF neurons. 

(F-H) Cumulative fraction of sIPSC IEI in LF (F), MF (G), and HF (H) neurons before (black) and after (color) DTX-I. 

(I) No significant differences in percent change in sIPSC peak after DTX-I treatment between LF (46.6 ± 17.7%), MF (33.7 ± 18.2%), and HF (26.1 ± 12.2%) neurons. 

(J) The sIPSC decay time constant is significantly increased in LF (control: 6.0 ± 0.4 ms, DTX-I: 8.0 ± 0.7 ms, p = 0.022) but not in MF (control: 9.1 ± 1.5 ms, DTX-I: 9.3 ± 1.2 ms) or HF (control: 9.7 ± 0.8 ms, DTX-I: 10.9 ± 1.4 ms) neurons. 

(K) No significant differences in percent change in the sIPSC decay time constant were observed between LF, MF, and HF neurons after DTX-I treatment. 

(L) The amount of charge transferred per sIPSC (Q) is significantly increased in LF (control: 0.5 ± 0.1 pC, DTX-I: 0.8 ± 0.1 pC) and HF (control: 0.9 ± 0.1 pC, DTX-I: 1.2 ± 0.2 pC) but not in MF (control: 1.1 ± 0.2 pC, DTX-I: 1.4 ± 0.3 pC) neurons. 

(M) No significant differences in percent change in sIPSC area after DTX-I treatment across CF regions.
Because ionotropic glutamate receptors were blocked throughout these experiments, the spontaneous spikes occurred on the top of depolarizing IPSPs. Under control condition, sIPSP varied widely in amplitude, with maximal membrane depolarization of up to several mV without spike activity (Fig. 2.10D). DTX increased the amplitude of sIPSP and transformed some into spikes (Fig. 2.10E, 2.10F, n=9). We further studied the effects of DTX on evoked IPSP and GABA-induced spikes. Train stimulations at different frequencies were applied to evoke GABA responses. At 50 and 100 Hz, IPSP summated temporally, forming a sustained membrane depolarization of about 15 mV. GABA-induced spikes were seen occasionally (Fig. 2.10G). Under DTX, more spikes were seen at all three frequencies tested, and bursts of spikes occurred at the beginning of the simulation. Significant increase in AP probability was detected for the stimulations at frequency of 10 and 50 Hz (Fig. 2.10H, 2.10I, n = 9). These data confirm that LTK currents prevent GABA-induced excitation, providing a critical role for LTK currents in maintaining synaptic inhibition in NL.

**Discussion**

This study sought to determine the interplay between intrinsic LTK currents and extrinsic synaptic inhibition in NL neurons across the tonotopic axis. We characterized the LTK currents in LF, MF, and HF neurons, and then demonstrated a tonotopic relationship between LTK currents and inhibitory synaptic input. Interestingly, the data suggest that while MF and HF neurons possess larger postsynaptic LTK currents than LF
Figure 2.10 Regulation of GABA responses in NL neurons by LTK channels. (A-C) Effects of DTX-I (0.1 µM) on intrinsic firing properties of NL neurons. Under control condition (A), the cell fires one single AP in response to prolonged suprathreshold current injections (threshold current indicated by the arrow, 1.2 nA in this case). DTX-I slightly depolarizes the RMP, substantially lowers the threshold current to 0.2 nA and changes the phasic firing pattern to a tonic mode (B). Pooled data show a dramatic increase in the number of APs in response to DTX-I application (C, n = 9). Furthermore, spontaneous APs, which are absent under control condition, appear prior to and after the current injections (indicated by # in B). Because ionotropic glutamate receptors were blocked throughout the experiments, these two spontaneous spikes likely occurred on the top of two depolarizing IPSPs. (D-F) Effects of DTX-I on sIPSP. Chart recordings show sIPSP with amplitude of up to several mV, without spike activity (D). DTX-I increases the amplitude of sIPSP and transforms some into spikes (E, one spike indicated by # is shown at an enlarged time scale). Spike frequency is significantly higher in DTX-I than control (F, n = 9). (G-K) Effects of DTX-I on evoked IPSP and GABA-induced spikes. Train stimulations at different frequencies were applied to evoke GABA responses. At 50 and 100 Hz, IPSP sum, forming a sustained membrane depolarization of about 15 mV. GABA-induced spikes are seen occasionally (G). Under DTX-I, more spikes are seen at all three frequencies tested (H), and bursts of spikes are noted (a burst of 4 APs shown at an enlarged time scale). Significant increase in AP probability is detected for the stimulations at frequency of 10 and 50 Hz (I and J, n = 9).
neurons, robust presynaptic LTK currents in LF neurons may compensate for the relatively lower postsynaptic LTK counterpart, leading to equally strong LTK influences equally strongly influences of LTK currents in all NL neurons.

One potential problem would be errors in current measurement arose due to poor space clamping, particularly in LF neurons, which are known to have longer dendrites than MF and HF neurons. While long dendrites can introduce space clamp errors, the number of dendritic bifurcations, and the diameter of primary dendrites can also dramatically influence space clamp. Specifically, thin primary dendrites with numerous bifurcations will also introduce relatively large space clamp errors. Furthermore, the membrane resistance is also a major factor in determining the degree of space clamp errors. (Bar-Yehuda and Korngreen 2008; Poleg-Polsky and Diamond 2011). We do not believe space clamp errors alone can account for differences in LTK amplitude across the tonotopic axis because the dendritic gradient in the NL is such that LF neurons have longer, thicker primary dendrites with relatively few bifurcations but as the CF region increases, $R_{\text{in}}$ decreases and dendritic length and diameter decrease, while the number of primary dendrites and bifurcations increase (Smith and Rubel 1979; Kuba et al. 2005). Therefore, given the gradient of $R_{\text{in}}$, dendritic length, width and branching, we expect a similar degree of space clamp errors across the tonotopic axis.

Given the tonotopic arrangement of LTK currents in NL, we expected to find more robust change in IPSP shape and size in MF and HF neurons than in LF neurons after application of $K_{V1}$ channel blocker DTX. To the contrary, the effect of DTX was
about equally evident in all CF regions. We found increases in IPSP and sIPSP peak amplitude and half width after DTX treatment in most CF regions. Although MF neurons have more LTK currents active at rest, there were no significant differences in the percent changes caused by DTX in the size and shape of IPSP and sIPSP between LF, MF, and HF neurons. The concurrent increase in sIPSP frequency (reduction in sIPSP IEI) in LF but not MF and HF neurons suggests that presynaptic LTK currents may have contributed to the changes in IPSP amplitude in LF neurons. We tested this hypothesis by conducting voltage clamp experiments, which should minimize the influence of postsynaptic LTK currents and therefore allow us to assess to what extent presynaptic LTK currents contributed to DTX-induced changes in the size and shape of the synaptic inhibitory responses. Our data were suggestive of a tonotopic arrangement of presynaptic LTK currents in NL, which is supported by the observation that DTX induced an increase in sIPSC frequency preferentially in LF neurons. Furthermore, DTX caused significant changes in the peak, decay, and amount of charge (Q) of IPSCs and sIPSCs in LF neurons, reflecting a combined effect of both presynaptic and postsynaptic LTK currents on the synaptic inhibition in LF neurons. Fewer parameters of IPSC were affected by DTX in MF neurons, and the effects of DTX on IPSCs in HF neurons were even less significant. These results suggest that presynaptic LTK currents are more prevalent in LF than in MF and HF neurons. A recent study (Yamada et al. 2013) demonstrates that local GABAergic neurons project primarily to LF neurons. It remains to be determined whether these GABAergic terminals express stronger LTK channels than those that originate from the SON.
Presynaptic mechanisms can be confirmed with analysis of variability (1/CV²) of peak amplitude of evoked synaptic responses and paired pulse ratio (PPR) (del Castillo and Katz 1954; Oleskevich et al. 2000; Scheuss et al. 2002). PPR paradigm, however, is not effective in studying synaptic inhibition of NL neurons due to dramatic fluctuations in IPSC peak amplitude under control conditions (Kuo et al. 2009; Tang et al. 2013). Analyses of 1/CV² of IPSPs (Fig. 2.6H, 2.6I) and IPSCs (Fig. 2.8J) did not show significant differences in the percent change of 1/CV² caused by DTX between CF regions. However, DTX tended to substantially reduce 1/CV² in LF neurons while slightly increasing 1/CV² in MF and HF neurons (Figs. 2.6, 2.8), suggesting that presynaptic LTK currents preferentially influence inhibitory synaptic input in LF neurons. We speculate that there may be a tonotopic arrangement of presynaptic LTK currents opposing the postsynaptic arrangement of LTK currents. In other words, postsynaptic LTK currents are largest in HF neurons while presynaptic LTK currents are largest in LF neurons. To support this hypothesis, further research will need to demonstrate the presence of LTK-subfamily channels (e.g.; Kv1, Kv4, Kv7) on inhibitory synapses in the NL.

The presence of presynaptic LTK channels on inhibitory terminals of LF neurons provides an intriguing possibility for interplay between these two neuronal properties that may be critical for forming fast inhibition in LF neurons. The fast kinetics of IPSCs in LF NL neurons can be attributed to both a presynaptic release profile with strong synchronization (Tang and Lu 2012) and a postsynaptic enrichment of the fast α1-GABA_A receptor subunit (Yamada et al. 2013). We propose that in addition to these
mechanisms, presynaptic LTK currents also contribute to accelerating IPSCs in LF neurons. In fact, Kv1-containing channels have been shown to regulate presynaptic spiking activity in a variety of structures, including the cerebellum, hippocampus, and motor nerve (for review see: Trussell and Roberts 2008). This suggests that LF neurons may utilize similar mechanisms including intrinsic voltage-gated conductances and fast synaptic inhibition to code ITD as observed in mammalian medial superior olive neurons (Grothe and Sanes 1993; 1994; Brand et al. 2002; Dodson et al. 2002, 2003; Grothe 2003; Roberts et al. 2013). While presynaptic LTK currents may regulate fast phasic inhibition in LF neurons, postsynaptic LTK currents prevent GABA-induced excitation. In NM neurons, GABA-driven excitation is prevalent in E14 chicks, but decreases during development and becomes predominantly inhibitory at E18. The decrease in GABA-induced excitation coincides with the increase in LTK currents (Howard et al. 2007). The same principle may apply to the maturation of GABAergic inhibition in NL neurons. The LTK currents are thus critical not only in switching the sign of GABA inputs from excitation to inhibition but also maintenance of synaptic inhibition in coincidence detector neurons. The interactions between these two critical neuronal properties along the tonotopic axis help create optimal ITD coding strategies dependent upon the frequency of the auditory inputs.
CHAPTER III

INTRINSIC PLASTICITY INDUCED BY GROUP II METABOTROPIC GLUTAMATE RECEPTORS VIA ENHANCEMENT OF HIGH THRESHOLD Kv CURRENTS IN AVIAN SOUND LOCALIZING NEURONS

Neuromodulation by G-protein coupled receptors (GPCRs) is an important component of neuronal communication that regulates cellular properties. Signaling via GPCRS ultimately governs epiphenomial processes such as sensory processing and behavior of an animal. Presynaptic GPCRs regulate neurotransmission by modulating ion channels and vesicular release machinery. (review in Starke et. al. 1989; Wu and Saggau 1997; Engelman and MacDermott 2004; Catterall and Few 2008). Recently, a growing body of research has begun to elaborate on the importance of postsynaptic GPCRs and their role in intrinsic plasticity, a process through which postsynaptic neuronal properties are regulated dynamically in response to sensory stimuli and behavioral training (review in Zhang and Linden 2003; Brown and Kaczmarek 2011; Kourrich et al. 2015).

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As the predominant excitatory neurotransmitter, glutamate acts on ionotropic receptors as well as on G protein-coupled metabotropic glutamate receptors (mGluRs). Presynaptic mGluRs, functioning as autoreceptors at glutamatergic synapses, or as heteroreceptors at non-glutamatergic synapses, modulate synaptic transmission and thus influence auditory processing in each and every level of the auditory system (review in Lu 2014). In timing-coding neurons in the avian auditory brainstem, presynaptic mGluRs induce short- and long-term plasticity of the inhibitory inputs to the cochlear nucleus magnocellularis (NM) (Lu 2007; Tang et al. 2013). In neurons of the nucleus laminaris (NL) where the sound localization cue interaural time difference (ITD) is first computed by detecting converging glutamatergic inputs from the NM, presynaptic mGluRs have been shown to improve coincidence detection via modulation of both the inhibitory (Tang et al. 2009; Tang and Lu 2012) and excitatory inputs to NL (Okuda et al. 2013). However, very little is known about whether and how postsynaptic mGluRs modulate the intrinsic properties of these neurons.

One intrinsic postsynaptic property of great interest in the timing-coding neurons is the voltage-gated potassium (K_V) channels, the protein expression of which in auditory brainstem timing-coding neurons is possibly the strongest among brain tissues. The low threshold and the high threshold K_V (LTK and HTK) components are critical intrinsic determinants of the generation and repolarization of action potentials (APs) respectively (review in Bean 2007), and both are particularly important for neurons that encode temporal information of auditory stimuli (review in Trussell 1999; Johnston et al. 2010). The LTK currents activate at membrane voltages near the resting membrane potential,
suppressing excitability and creating a narrow time window during which membrane depolarization can reach the spike threshold (review in Golding 2012). In contrast, the HTK currents activate at membrane voltages near the peak of an AP, narrowing spike width and fastening membrane repolarization and allowing a cell to fire repetitively at high discharge rates (review in Rudy and McBain 2001). These currents are largely produced by \( K_{V3} \)-subunit containing channels and are prominent in cells that fire APs at high frequencies in the auditory brainstem including NL neurons (Parameshwaran et al. 2001; Parameshwaran-Iyer et al. 2003; Lu et al. 2004). Although multiple \( K^+ \) conductances, such as \( Ca^{2+} \)-activated \( K^+ \) channels, \( K_{V4} \)-containing channels, and \( K_{V3} \)-containing channels can contribute significantly to the fast repolarization of neuronal membrane (review in Bean 2007), in NL neurons, \( K_{V3} \)-containing channels are the primary player in AP repolarization (Gao and Lu 2008). Here, we examined the modulatory role of mGluRs on postsynaptic \( K_{V} \) currents of NL neurons, with a focus on group II mGluRs (mGluR II), which have been shown to induce intrinsic plasticity via modulating voltage-gated \( Ca^{2+} \) channels (review in Zhang and Linden 2003), and have well-characterized presynaptic effects on synaptic transmission in the NL (Tang et al. 2009; Tang and Lu 2012; Okuda et al. 2013). We found that activation of mGluR II directly influenced their firing properties, leading to an improved ability to follow high frequency inputs with greater reliability.
Methods

Slice preparation and in vitro conventional whole-cell recordings (WCR)

Brainstem slices (250–300 μm in thickness) were prepared from chick embryos E17–E18 as described previously (Tang et al. 2009). The ice-cold artificial cerebrospinal fluid (ACSF) used for dissecting and slicing the brain tissue contained the following (in mM): 250 glycerol, 3 KCl, 1.2 KH₂PO₄, 20 NaHCO₃, 3 HEPES, 1.2 CaCl₂, 5 MgCl₂, and 10 dextrose, pH 7.4, when gassed with 95% O₂ and 5% CO₂. The procedures have been approved by the Institutional Animal Care and Use Committee at Northeast Ohio Medical University, and are in accordance with National Institutes of Health policies on animal use. Slices were incubated at 34–36°C for approximately 1 h in normal ACSF containing the following (in mM): 130 NaCl, 26 NaHCO₃, 3 KCl, 3 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, and 10 dextrose, pH 7.4. For recording, slices were transferred to a 0.5 mL chamber mounted on an upright BX51 microscope (Olympus) with a 40x water-immersion objective. The chamber was continuously superfused with ACSF (1–2 mL/min) by gravity. Recordings were performed at room temperature (23-25 °C).

Patch pipettes were drawn on an Electrode Puller PP-830 (Narishige) to 1–2 μm tip diameter using borosilicate glass micropipettes (inner diameter of 0.86 mm; outer diameter of 1.60 mm) (VWR Scientific). Placement of recording electrodes was controlled by a micromanipulator NMM-25 (Narishige). The electrodes had resistances between 2 and 4 MΩ when filled with a solution containing the following (in mM): 105 K-gluconate, 35 KCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 4 ATP-Mg, and 0.3 GTP-Na, with
pH of 7.2 (adjusted with KOH) and osmolarity between 280 and 290 mOsm/L. The Cl⁻ concentration (37 mM) in the internal solution approximated the physiological Cl⁻ concentration in NL neurons (Tang et al., 2009). The liquid junction potential under conventional WCR was 10 mV, and data were corrected accordingly. In voltage clamp WCR, series resistance (Rₛ) averaged at 6-8 MΩ, and was compensated by 65-75%. Cells were clamped at a membrane potential of -60 mV. All experiments were conducted using an Axopatch 200B amplifier (Molecular Devices). Data were low-pass filtered at 10 kHz and digitized with a Data Acquisition Interface ITC-18 (InstruTECH) at 50 kHz. Recording protocols were written and run using the acquisition and analysis software AxoGraph X (AxoGraph Scientific).

**Establishment of perforated patch clamp recordings**

Dialysis of intracellular soluble signaling molecules has been well understood to be a disadvantage of conventional WCR, particularly when studying GPCR function (Horn and Marty 1988; Neher 1988; Falke et al. 1989). We performed perforated patch clamp recordings (PPCR), using nystatin (100 µg/mL) or escin (40 µM) as the perforating substance, in order to preserve the intracellular environment and prevent possible intracellular dialysis of signaling molecules required for mGluR effects. The tip (first 50-100 µm) of the electrode was filled with normal internal solution and then backfilled with the internal solution containing the perforating antibiotic. Internal solutions were discarded after 2 hrs due to decay of perforating substance.
Given the nature of voltage clamp and space clamp errors in neurons with dendrites (Häusser 2003; Poleg-Polsky and Diamond 2011), particularly in recording modes with inevitable high series resistance ($R_S$), such as perforated patch clamp (Rall and Segev 1985; Bar-Yehuda and Korngreen 2008), establishment of a consistent and reliable PPCR configuration is critical for comparisons of current measurements before and after an experimental manipulation (e.g., drug application or electrical stimulation of presynaptic terminals). Maintenance of PPCR without break-in into WCR ensures that the composition of the cytosol is intact (Chung and Schlichter 1993; Yawo and Chuhma 1993; Strauss et al. 2001). Several steps were taken to obtain and maintain PPCR. First, we carefully monitored the uncompensated current responses to a -5 mV test pulse. A gradual decrease in access resistance ($R_a$) indicated possible successful perforation, while a sudden reduction in $R_a$ indicated WCR mode. Second, we included a non-membrane permeable dye Lucifer Yellow (LY, 5 mM) in the recording electrodes, so if the membrane ruptured during recording we could visually see the dye inside the neuron (Fig. 3.1A). In this case, the data were discarded. Finally, $K_V$ currents recorded in ACSF showed no differences compared to the $K_V$ currents recorded after bath application of a cocktail of drugs to block Na$_V$ channels, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (which mediate the $I_{h}$ current), AMPA receptors, and GABA$_A$ receptors with tetrodotoxin (TTX, 1 µM), 4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD7288, 80 µM), 6,7-Dinitroquinoxaline-2,3-dione (DNQX, 50 µM), and 6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (gabazine, 20 µM). This indicated that time- and voltage-dependent
changes in membrane conductance, if any, under the PPCR configuration, did not alter the $K_V$ currents of interest (Fig. 3.1B, n = 8). Therefore, in all the subsequent PPCR experiments, the cocktail of blockers (TTX, ZD7288, DNQX, and gabazine) was applied.

Under PPCR, cells were clamped at a membrane potential of -50 mV, without correction of junction potential. A number of considerations need to be made in order to correct for junction potentials in perforated patch configuration, including a possibility for a Donnan potential to develop in these conditions, or even no potential at all after the first 30s of obtaining a perforated configuration (Horn and Marty 1988; Kim and Trussell 2007). In fact, Kim and Trussell (2007) found that in gramicidin perforated recordings, the junction potential was likely negligible (approximately 1.4 mV in their study). In our voltage clamp PPCR experiments, series resistance ($R_s$) averaged at 31.1-36.5 MΩ, and was compensated by 60-70%.

**Drugs**

All chemicals were purchased from Sigma-Aldrich except for ZD7288 and gabazine, which were obtained from Tocris, DNQX, 3’,4’,5’,6’-Tetrahydroxyspiro[isobenzofuran-1(3H),9’-(9H)xanthen]-3-one (Gallein), 3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (Go6983), and 1-[6-[[17β]-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), which were obtained from Abcam, and Lucifer Yellow-Li salt, which was obtained from Biotium.
Identification of tonotopic characteristic frequency (CF) regions

Due to dramatic tonotopic differences of various intrinsic properties of neurons in the NL (review in Wang et al. 2010), we paid close attention to the CF region from which each neuron was recorded. However, it is not possible to precisely define the CF of NL neurons in an in vitro slice preparation. Therefore, to categorize neurons into low-, middle-, and high-frequency (LF, MF, and HF) regions, we adapted an approach modified from Kuba et al. (2005), by using the rostral-caudal and medial-lateral position as an indicator of CF. Generally 5 slices of brainstem tissue containing relevant nuclei were collected, where slice #1 was most caudal and slice #5 was most rostral. CF region was also determined by the presence of a monolayer of cells, which is characteristic of MF and HF but not LF regions (Rubel and Parks 1975).

Data processing and analysis

For $K_v$ current analysis, leak subtraction was implemented offline using a linear regression to negative voltage steps (-80 to -65 mV) for all experiments where HCN channels were blocked. The presence of $I_h$ created nonlinearities in the current measurement around resting membrane potential (RMP), making linear-regression based leak subtraction difficult. Therefore, in one set of experiments (Fig. 3.4), where the effects of endogenous glutamate on mGluRs and $K_v$ currents were studied, $I_h$ was not blocked and leak subtraction was not performed.
The RMP was constantly monitored during experiments. The input resistance ($R_{in}$) was calculated from the voltage responses to a somatic current injection (-50 pA), using Ohm’s Law. Voltage activation curves were fitted by a Boltzmann function to reveal the voltage activation properties, where $G$ is the conductance at a given voltage, $G_{max}$ is the maximum conductance during the recording, $E$ is the holding potential (a.k.a., voltage command or $V_{command}$), $V_{1/2}$ is the voltage where half of the conductance is activated, and $K$ is the slope factor.

$$G/G_{max} = \frac{1}{1 + e^{-(E+V_{1/2})/K}}$$

Current activation was measured by a single exponential function in which $A$ stands for current amplitude, $t$ for time and $\tau$ for time constant.

$$f(t) = A*e^{(-t/\tau)}$$

Data were processed using custom written script for MATLAB (v13, MathWorks). Data were analyzed using PASW (v18, SPSS) and plotted using Igor Pro (v6.01, Wavemetrics) Mean and SEM were reported and plotted. Correlations were determined using Pearson's regression analysis. Statistical differences were determined by either paired t-tests, one way Analysis of Variance (ANOVA), or repeated measures Analysis of Variance (RM-ANOVA), using a Greenhouse-Geisser correction. When significant differences were observed in an ANOVA a Tukey's HSD post hoc analysis was conducted to elucidate individual group differences, and when significant differences were observed in an RM-ANOVA, a Bonferroni-corrected paired-comparison was conducted for individual sample comparisons.
Results

**PPCR uncovers $K_V$ modulation by mGluR II**

After establishing stable perforated recordings (Fig. 3.1A, 3.1B), we sought to examine the effects of mGluR agonism on $K_V$ currents. A robust increase in $K_V$ currents was observed following bath application of DCG-IV (2 µM), a selective mGluR II agonist, in NL neurons across all CF regions in PPCR ($n = 16$) but not in conventional WCR ($n = 8$) configuration (Fig. 3.1C, 3.1D). The DCG IV-sensitive $K_V$ current activated at relatively positive voltage commands (about -30 mV), with little to no currents at voltage commands close to RMP (about -60 mV for NL neurons; Fig. 3.1D inset). This strongly suggests that DCG-IV caused an increase in HTK currents.

The detection of mGluR-mediated modulation of $K_V$ currents under PPCR but not WCR may be interpreted by either of the two following scenarios. First, the relatively high $R_S$ and $R_a$ during PPCR may lead to voltage- and space-clamp errors that change over the course of our recording. To confidently exclude this possibility, we compared $R_{in}$ and $R_S$ between control and DCG-IV conditions, because changes in $R_{in}$ and $R_S$ can indicate changes in $R_a$, which was expected to be stable throughout the duration of PPCR. We observed stable $R_{in}$ (ctr: 86.4 ± 7.6 MΩ, DCG-IV: 83.4 ± 9.9 MΩ) and $R_S$ (ctr: 34.3 ± 2.2 MΩ, DCG-IV: 34.1 ± 2.1 MΩ) across recordings before and after DCG-IV application (Fig. 3.1E, $n = 16$). To ensure the stability of $R_S$ during PPCR, we continuously monitored the current responses to a test pulse (-5 mV) in voltage clamp mode. Cells with a change exceeding 15% in the decay time constant (approximation of
**Figure 3.1** Perforated patch clamp recordings (PPCR) but not conventional whole-cell recording (WCR) uncovers K\textsubscript{V} modulation by mGluR II. (*A*), *top*: Sample cell that spontaneously ruptured into WCR configuration. Note the sudden jump increase in the test pulse response and filling of cell with the fluorescent dye Lucifer Yellow (LY). *bottom*: Sample cell that had perforated and maintained stable perforation configuration. Note the gradual increase in the test pulse response and absence of LY inside the cell. The soma of the recorded cell is delineated by the line. (*B*), Voltage clamp recordings before (black) and after (orange) bath application of a cocktail of blockers to isolate K\textsubscript{V} currents revealed that stable PPCR was attainable. The subtraction trace (grey) and current-voltage (IV) curves show no change in steady state K\textsubscript{V} currents revealed that stable PPCR was attainable. The subtraction trace (grey) and current-voltage (IV) curves show no change in steady state K\textsubscript{V} currents (n = 8). (*C*), Sample conventional WCR of K\textsubscript{V} currents using the protocol shown on the left (V\textsubscript{hold} = -50 mV, V\textsubscript{command} = -70 to +5 mV for PPCR and -80 to -5 mV for WCR, 100 ms duration). Steady state K\textsubscript{V} IV curves before (black) and after mGluR II agonist DCG-IV (2 µM, green) application were not different (n = 8). (*D*), Sample PPCR of K\textsubscript{V} currents before, during, and after DCG-IV (2 µM) application. Steady IV curve show that DCG-IV increased K\textsubscript{V} currents. *inset*: IV curve of DCG-sensitive K\textsubscript{V} currents (n = 16). (*E*), DCG-IV application did not change input resistance (R\textsubscript{in}) or series resistance (R\textsubscript{s}) of perforated NL neurons (n = 16). (*F*), The percent change in membrane time constant (\(\tau\text{M}\)) is not correlated to the percent increase in K\textsubscript{V} currents (n = 16, r = 0.047, p = 0.862). All the data in the rest of the study, except for those in Figure 3.2, were obtained with PPCR. In this and subsequent figures, Means ± SEM are shown. *, **, and *** indicate p < 0.05, p < 0.01, and p < 0.001, respectively.
τ_M) of the test-pulse evoked current before and after DCG-IV were discarded. More importantly, we found no correlation between changes in τ_M and changes in maximum change in K_V currents (Fig. 3.1F; n = 16, r = 0.047, p = 0.862). This indicates that within the accepted level (<15%) for alteration in R_S, the modulation of K_V was not attributed to voltage- or space-clamp errors due to changes in R_a. Therefore, we considered the second scenario, which is the possible washout, during WCR, of one or multiple critical signaling molecules required for second messenger system (Trussell and Jackson 1987; Horn and Marty 1988; Vargas et al. 1999). Supporting this notion, perforated but not conventional WCR, disclosed mGluR-mediated modulation of K^+ currents (e.g.; leak K^+ currents, delayed rectifying outward currents, and Ca^{++}-activated K^+ currents), in a large variety of neurons (e.g. Shirasaki et al. 1994; Takeshita et al. 1996; Katayama et al. 2003; Zhang et al. 2015). Therefore, it is likely that under our WCR configuration dialysis of one or more important components for mGluR II intracellular signaling pathway occurred, resulting in the observation of no modulation in K_V currents.

**MF and HF neurons have stronger K_V currents than LF neurons**

Prior to further determining the mechanism and function of mGluR-mediated modulation of K_V currents, it is important to first address the tonotopic distribution of K_V currents in the NL. Anatomical and physiological evidence has shown that low threshold K_V (LTK) currents, mediated by K_V1-subunit containing channels, are strongly expressed and highly active in the MF and HF regions, with less expression and activity in the LF region (Kuba et al. 2005; Hamlet et al. 2014). Furthermore, anatomical evidence has
shown that Kv3-subunit containing channels in NL neurons are more densely expressed in the MF and HF regions compared to the LF region, suggesting that HTK currents are also tonotopically distributed in the NL (Parameshwaran et al. 2001). We determined the tonotopic distribution of Kv currents without isolating either component to obtain a more physiologically relevant description of the tonotopy of total Kv currents in the NL. We found that MF neurons had overall larger Kv currents compared to LF and HF neurons (Fig. 3.2A, 3.2B). Kv currents at V_{command} of 0 mV were significantly larger in MF neurons (14.09 ± 2.09 nA, n = 16, p = 0.010) compared to LF neurons (8.02 ± 1.04 nA, n = 12). HF neuron Kv currents (9.93 ± 0.91 nA, n = 16) were slightly but significantly larger than LF neurons at 0 mV (p = 0.049), while no difference was observed between MF and HF neurons. However, it is important to account for the tonotopic gradient of membrane area along the frequency-coding axis due to differences in dendritic length and size of NL neurons. LF neurons have the longest dendrites, and the dendritic length decreases with increasing CF (Rubel and Parks 1975; Smith and Rubel 1979). This leads to a corresponding gradient in membrane capacitance (C_m), with large C_m in LF neurons and small C_m in HF neurons. Therefore, we normalized Kv currents to C_m, deriving the Kv current density in LF, MF, and HF neurons. Kv current density measurements showed the same trend as the total current amplitude. Kv current density at 0 mV in MF (0.303 ± 0.039 nA/pF, p = 0.001) and HF (0.270 ± 0.025 nA/pF, p = 0.026) neurons was significantly higher than in LF (0.16 ± 0.03 nA/pF) neurons (Fig. 3.2C). Finally, the activation kinetics of Kv currents across the tonotopic axis were assessed using G/G_{max}
Figure 3.2 MF and HF neurons have stronger $K_V$ currents than LF neurons. (A) Sample recordings of $K_V$ currents from LF, MF, and HF NL neurons. (B) IV curves reveal that MF neurons have larger $K_V$ currents than LF but not HF neurons. Inset: MF neurons (14.09 ± 2.09 nA, n = 16) and HF (9.93 ± 0.91 nA, n = 16) neurons have significantly more $K_V$ current than LF (8.21 ± 1.04 nA, n = 12) ($p = 0.010$, $p = 0.049$, respectively) while no differences were observed between MF and HF neurons. (C) Current density of MF and HF neurons is larger than that of LF neurons. Inset: At $V_{\text{command}} = 0$ mV, MF (0.303 ± 0.039 nA/pF) and HF (0.270 ± 0.025 nA/pF) neurons have significantly larger current densities than LF (0.160 ± 0.028 nA/pF) neurons ($p = 0.001$ and $p = 0.026$, respectively). (D) Current activation plots reveal that $K_V$ currents of MF and HF neurons are activated at more hyperpolarized potentials than LF neurons. Inset: MF (-18.9 ± 2.6 mV) and HF (-21.7 ± 3.2 mV) neurons have significantly more hyperpolarized $V_{1/2}$ than LF (-2.6 ± 3.9 mV) neurons ($p < 0.001$ and $p = 0.001$, respectively).
plot fitted to a Boltzmann function to reveal $V_{1/2}$, the voltage at which half of the $K_V$ conductance is active. $V_{1/2}$ of MF (-18.9 ± 2.6 mV, $p = 0.001$) and HF (-21.7 ± 3.2 mV, $p < 0.001$) neurons was significantly more hyperpolarized than that of LF neurons (-2.6 ± 3.9 mV) (Fig. 3.2D), consistent with previous reports (Kuba et al. 2005; Hamlet et al. 2014). Together these data show that $K_V$ currents in MF and HF neurons are not substantially different but are significantly larger in amplitude and kinetics than those in LF neurons.

**mGluR II enhancement of $K_V$ currents is most prominent in LF neurons**

Okuda et al. (2013) reported a tonotopic distribution of mGluR II in posthatchling chicks (P1-P4), showing that LF neurons had as much as a 5-fold higher intensity of immunohistochemistry staining compared to MF and HF neurons. Because we used animals of different age (E17-18), we confirmed the expression of mGluR II in the NL by using a polyclonal antibody specific for both members of mGluR II (mGluR2 and mGluR3). We also found dense staining of mGluR II in the LF region with less immunoreactivity in the MF and HF regions of E18 chick embryos (data not shown). MF and HF neurons had similar staining intensity, so we compared the amount of mGluR II-enhancement of $K_V$ currents between LF neurons ($n = 9$) and MF and HF neurons ($n = 7$, Fig. 3.3). We observed increased $K_V$ currents following DCG-IV application in 16 out of 17 neurons (LF, $n = 9$ of 9; MF and HF, $n = 7$ of 8). A predominantly high threshold current was enhanced by DCG-IV (Fig. 3.3A-D). Specifically, in LF neurons, a significant increase in $K_V$ currents was observed at $V_{\text{command}} (V_C)$ of -5 mV (ctr: 0.58 ±
0.12 nA, DCG-IV: 1.72 ± 0.22 nA, \( p = 0.002 \) and +5 mV (ctr: 1.67 ± 0.24 nA, DCG-IV: 3.95 ± 0.28 nA, \( p < 0.001 \)), but not at -30 mV (ctr: 0.13 ± 0.05 nA, DCG-IV: 0.24 ± 0.08 nA; Fig. 3.3B). A similar but less dramatic enhancement of \( K_V \) currents was observed in MF and HF neurons. There was a significant increase in \( K_V \) currents at \( V_{\text{command}} \) of -5 mV (ctr: 2.21 ± 0.34 nA, DCG-IV: 2.75 ± 0.37 nA, \( p = 0.034 \)) and +5 mV (ctr: 3.04 ± 0.50 nA, DCG-IV: 3.78 ± 0.58 nA, \( p = 0.009 \)), while no significant differences were observed at -30 mV (ctr: 0.50 ± 0.19 nA, DCG-IV: 0.69 ± 0.20 nA; Fig. 3.3C, 3.3D).

To examine the properties of the DCG-IV-sensitive \( K_V \) current, we subtracted the \( K_V \) currents recorded before DCG-IV from the \( K_V \) currents recorded after DCG-IV application in LF neurons (Fig. 3.3E). The DCG-IV-sensitive current appeared to be relatively slow, as shown by the activation \( \tau \) (16.3 ± 6.3 ms, \( n = 7 \)) calculated from the currents activated at -5 mV (Fig. 3.3F). It should be noted that the speed of activation as well as the amplitude of the mGluR-sensitive current would likely be increased under physiological temperatures (Kiernan et al. 2001; Cao and Oertel 2005). We compared the mGluR-enhancement of \( K_V \) currents in LF to the \( K_V \) enhancement in MF/HF neurons to assess to what extent this modulation varied across the tonotopic axis. Comparison of the percent change in \( K_V \) currents revealed that there was a significantly larger increase after DCG-IV application in LF compared to MF/HF neurons at the most depolarized voltage commands, -5 mV (LF: 64.0 ± 14.7%, MF/HF: 14.4 ± 6.7%, \( p = 0.014 \)) and +5 mV (LF: 82.7 ± 14.9%, MF: 17.7 ± 7.8%, \( p = 0.003 \)) but not at -30 mV (LF: 14.9 ± 13.6%, MF/HF: 6.7 ± 8.6%; Fig. 3.3G). These data show that mGluR-II-enhancement of HTK currents is most prominent in the LF region.
Figure 3.3 mGluR II enhancement of K\textsubscript{V} currents is most prominent in LF neurons. (A) DCG-IV (2 µM) increased K\textsubscript{V} currents in LF neurons (n = 9). (B) The predominant change in K\textsubscript{V} currents was observed in the most depolarized voltage commands, indicating a high threshold K\textsubscript{V} (HTK) current was affected by DCG-IV. (C) DCG-IV produced a slight increase in K\textsubscript{V} currents in MF and HF neurons (n = 7). (D) The change in K\textsubscript{V} currents in MF and HF NL neurons was significantly increased at V\textsubscript{command} of -5 and +5 mV but not at -30 mV. (E) The DCG-IV-sensitive current (DCG-IV K\textsubscript{V} currents minus control K\textsubscript{V} currents) has noticeable activation at about -20 mV, further confirming it is the HTK component. (F) Activation τ at V\textsubscript{command} of -5 mV reveals the DCG-IV-sensitive current is relatively slow (16.3 ± 6.3 ms, n = 7), indicating it is a delayed rectifying current. (G) Percent change in K\textsubscript{V} was significantly larger for LF (open bars) compared to MF/HF (filled bars) neurons at V\textsubscript{command} of -5 and +5 mV but not at -30 mV.
Endogenous glutamate enhances of $K_V$ currents in NL neurons

The enhancement of $K_V$ currents by pharmacological activation of mGluR II is interesting, but it is necessary to know whether the modulation is physiologically relevant. To address this issue, we electrically activated the glutamatergic input to NL from the contralateral NM by a bipolar tungsten electrode placed at the midline of the slice where the contralateral NM fibers travel, and examined the effects on $K_V$ currents in LF neurons under PPCR (Fig. 3.4A). Electron microscopy data have shown that mGluR II tend to be located distally from glutamate release sites in a large variety of neurons (Oshigi et al. 1998; Tamaru et al. 2001), such as the hippocampus (Shigemoto et al. 1997), spinal cord (Tang and Sim 1999), and cerebellar cortex (Ohishi et al. 1994), indicating that these receptors are expressed extrasynaptically. Furthermore, work from our lab has demonstrated that activation of mGluR II by endogenous glutamate in the NL in brain slice preparations is facilitated by blocking glutamate reuptake (Tang et al. 2009), suggesting that mGluR II are located distal from the active zone, thus being active when glutamate spillover occurs. Therefore, we bath applied TFB-TBOA (1 µM), a blocker of astrocyte glutamate reuptake, to reduce potential washout of synaptically released glutamate during perfusion and to enhance the likelihood of detecting any effect the released glutamate may have on $K_V$ currents. Gabazine (20 µM) was applied to block inhibitory conductances. Once we confirmed that stimulation of the contralateral fibers produced an EPSC in the recorded neuron, we then recorded whole-cell currents, without blocking most ionic conductances (e.g. $Na_V$, $I_h$, AMPAR current), and measured the steady state $K_V$ current. To potentially maximize the release of endogenous glutamate, we
applied a train stimulation (500 Hz, 500 pulses) 5 times (with 10 s inter-stimulation interval; ISI), to the contralateral NM fibers (Fig. 3.4B). K_V currents were recorded immediately after the stimulation, and again 5 mins later. Immediately after the stimulation we observed a significant increase in steady state K_V currents, and the currents recovered to baseline 5 mins later (Fig. 3.4C, 3.4D, n = 10). There was a significant difference in K_V currents before, immediately after stimulation, and 5 mins after stimulation at -5 mV (ctr: 0.752 ± 0.073 nA; stimulation: 0.873 ± 0.090 nA; recovery: 0.780 ± 0.085 nA; p = 0.003) and at +5 mV (ctr: 1.124 ± 0.104 nA; stimulation: 1.358 ± 0.132 nA; recovery: 1.164 ± 0.119 nA, p < 0.001) but not at -30 mV (ctr: 0.142 ± 0.023 nA; stimulation: 0.150 ± 0.024 nA; recovery: 0.151 ± 0.022 nA). Bonferroni-corrected pairwise comparisons revealed that there was a significant increase in K_V at -5 mV and +5 mV (p = 0.006, p = 0.001, respectively) after stimulation and recovery of K_V currents at both holding potentials (p = 0.05, p = 0.003, respectively) approximately 5 mins after stimulation (Fig. 3.4E). These data demonstrate that endogenously released glutamate is sufficient to enhance steady state K_V currents. Moreover, the increased K_V current is similar to that observed by pharmacological mGluR II activation in that the enhancement was most pronounced at the most depolarized voltage commands, suggestive of an enhanced HTK current.

**Signaling molecules involved in mGluR II enhancement of K_V currents**
Figure 3.4 Endogenous glutamate enhances K_V currents in NL neurons. (A) Schematic showing the auditory circuit in the chicken brainstem, and the placement of the stimulating and recording electrodes. NA: nucleus angularis; NM: nucleus magnocellularis; SON: superior olivary nucleus. (B) Sample voltage clamp recording (V_{hold} = -50 mV) of EPSCs in response to train stimulation (500Hz, 500 pulses), with the response to the first 10 pulses of the train stimulation shown at an enlarged time scale. (C) Sample recording from a neuron before (black), immediately after (blue), and 5 mins (grey) after 5 repetitions of the train stimulation (10 s inter-stimulation interval, ISI). An overlay of the currents at V_{command} of +5 mV is shown on the right. (D) IV curves show an increase in the steady state K_V currents immediately after the stimulation, and recovery to baseline levels in 5 mins (n = 10). Inset: The stimulation-sensitive K_V current, obtained by subtracting the baseline recordings (black) from the post-stimulation recordings (blue). (E) There was a significant increase in the steady state K_V currents immediately after the stimulation at -5 mV and +5 mV, and the currents returned to the baseline level 5 min after the stimulation was terminated.
To further support the hypothesis that activation of mGluR II was responsible for the increase in HTK currents, we applied an mGluR II-selective antagonist, LY341495 (1 µM), prior to application of DCG-IV. LY341495 eliminated the enhancement of $K_v$ currents by DCG-IV (Fig. 3.5A, n = 4), with unchanged $K_v$ currents at $V_{\text{command}}$ of -30 mV (ctr: 0.26 ± 0.04 nA, DCG-IV: 0.27 ± 0.05 nA), -5 mV (ctr: 1.00 ± 0.22 nA, DCG-IV: 1.03 ± 0.23 nA), and +5 mV (ctr: 1.47 ± 0.41 nA, DCG-IV: 1.39 ± 0.34 nA) (Fig. 3.5B). GPCRs can modulate intrinsic currents via the $G_{\alpha}$ subunit or $G_{\beta/\gamma}$ complex (review in Hille 1994; Dascal 2001). Application of the cell permeable $G_{\beta/\gamma}$ blocker gallein (5 µM) to brain slices for 30 mins was sufficient to block $G_{\beta/\gamma}$ complex activation (Lehmann et al. 2008; Belkouch et al. 2011; Seneviratne et al. 2011). Therefore, we incubated slices in gallein (5 µM) for >30 mins prior to recording $K_v$ currents. No changes in $K_v$ currents were observed after DCG-IV application in the presence of gallein (Fig. 3.5C, 3.5D, n = 4), at $V_{\text{command}}$ of -30 mV (ctr: 0.40 ± 0.13 nA, DCG-IV: 0.46 ± 0.19 nA), -5 mV (ctr: 2.48 ± 0.79 nA, DCG-IV: 2.68 ± 0.97 nA), and +5 mV (ctr: 3.59 ± 1.15 nA, DCG-IV: 3.47 ± 1.12 nA).

The $G_{\beta/\gamma}$ complex can activate various signaling pathways including phospholipase C (PLC) (review in Clapham and Neer 1997; Smrcka 2008). Therefore, we attempted to determine whether PLC activity was involved in the mGluR-enhancement of $K_v$ currents by incubating slices in cell-permeable PLC blocker, U73122 (10 µM), 10 mins before and during DCG-IV application. When DCG-IV was applied in the presence of U73122, there was no significant change in $K_v$ currents (Fig. 3.5E, 3.5F; n = 7) at -30 mV (ctr: 0.36 ± 0.07 nA, DCG-IV: 0.38 ± 0.07 nA), -5 mV (ctr: 0.90 ± 0.10 nA, DCG-
IV: 0.93 ± 0.11 nA), or +5 mV (ctr: 1.17 ± 0.12 nA, DCG-IV: 1.24 ± 0.17 nA), suggesting that the Gβγ complex may activate PLC to enhance HTK currents. One way PLC may influence Kv currents is via protein kinase C (PKC) because PLC is known to modulate PKC activity via degradation of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Previous work on Kv modulation in the mammalian sound localization circuit, as well as in many other brain regions, has shown that PKC can modulate HTK currents (Atzori et al. 2000; Sun et al. 2003), including HTK currents mediated by Kv3.1 channels (Critz et al. 1993; Liu and Kaczmarek 1998; Macica et al. 2003; Song et al. 2005; Song and Kaczmarek 2006). To test whether PKC was involved, slices were pre-incubated for at least 30 mins in a cell permeable PKC blocker, Go6983 (20 nM), which non-selectively blocks all PKC subunits with the exception of PKCµ (Gschwendt et al. 1996; Peterman et al. 2004). Application of Go6983 before and during DCG-IV exposure prevented Kv modulation (Fig. 3.5G, 3.5H, n = 7), with no significant changes observed at Vcommand of -30 mV (ctr: 0.37 ± 0.08 nA, DCG-IV: 0.36 ± 0.10 nA), -5 mV (ctr: 1.05 ± 0.17 nA, DCG-IV: 1.01 ± 0.13 nA), or +5 mV (ctr: 1.42 ± 0.23 nA, DCG-IV: 1.37 ± 0.16 nA). This suggests that mGluR II enhancement of Kv currents requires the Gβγ complex pathway associated with PLC and PKC.

**mGluR II affect the intrinsic properties of LF neurons**

To address the functional relevance of the mGluR II enhanced Kv current, it is critical to determine how the increased Kv currents modulate the output of NL neurons.
Figure 3.5 mGluR II enhancement of $K_V$ currents is dependent on $G_{\beta\gamma}$, phospholipase C (PLC), and protein kinase (PKC). (A) mGluR II antagonist LY341495 (5 µM) blocked DCG-IV enhancement of $K_V$ currents ($n = 4$). (B) No change in $K_V$ currents was observed at $V_{command}$ of -30, -5, and +5 mV in the presence of LY341495. (C, D) Blockade of $G_{\beta\gamma}$ subunits using gallein (5 µM) prevented DCG-IV enhancement of $K_V$ currents ($n = 4$). (E, F) Cell permeable PLC blocker U73122 (10 µM) eliminated DCG-IV enhancement of $K_V$ currents ($n = 7$). (G, H) Blockade of PKC using Go6983 (20 nM) prevented the DCG-IV enhancement of $K_V$ currents ($n = 7$).
Because mGluR II exerted their modulation on $K_V$ currents in LF neurons most prominently, we examined voltage deflections and action potentials (APs) in response to current injections in LF neurons. Under current clamp, we injected somatic currents using a prolonged pulse protocol (200 ms pulse duration, $I_{\text{injection}} = -0.10$ to $+0.40$ pA) to evoke subthreshold as well as suprathreshold voltage responses. DCG-IV (2 µM) resulted in little to no changes in the RMP (ctr: $-56.2 \pm 1.2$ mV, DCG-IV: $-55.5 \pm 1.1$ mV, $n = 16$) (Fig. 3.6A). However, we revealed subtle but significant reductions in the outward rectification (ctr: $0.41 \pm 0.05$, DCG-IV: $0.32 \pm 0.04$, $p = 0.040$), reflecting a reduction in membrane voltage to the most positive current injections (Fig. 3.6B). In line with an increase in HTK currents, the absolute maximum rate of fall of APs was significantly increased (ctr: $-116.2 \pm 8.5$ mV/s, DCG-IV: $-133.8 \pm 9.7$ mV/s, $p = 0.017$; Fig. 3.6C), without changing the maximum rate of rise (ctr: $162.9 \pm 18.4$ mV/s; DCG-IV: $171.4 \pm 21.4$ mV/s; data not shown). Spike threshold remained unchanged, in either $I_{\text{threshold}}$ (ctr: $0.196 \pm 0.034$ nA, DCG-IV: $0.201 \pm 0.033$ nA) or in $V_{\text{threshold}}$ (ctr: $26.9 \pm 2.1$ mV, DCG-IV: $27.0 \pm 1.9$ mV) in response to long current pulses (data not shown). To further explore whether DCG-IV changed neuronal excitability, we examined the variability in $I_{\text{threshold}}$ (rheobase) to short pulse current injections (2 ms of duration, $I_{\text{injection}} = +0.20$ to $+0.45$ nA). No differences in the rheobase were observed after DCG-IV application (ctr: $0.357 \pm 0.038$ nA, DCG-IV: $0.347 \pm 0.043$ nA, $n = 6$, data not shown). To examine how DCG-IV modulates individual APs, the AP height and half width at threshold and at $I = 0.25$ nA were analyzed (Fig. 3.6D). No change in AP height was observed (at threshold, ctr: $91.9 \pm 4.5$ mV, DCG-IV: $94.9 \pm 4.2$ mV; at 0.25 nA, ctr: $102.1 \pm 4.6$ mV, DCG-IV: ...
102.0 ± 5.0 mV; Fig. 3.6E), while DCG-IV caused a significant reduction in spike half width at 0.25 nA (ctr: 1.15 ± 0.08 ms, DCG-IV: 1.09 ± 0.09 ms, \( p = 0.010 \)) but not at threshold (ctr: 1.13 ± 0.08 ms, DCG-IV: 1.18 ± 0.13 ms; Fig. 3.6F). In summary, DCG-IV produced subtle but significant changes in membrane rectification, AP rate of fall, and AP half width of NL neurons, without affecting RMP, AP rate of rise, or neuronal excitability. These results are consistent with the selective enhancement of HTK but not LTK currents by mGluR II in NL neurons.

**mGluR II enhances the high frequency following ability of LF neurons**

The enhancement of HTK currents by mGluRs presents a feedforward modulatory mechanism to dynamically regulate the output of neurons, particularly when the neurons are undergoing periods of high activity. One major role of HTK currents is to maintain high frequency firing following ability by allowing a neuron to repolarize faster and thus producing narrow APs (review in Rudy et al. 1999; Rudy and McBain 2001; Johnston et al. 2010). Therefore, we predicted that enhancement of \( K_v \) currents by mGluR II in NL neurons would enable them to fire at high rates due to increased rate of membrane repolarization and reduced AP width. To test this hypothesis, we injected trains of 50, 100, and 200 Hz square current pulses (1 ms pulses, 500 ms train stimulation duration) to the recorded cells and examined the effects of DCG-IV (2 µM) (Fig. 3.7A). The amplitude of the current pulses was set to 10 times the current threshold measured with the long pulse current protocol. Because the HTK currents were increased during DCG-IV application, the relatively slowly activating HTK currents should progressively
Figure 3.6 Effects of mGluR II activation on the intrinsic properties of LF NL neurons.  
(A) Sample membrane potential recordings using a protocol with prolonged somatic current injections (200 ms pulse duration, \(I_{\text{injection}} = -0.1 \) to \(+0.4 \text{ nA}\)). The voltage-current (V/I) curves show that DCG-IV reduced membrane potentials to the most positive current injection (\( n = 16 \)). No change in RMP was observed (ctr: \(-56.2 \pm 1.2 \text{ mV}\), DCG-IV: \(-55.5 \pm 1.1 \text{ mV}\)).  
(B) DCG-IV significantly reduced the rectification index (slope of voltage deflections to depolarized current injections divided by slope of voltage deflections to hyperpolarized current injections).  
(C) The absolute AP rate of fall was significantly increased after DCG-IV application.  
(D) Sample APs at threshold and at \(I = 0.25 \text{ nA}\).  
(E) No significant change in AP height was observed after DCG-IV application.  
(F) DCG-IV reduced AP half-width significantly at \(I = 0.25 \text{ nA}\) (\( n = 16 \)).
decrease AP peaks during high frequency current injections. Indeed, when we compared the height of the first and last AP, we found that DCG-IV decreased AP height significantly at all frequencies tested (Fig. 3.7B-D, left panels): 50 Hz (ctr: -3.7 ± 4.1%, DCG-IV: -12.1 ± 3.0%, p = 0.012), 100 Hz (ctr: -10.2 ± 4.4%, DCG-IV: -22.4 ± 2.6%, p = 0.005), and 200 Hz (ctr: -22.8 ± 4.5%, DCG-IV: -36.1 ± 5.7%, p < 0.001). Additionally, with increased HTK currents, neurons should fire APs more reliably in response to high frequency somatic current injections. To assess this, we examined the number of failures and the variability of time between spikes. DCG-IV significantly decreased the number of failures at all frequencies tested (Fig. 3.7B-D, middle panels): 50 Hz (ctr: 11.3 ± 2.5, DCG-IV: 8.8 ± 2.5, p = 0.013), 100 Hz (ctr: 21.1 ± 3.2, DCG-IV: 13.4 ± 2.9, p = 0.004), and 200 Hz (ctr: 65.8 ± 7.7, DCG-IV: 53.5 ± 7.3, p = 0.023). DCG-IV also significantly reduced the coefficient of variation of inter-spike interval (ISI CV) during train stimulation at 50 Hz (ctr: 0.051 ± 0.020, DCG-IV: 0.030 ± 0.015, p = 0.004) and 100 Hz (ctr: 0.061 ± 0.012, DCG-IV: 0.043 ± 0.009, p = 0.013). A small but consistent reduction in ISI CV at 200 Hz was observed (ctr: 0.146 ± 0.071, DCG-IV: 0.062 ± 0.034, p = 0.057) (Fig. 3.7B-D, right panels). Together, these data further confirm voltage clamp observations that DCG-IV increases an HTK current in NL neurons. The increased HTK current decreased AP height during the course of somatic train stimulation and increased the reliability of an NL neuron to follow high rates of stimulation.
Figure 3.7 Activation of mGluR II enhances ability of LF NL neurons to follow high frequency inputs. (A) Sample membrane potential recordings in response to current injections (2 ms pulse duration, 500 ms train duration) at 50 (50 pulses), 100 (100 pulses), and 200 Hz (200 pulses) before and after DCG-IV application. (B) left: At 50 Hz, significant percent reduction in peak AP amplitude (first minus last AP amplitude) was observed after DCG-IV application (n = 12). middle: There was also a significant reduction in the number of failures after DCG-IV application. right: The coefficient of variation in inter-spike interval (ISI CV) was significantly reduced by DCG-IV. (C) At 100 Hz, similar significant changes in peak AP amplitude, number of failures, and ISI CV were observed. (D) At 200 Hz, there was a significant reduction in peak AP amplitude and the number of failures. The ISI CV remained unchanged (p = 0.057), possibly due to the relatively large variability in control conditions.
Discussion

The results of this study reveal that mGluR II selectively enhance the HTK currents in NL neurons, providing a novel intrinsic feedforward modulatory mechanism regulating postsynaptic neuronal properties in the sound localization circuit. This modulation is most prominent in LF neurons, in which the baseline $K_V$ currents are relatively weaker while the expression of mGluR II is stronger compared to MF/HF neurons. The modulation is dependent on activity of the $G_{\beta\gamma}$ complex, PLC, and PKC. The enhancement of the HTK currents improves NL neuron's ability to follow high frequency inputs. Our discussion below focuses on the tonotopic nature of this modulation, the signaling pathway, and the functional implications.

Tonotopic nature and selective enhancement of the HTK currents by mGluR II

The $K_V$ currents in NL neurons are tonotopically distributed, with MF and HF neurons having stronger baseline $K_V$ currents compared to LF neurons (Fig. 3.2). Interestingly, the HTK but not the LTK component is subject to mGluR II modulation. This observation is unlikely the results of voltage clamp errors. Due to relatively high $R_S$ ($34.3 \pm 2.2 \text{ M}\Omega$) in PPCR (approximately 5-10 M$\Omega$ in WCR) in addition to extensive dendrites in LF neurons, space clamp and voltage clamp errors may have arisen (Häusser 2003; Bar-Yehuda and Korngreen 2008), leading to ambiguous determination of activation voltages for LTK versus HTK currents. However, the LTK currents are strongly active at around RMP in NL neurons (Hamlet et al. 2014), and are readily
activated by small membrane depolarization. At $V_{\text{command}}$ of -30 mV, the LTK currents were expected to be highly active, and no mGluR II modulation was observed. At -5 and +5 mV, the HTK currents were active, and mGluR II enhancement of the currents was significant (Fig. 3.3), clearly indicating that the modulation was selective for the HTK component. This conclusion is further strongly supported by our current clamp recordings. Activation of mGluR II did not change the neuronal properties (e.g., RMP, $I_{\text{threshold}}$, $V_{\text{threshold}}$, and rheobase of excitability) that are regulated by LTK currents (review in Trussell 1999; Johnston et al. 2010; Golding 2012). In contrast, the neuronal properties that are regulated by HTK currents (e.g., membrane outward rectification, AP rate of fall, AP width, and high frequency following ability) (Brew and Forsythe 1995; Wang et al. 1998; Rudy et al. 1999; Rudy and McBain 2001) were significantly altered. Therefore, despite the limitations of voltage control in voltage clamp recordings under PPCR configuration, our data strongly suggest that the HTK channels are the primary modulatory targets of mGluR II in NL neurons.

The tonotopy of $K_V$ currents opposed the tonotopic distribution of mGluR II. The protein expression of mGluR II is the strongest in LF region (Okuda et al. 2013; our unpublished data), where the $K_V$ currents are the weakest but the enhancement by mGluR II is the most prominent. Such a dynamic HTK current in LF neurons was also reported for the mammalian medial nucleus of the trapezoid body (MNTB), a critical nucleus involved in sound localization circuit. In MNTB, while there is no gradient in protein expression of mGluR II (Elezgarai et al. 2001), somatic $K_V3.1$ channels are tonotopically arranged, with LF neurons having weaker $K_V$ currents than HF neurons (Wang et al.
1998; Li et al. 2001; Brew and Forsythe 2005; Leão et al. 2006). Interestingly, sound or electrical activation of afferent fibers to MNTB induces an increase in HTK currents, and the increase is larger in LF neurons (Leão et al. 2010), suggesting that the HTK currents in LF neurons are more dynamical (Song et al. 2005; Leão et al. 2010). Why would LF NL neurons require highly dynamic HTK currents? A plausible interpretation may reside in the patterns of their synaptic inputs. MF and HF neurons possess higher spontaneous synaptic activity than LF neurons in vitro (Kuba et al. 2005; Tang and Lu 2012; Hamlet et al. 2014) and in vivo (Nishino et al. 2008). Thus, at rest (a "quiet" state) LF neurons receive fewer synaptic inputs, and mGluR II activity is presumably low. During sound stimulation (an “active” state), LF neurons receive strong excitatory inputs from NM (Nishino et al. 2008). Therefore, a mechanism may have evolved to allow LF neurons to adapt to the dramatic change in the amount of synaptic inputs. As one key mechanism, mGluR-enhanced Kv currents would enable them to follow the excitatory inputs with greater reliability. In contrast, MF and HF neurons constantly receive relatively more numerous and stronger synaptic inputs, even at rest (Kuba et al. 2005; Nishino et al. 2008; Sanchez et al. 2010; Hamlet et al., 2014), reducing the need for such a dynamic HTK current. These data are in line with the idea that the tonotopic distribution of synaptic inputs may be complemented by tonotopic distribution of mGluR modulation (review in Lu 2014).

A potential mechanism for Kv enhancement by mGluR II
mGluR II can cause intracellular changes through the \( G_{\alpha} \), \( G_{\beta\gamma} \) complex, or a combination of both (review in Niswender and Conn 2010; Nicoletti et al. 2011). While we were able to show that activity of \( G_{\beta\gamma} \), PLC, and PKC are critical factors for mGluR II-enhancement of KV currents, the interactive nature of intracellular signaling molecules make it difficult to confidently assert the exact pathway. Our data show that blocking the \( G_{\beta\gamma} \) complex prevented the DCG-IV enhancement of KV currents in NL neurons, indicating signaling transduction via the \( G_{\beta\gamma} \) complex. Downstream to the \( G_{\beta\gamma} \) complex is the activation of PLC, followed by the cleavage of \( PIP_2 \) in to DAG and IP_3, which can change the activity of PKC (review in Clapham and Neer 1997; Smrcka 2008). Our data support that PLC and PKC are critical factors for the modulation of KV currents by mGluR II.

In fact, there is a large body of literature showing that HTK currents can be modulated by the phosphorylation state of specific subunits (e.g. Augustine and Bezanilla 1990; Atzori et al. 2000; Strumbos et al. 2010). Like our work has shown, much of the literature concerning phosphorylation-induced changes in KV3 currents has shown that PKC is intimately involved (Liu and Kaczmarek 1998; Macica et al. 2003; Sun et al. 2003; Song et al. 2005; Song and Kaczmarek 2006).

However, as stated earlier, these intracellular signaling pathways can communicate and interact. As a result, we cannot completely rule out the involvement of \( G_{\alpha} \), cAMP, or other signaling molecules in mGluR II enhancement of KV currents. Specifically, PKC has been shown to directly inhibit interactions between \( G_{\alpha} \) subunit and
Figure 3.8 Schematic showing the proposed mechanism for mGluR II enhancement of $K_V$ currents in NL neurons. (A) Biocytin stained NL neuron with bipolar dendrites receiving separate and independent inputs from the ipsi- and contralateral glutamatergic inputs from the NM, which receive direct input from the ipsilateral ear. (B) top: During periods of low activity from the NM, mGluR II are inactive and $K_V$ currents are functional at the baseline level. bottom: NL neurons, specifically LF neurons, follow high frequency inputs imperfectly, with irregularity and failures present. (C) top: During periods of high activity from the NM, mGluR II are active and $K_V$ currents are increased. bottom: NL neurons follow high frequency inputs more reliably with fewer failures.
adenylate cyclase (Gordeladze et al. 1989; García-Sáinz and Gutiérrez-Venegas 1989), which may influence K\textsubscript{v} currents as well (Chung and Kaczmarek 1995).

Taken together, we propose that in NL neurons, activation of mGluR II leads to activation of the G\textsubscript{βγ} complex, which changes PKC activity, likely via PLC, and ultimately causes an enhancement of HTK currents (Fig. 3.8). Furthermore, given that PKC activity also alters HTK currents in neurons of the mammalian sound-localizing circuit (review in Brown and Kaczmarek 2011); it is possible that postsynaptic mGluR II may be a cellular mechanism shared by neurons that require a dynamic ability to fire APs at high rates.

**Functional Implications**

One of the critical findings of this study is that via mGluR II the glutamatergic input to the NL can provide a feedforward modulatory mechanism to improve the high frequency following ability of NL neurons. The increased ability to follow high frequency inputs due to enhanced HTK conductance seems to be counterintuitive, because suppression of HTK conductance has been shown to enhance spike generation (Dong et al. 2005). However, in fast spiking neurons the enhanced HTK currents may allow faster recovery of Na\textsubscript{v} channels from inactivation and promote activation of resurgent Na\textsubscript{v} currents (Akemann and Knöpfel 2006), producing more and faster APs. Importantly, mGluRs reduced the temporal variability of NL spikes, suggesting that NL neurons can dynamically regulate their ability to follow high
frequency inputs depending on the history of previous inputs. LF neurons have a large dynamic range in their synaptic inputs from the NM, and enhancement of $K_V$ currents may allow these neurons to adapt to the dramatic changes in the strength of inputs from resting to active state. Song et al. (2005) proposed that in quiet environments neurons have improved temporal precision but a reduced ability to follow high frequency stimulation, while in noisy environments the ability to follow high frequency stimulation is improved at the expense of temporal precision. This allows adaptive ion channel changes to sensory stimuli (Song et al. 2005; Strumbos et al. 2010). While we did not observe a loss of temporal fidelity after mGluR II induced $K_V$ enhancement in NL neurons, our data do provide strong evidence for a mechanism by which HTK currents can be enhanced by glutamatergic input, an intrinsic plasticity that may be shared by mammals and birds in their temporal processing.
CHAPTER IV

CONCLUSIONS

The overarching aim of this dissertation was to uncover cellular mechanisms that enable sound localization to sounds at different frequencies. The specific aims addressed covariation of LTK currents and synaptic inhibition, and mGluR II modulation of HTK currents across the tonotopic axis of the chicken NL. The results revealed how tonotopic variations of intrinsic, synaptic, and modulatory mechanisms interact in the chicken NL, showing that ITD-coding neurons have specialized properties arranged tonotopically, which enable coincidence detection to different sound frequencies.

Chapter II addressed the influence of tonotopically organized LTK currents and their interaction with tonotopically organized synaptic inhibition. LTK currents varied along the tonotopic axis in presynaptic inhibitory terminals and in postsynaptic NL neurons in an opposing manner, such that LF inhibitory inputs had more robust LTK current regulation of presynaptic inhibition than in MF and HF inhibitory inputs. However, postsynaptic LTK currents varied in an opposite manner, with LF neurons possessing weaker LTK currents than MF and HF neurons.

Chapter III addressed the covariation of modulatory mGluR II and HTK currents. It is known that both the expression of mGluR II (Okuda et al. 2013; unpublished data from our lab) and presynaptic mGluR II modulation of excitatory neurotransmission (Okuda et al., 2013) are more pronounced in LF neurons than MF and HF neurons. Our work shows that postsynaptic modulation by mGluR II is also more pronounced in LF neurons. This was revealed by the use of a more advanced electrophysiological recording
technique that preserves the intracellular composition of the postsynaptic neuron. The work serves to show that A) postsynaptic mGluR modulation could be overlooked by traditional whole-cell recordings and B) mGluR IIIs can act to provide feedforward plasticity to enhance the output of neurons during periods of high activity in a CF-dependent manner. Therefore, mGluR II can provide LF neurons an activity-dependent mechanism to change their intrinsic properties.

**Tonotopic interplay between LTK currents and synaptic inhibition**

Chapter II addressed the interactions between LTK currents and synaptic inhibition along the tonotopic axis in NL. It was believed that synaptic inhibition in the chicken ITD-coding circuit was universally slow and sustained (Hyson et al. 1995; Yang et al. 1999; Lu and Trussell 2000; Kuo et al. 2009) such that inhibitory inputs would serve to shunt excitatory inputs through the widespread activation of GABA<sub>A</sub> and LTK currents (Funabiki et al. 1998; Howard et al. 2007). Only recently has work shown that slow and sustained inhibition, and even tonic (or continuous) inhibition, was a property of MF and HF neurons, while LF neurons tended to have faster and more phasic inhibitory inputs (Tang et al. 2011; Tang and Lu 2012; Yamada et al. 2013).

Results from chapter II confirmed that LF neurons had inhibitory inputs of smaller amplitude and faster kinetics. The work added to previous research by showing that LF neurons had faster and smaller inhibitory inputs not only due to GABA<sub>A</sub> channels with a unique subunit composition (Yamada et al. 2013) but also due to more robust presynaptic LTK currents on the presynaptic terminals of inhibitory inputs (Hamlet et al. 2013).
This finding suggests that ITD-coding in LF neurons may resemble ITD-coding in mammalian MSO neurons, which also receive fast phasic inhibition (Grothe and Sanes 1993; 1994; Brand et al. 2002; Dodson et al. 2002; 2003; Grothe 2003; Roberts et al. 2013). While it is generally believed that ITD-coding is utilized differently in birds and mammals, it is possible that at least for low frequency sounds, similar ITD-coding strategies may be adapted.

Modulation of \( K_v \) currents across the tonotopic axis

While work in mammals has shown that HTK currents can be modulated by acoustic input (Song et al. 2005), \( K_v \) currents are generally thought of as non-dynamic elements in ITD-coding neurons. One reason for this idea may be due to the use of a conventional recording technique called whole cell patch clamp (WCR). However, it has long been known that this recording technique can disrupt GPCR modulation of ionic currents (Horn and Marty 1988), so we examined whether mGluR II, which is known to presynaptically regulate NL neuron function (Okuda et al. 2013), could modulate postsynaptic \( K_v \) currents.

The results from chapter III show that using WCR we did not detect mGluR II modulation of \( K_v \) currents in NL neurons. However, by using PPCR, which leaves the intracellular milieu intact, we showed postsynaptic mGluR II activation enhanced \( K_v \) currents. Specifically, mGluR II enhanced HTK currents, which was confirmed by recording the changes in neuronal firing. HTK currents make APs narrower, and therefore higher HTK conductances allow a neuron to produce more APs in a given
period of time (Rudy et al. 1999; Rudy and McBain 2001; Johnston et al. 2010). We found that mGluR II activation similarly improved the NL neuron's ability to follow trains of stimulation up to 200 Hz with fewer failures and less variability in the timing between APs. Furthermore, we were able to evoke similar changes in HTK currents with endogenous glutamate by repetitively stimulating presynaptic NM inputs and blocking glutamate reuptake. Because mGluR IIs are generally found in extrasynaptic locations (Shigemoto et al. 1997; Tang and Sim 1999; Tamaru et al. 2001), it appears that this modulation could occur in vivo to drive a form of intrinsic feedforward plasticity to enhance the ability of NL neurons to produce APs at high rates.

The study also highlighted the tonotopic organization of mGluR II enhancement of HTK currents. Unpublished data from our lab and from Okuda et al. (2013) has shown mGluR II expression is most dense in the LF region. Using WCR, Okuda et al. (2013) found that mGluR II modulated predominantly presynaptic NM terminals in the LF region of the NL, acting as autoreceptors to reduce synaptic depression and improve coincidence detection. We found that the postsynaptic effects of mGluR II were also tonotopically organized. While mGluR II activation enhanced HTK currents in all CF regions, the enhancement was most robust in LF NL neurons.

Using pharmacological tools to explore the signaling pathway, we found that modulation of HTK currents in chick bore some resemblance to modulation of HTK in mammal. Specifically, the signaling molecule PKC reduces HTK conductances in neurons of the ILD-coding circuit in the mouse (Critz et al. 1993; Liu and Kaczmarek 1998; Macica et al. 2003; Sun et al. 2003; Song et al. 2005; Song and Kaczmarek 2006).
PKC is also important for mGluR II enhancement of HTK currents in chick. Additionally, the tonotopic nature of HTK enhancement in chicken was also similar to HTK enhancement in mammals (Song et al. 2005; Leão et al. 2010). Although the specific signaling mechanism for HTK enhancement in mammal was not defined, given the ubiquity of mGluRs in the auditory system, it is possible that LF neurons in ITD-coding circuits require dynamically regulated HTK conductance allowing for ITD coding to dynamic acoustic environments.

Taken together, these data highlight the vast variations of cellular properties of ITD-coding neurons, which enable accurate localization of sounds of different frequencies.
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