NEUROMODULATION OF INTRINSIC AND SYNAPTIC PLASTICITY IN AUDITORY CORTEX

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ABSTRACT

DEEPTI RAO: Neuromodulation of Intrinsic and Synaptic Plasticity in Auditory Cortex
(Under the direction of Paul B. Manis)

The study of auditory system development and plasticity is important. Large populations of people suffer from hearing loss throughout their lifetime. To optimize treatment for children with hearing loss, it is crucial to understand how early hearing loss affects the development and processing of the central auditory system. Equally important is the understanding of how changes in neural activity in the auditory pathways can have an effect on its function. In this thesis, I studied 1) how hearing loss affects auditory cortical activity and 2) the mechanisms that could underlie learning and memory of sound information. In the first study, I found that hearing loss enhances auditory cortical activity and alters the manner in which the auditory cortex responds to the neuromodulator serotonin. In the second study, I found that the auditory cortex follows unique cellular learning and memory rules and these rules are altered by the neuromodulator acetylcholine.

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ABBREVIATIONS

SNHL	Sensorineural hearing loss
STDP	Spike-timing dependent plasticity
bAP	Back propagating action potential
A1	Primary auditory cortex
LTP	Long term potentiation
LTD	Long term depression
tLTP	Timing dependent long term potentiation
tLTD	Timing dependent long term depression
EPSP/C	Excitatory post synaptic potential / current
AP	Action potential
5HT	5-hydroxytryptamine (serotonin)
BF	Best frequency
MGv	Ventral division of the medial geniculate nucleus
NMDAR	*N*-methyl *D*-aspartate receptor
mGluR	Metabotropic glutamate receptor
AIS	Axon initial segment
mAChR	Muscarinic acetylcholine receptor

CHAPTER 1

INTRODUCTION

Arising from the sound source, hearing starts as sound transduction in our ears and processing in our brain. Our ears capture vibrations of sound waves, which are detected and amplified by the cochlea. The basilar membrane of the cochlea responds most strongly to sound of a specific frequency. The places responding to high frequencies are at the basal end of the cochlea, and the places responding to low frequencies are at the apex, giving rise to a topographical mapping of frequency referred to as cochleotopy. The vibration of the basilar membrane initiates sensory transduction by displacing the stereocilia of the hair cells, opening transduction channels, and generating a receptor potential that drives the release of the neurotransmitter glutamate. Glutamate in turn depolarizes the dendrites of spiral ganglion cells, initiating action potentials that travel along the auditory nerve to the cochlear nuclei (reviewed in (Hudspeth 1997)). The targets of cochlear nuclei neurons include the superior olivary complex, where the binaural cues for sound localization are processed (Cant and Benson 2003), and nuclei of the lateral lemniscus. These nuclei, along with the cochlear nucleus, converge on the inferior colliculus which processes sounds with complex temporal structures and sounds of particular significance (Suga 1969; Suga et al. 2002). The output of the inferior colliculus ascends to the medial geniculate nucleus, which in turn projects onto the auditory cortical areas. The auditory cortex is subdivided into primary and belt areas (Merzenich and Brugge 1973). The belt areas of the auditory cortex process complex sounds that mediate communication (Recanzone 2008).

The primary auditory cortex (A1) in humans is located in the superior temporal gyrus in the temporal lobe. The function of A1 is to process sound pitch, volume and location of sound, the temporal order and patterns of sounds and to deconstruct complex sound patterns such as human speech (reviewed in (Rauschecker and Scott 2009)). A1 also acquires and retains specific memory traces about the behavioral significance of relevant sounds (Weinberger 2004).

1. Functional architecture of A1

Tonotopy established in the cochlea is maintained throughout the auditory pathway including in A1. The first tonotopic auditory cortical map was demonstrated by Woolsey and Walzl (1942). These investigators performed localized electrical stimulation of auditory nerve fibers in the cochlea and mapped the patterns of evoked responses on the auditory cortex. They found that the frequency tuning varied smoothly across cortex of cats and monkeys. However, recent evidence indicates that tonotopy in A1 exists only on a global scale, such that neurons with different tuning properties are more likely to be neighbors than neurons with similar tuning properties. Therefore, although tonotopy can be seen globally, it is fractured on a local scale (Bandyopadhyay et al. 2010; Rothschild et al. 2010).

Neurons in A1 receive excitatory input from two major sources: the feedforward thalamocortical projections, and recurrent intracortical inputs. Feedforward thalamocortical information comes primarily from the ventral division

of the medial geniculate nucleus (MGv). The tonotopic organization of A1 is established during development at the time when thalamocortical axons arrive in cortex. Tonotopy is initiated by spontaneous thalamocortical activity and then is refined by sensory-driven thalamocortical activity (Chang and Merzenich 2003; Zhang et al. 2001). This thalamocortical input directly mediates cortical responses to best frequency (BF, stimulus frequency eliciting the largest magnitude response) and stimuli that are spectrally close to BF. Thalamocortical neurons project to restricted cortical regions that have similar BF (Winer et al. 1999). However, there is also evidence that individual neurons in A1 receive inputs from most of the audible spectrum (Kaur et al. 2004). Cortical silencing studies reveal that although thalamocortical inputs determine the shape of the frequency tuning curve of a neuron, intracortical excitatory inputs refine the frequency tuning by selectively amplifying responses at BFs of cortical neurons (Kaur et al. 2005; Liu et al. 2007). These results suggest that both intracortical and thalamic inputs contribute to the tonal response map of A1 neurons.

1.1 Recurrent synapses of Layer 2/3 pyramidal neurons

A1 possesses six layers of organization, numbered: Layers 1 through 6. Layers 3 and 4 receive thalamocortical input and send their projections to pyramidal neurons in L2/3. The major intracortical connections arise from L2/3 neurons. L2/3 neurons make connections within the same layers and to contralateral L2/3 neurons (Code and Winer 1985; Linden and Schreiner 2003; Ojima et al. 1991; Winer 1984; 1985). L2/3 neurons extend their axons laterally and are aligned along the tonotopic axis, linking columns of neurons with

different frequency tuning (Clarke et al. 1993; Matsubara and Phillips 1988; Ojima et al. 1991; Read et al. 2002; Song et al. 2006). In support of this, a recent study measured sensory evoked-calcium transients in individual dendritic spines in L2/3 of A1 *in vivo* and found that sounds played at different frequencies revealed spines on the same dendrite are heterogeneously tuned (Chen et al. 2011). These results suggest that synapses onto L2/3 neurons play a crucial role in determining frequency tuning on a local scale (Bandyopadhyay et al. 2010; Rothschild et al. 2010).

Frequency tuning of A1 neurons is determined by a suprathreshold response to a sound frequency. However, studies using intracortical disinhibition reveals an expansion of receptive fields to include more frequencies, suggesting the presence of subthreshold excitatory postsynaptic potentials (Kaur et al. 2005) that are inhibited by intracortical circuits (Foeller et al. 2001; Muller and Scheich 1988). Intracortical subthreshold receptive fields can span five octaves or more (Kaur et al. 2004). Subthreshold receptive fields are also observed in visual and somatosensory cortices (Bringuier et al. 1999; Li and Waters 1996). What is the function of the intracortical subthreshold receptive field in A1? Spectrotemporally complex stimuli could evoke integration of subthreshold excitatory potentials resulting in suprathreshold neural activity (Weinberger and Bakin 1998). Studies reveal that recurrent connections among L2/3 pyramidal neurons broaden sub-threshold receptive fields (Ojima and Murakami 2002). Subthreshold receptive fields could play an important role in integrating responses to spectrotemporally complex stimuli, such as frequency modulated sounds, and offer a substrate for

plasticity of the tonotopic map (Kilgard and Merzenich 1998). It has long been known that the auditory system is capable of precisely timed responses to acoustic stimuli. Precise timing of synaptic onsets in A1 may be important for spectral integration. The synaptic response latency, for a neuron, increases with increasing spectral distance from BF (~1 or 4 ms/octave at suprathreshold intensities) (Kaur et al. 2004). Therefore, A1 responses to non-BF stimuli occur before thalamocortical neurons spike in response to the same stimulus. Conduction delays in intracortical pathways, from distant neurons for which the stimulus is BF, increase with increasing spectral distance. These delays might account for increasing synaptic onset latencies with increasing spectral distance from BF. Summation of excitatory postsynaptic potentials evoked by tones of different frequencies could increase when the tones are presented asynchronously. Larger summation is evoked when tones are staggered as in a frequency modulated sweep. Given the precise timing of synaptic onset latencies of intracortical synapses, would plasticity at these synapses influence spectral tuning of these neurons?

Intrinsic excitability, synaptic dynamics and the anatomic organization of L2/3 neurons all influence cortical auditory processing, including basic features such as frequency tuning. Differences in developmental regulation of intrinsic and synaptic properties might reflect changes in mechanisms required to process sound at different ages (Rao et al. 2010). Furthermore, if the intrinsic and synaptic properties are regulated by neuromodulatory systems that are activated

during behavioral states, then L2/3 neurons could alter their responses to auditory stimuli and, consequently, auditory information processing.

Given the importance of recurrent connections between L2/3 neurons, we wanted to test the hypothesis that L2/3 neurons and their connections are regulated by auditory experience. In this thesis, I will discuss two fundamental questions. First, how does hearing loss affect the intrinsic electrical excitability of L2/3 neurons? Second, are intrinsic and synaptic plasticity of L2/3 neurons regulated by neuromodulators that physiologically activate during specific behavioral states? Below I discuss plasticity and neuromodulation of this plasticity in A1.

2. Developmental plasticity in auditory cortex

In rodent auditory cortex, the first 3 postnatal weeks is a time of rapid development of neural circuitry. Thalamic afferents to cortex appear in the first postnatal week (Ignacio et al. 1995; Robertson et al. 1991). Hearing onset, in rodents, occurs in the second week of life between postnatal day 10 (P10) and P12 (Ehret 1976). Soon after the onset of hearing, A1 is occupied by broadly tuned neurons that only respond to high frequency sounds and during a 2 to 3 week period (Zhang et al. 2001), A1 undergoes an experience-driven refinement of selective frequency tuning to acquire an adult-like organization. Thus, auditory experience during early postnatal development is important in shaping the

tonotopic map. Several manipulations results in plasticity of the tonotopic map. For example, restricted unilateral (Robertson and Irvine 1989) or bilateral cochlear lesions (Schwaber et al. 1993) results in a restructuring of auditory cortex: deprived cortical areas corresponding to the cochlear lesion are occupied by expanded representations of adjacent cochlear regions, and of the frequencies represented at those regions. Similarly, chronic noise exposure to a specific frequency results in a reduction in representation of exposed frequency but expansion in the representation of neighboring frequencies (Seki and Eggermont 2003). In contrast, aversive learning (Weinberger 2003) or extensive training of animals to discriminate tonal frequencies (Recanzone et al. 1993), sound levels (Polley et al. 2004) and temporal modulation rates (Bao et al. 2004) enlarges cortical representation of the experienced frequency.

The highly plastic critical period in the auditory cortex is known to begin at the onset of hearing, at P12 in rats, and extends through the first month of postnatal life (Zhang et al. 2001). However, several critical periods exist for various sound features, such as the best frequency, tuning bandwidth, and frequency modulation (Insanally et al. 2009). For example, the critical period for spectral tuning lasts during a 3-day window as pure tone exposure expands representation in cortex only if exposure occurs between P11-P13 (Zhang et al. 2001). The critical period also depends on the temporal structure of sensory inputs. Studies show that noise exposure delays both the development of spectral tuning and critical period for frequency map plasticity (Chang and Merzenich 2003). Further, critical period closure is controlled by local circuits in

A1, i.e. exposing rats to spectrally limited noise results in closure of critical period of circuits that correspond to noise-free frequencies and an open critical period of circuits corresponding to noise-exposed areas of cortex which are immature (de Villers-Sidani et al. 2008). Adult A1 also maintains a small degree of plasticity (Syka 2002). For example, between P20 and P35 the bandwidths of excitatory receptive fields continue to decrease and the ability of neurons to follow repetitive stimuli is enhanced (Chang et al. 2005; Syka 2002).

2.1 Cellular mechanisms of plasticity in auditory cortex

The mechanisms underlying experience-dependent changes in auditory response properties remain largely unexplored. One possible mechanism could be sensory-driven refinement of synaptic connections through long-term potentiation (LTP) and long-term depression (LTD) (Constantine-Paton et al. 1990; Goodman and Shatz 1993; Malenka and Bear 2004; Zhang and Poo 2001). Activity-dependent plasticity of sensory representations has been demonstrated to be mediated, at least in part, by a type of synaptic modification called spike timing dependent plasticity *in vivo* in visual, somatosensory and auditory cortices (Dahmen et al. 2008; Jacob et al. 2007; Yao and Dan 2001). Experience dependent changes require neurons to detect important information in the sensory environment and store this information as changes in synaptic dynamics or intrinsic excitability. Although both changes in synaptic strength and intrinsic excitability determine neuronal firing, learning induced refinement of cortical circuits has largely been ascribed to synaptic plasticity mechanisms

(Figure 1). However, intrinsic plasticity that alters the firing properties of a neuron can affect network function, and studies that show activity-dependent modulation of excitability in a variety of neurons (Gittis and du Lac 2006). How could activity or experience modulate excitability? Behavioral training with sensory inputs could decrease thresholds for neural activity thus enhancing the intrinsic excitability of neurons and, after extinction of learning, retraining could result in faster rates of acquisition. The change in intrinsic excitability could thus serve as a memory trace of the trained sensory input.

Below, I will discuss these two forms of plasticity, 1) Spike timing-dependent plasticity (STDP) and 2) Intrinsic plasticity.

2.1.1 Spike timing-dependent plasticity (STDP)

Synaptic strength can be modified by activity, in a way that depends on the timing of neuronal firing on either side of the synapse. Presynaptic activity that precedes postsynaptic firing, by up to tens of milliseconds, causes timing dependent LTP (tLTP), whereas reversing this temporal order causes timing dependent LTD (tLTD), a phenomenon called spike timing-dependent plasticity (STDP) (Debanne et al. 1994; Levy and Steward 1983). How does STDP bring about changes in cortical representations? The relative timing of sensory stimuli plays a crucial role in dynamic regulation of cortical function through STDP-like rules. For example, timed visual or whisker stimuli produces receptive field changes *in vivo* in visual and somatosensory cortices respectively through STDP rules (Jacob et al. 2007; Yao and Dan 2001). Interestingly, the critical time

window for STDP induction varies widely with brain region, cell and synapse type (Abbott and Nelson 2000; Larsen et al. 2010). Therefore, one might wonder what STDP rules A1 synapses follow.

Millisecond timing is functionally important in the auditory system for several reasons. First, A1 neurons can lock with millisecond precision to the fine timing of acoustic stimuli (Eggermont 2007). Second, millisecond differences in neural activity in A1 are sufficient to drive decisions (Yang et al. 2008). Third, interaural time delays of less than one millisecond are used for the spatial localization of sound (Harper and McAlpine 2004). To facilitate processing at such fast timescales, one may predict that A1 has unique timing rules for STDP. Only recently have studies emerged focusing on STDP in the auditory pathway.

Synapses in the cochlear nucleus (Tzounopoulos et al. 2004) and A1 (Karmarkar et al. 2002) have been shown to express STDP. In the dorsal cochlear nucleus, STDP is Hebbian or anti-Hebbian depending on the cell type (Tzounopoulos et al. 2004). At recurrent synapses in A1, repetitive pairing of pre→post activity with a 10 ms delay produces tLTP and post→pre at 40 ms delay produces tLTD (Karmarkar et al. 2002). How does STDP cause functional changes in A1? One way STDP can affect response properties of A1 neurons is by repetitive and asynchronous pairings of pure tones of different frequencies that produces shifts in the frequency selectivity of neurons (Dahmen et al. 2008). Dahmen et al, (2008) paired a non-BF tone with a BF tone with a 10 ms delay between the two tones. When the non-BF tone was presented before the BF tone, there was a shift in the neuronal BF toward the non-BF. Conversely, when

the non-BF tone was played after the BF tone, then the neuronal BF shifted away from the non-BF tone. These BF shifts were found to be restricted to neurons recorded from L2/3 and L4 (Dahmen et al. 2008). Thus, millisecond scale relationships within acoustic stimuli and between neuronal spiking can influence frequency responses and auditory processing and suggest that STDP is a relevant mechanism for experience-dependent plasticity in the auditory cortex.

The cellular mechanisms supporting STDP in A1 is unknown. Rate-dependent LTP and LTD have been observed at thalamocortical synapses and at excitatory intracortical synapses in A1 (Bandrowski et al. 2001; Kudoh and Shibuki 1997; 1994; 1996). Both experience and development regulate rate-dependent LTP (Speechley et al. 2007). STDP in A1 could also follow similar regulation. Activation of NMDARs is necessary for rate-dependent LTP of thalamocortical synapses (Kudoh and Shibuki 1994; 1996), while activation of mGluRs are necessary for LTD at the same synapse (Bandrowski et al. 2001). One might predict, in A1, that NMDARs are similarly required for tLTP induction and mGluR activation is required for tLTD. However, the receptors and signaling pathways supporting STDP in A1 is still unstudied.

STDP involves the crucial interplay between synaptic activation, elevation of postsynaptic dendritic spine calcium concentration and synaptic plasticity (Bi and Poo 1998; Debanne et al. 1998; Magee and Johnston 1997; Markram et al. 1997). STDP can occur either by requiring back propagating action potentials (bAPs) or dendritic spikes (Golding et al. 2002). A key function of bAPs or dendritic spikes in this process is the depolarization-induced relief of NMDAR

channels from Mg2+ block and subsequent increase in synaptic calcium influx. The calcium signaling profile is correlated to the polarity of synaptic plasticity (Ismailov et al. 2004). Spine calcium concentration can be regulated by dendritic ion channels. Changes in ion channel kinetics, opening probability, or voltage-dependence can have a profound effect on the magnitude and timing of calcium influx.

2.1.2. Intrinsic Plasticity

Intrinsic electrical excitability determines a neuron's characteristic firing pattern and the way in which neurons integrate synaptic input. The contribution of a neuron to circuit function can be enhanced or reduced by modifying the input-output function, independent of changes in synaptic input. The cellular mechanisms regulating intrinsic plasticity remain unclear. One possible mechanism involves signaling molecules that can regulate ion channel function through phosphorylation or dephosphorylation (Cantrell et al. 1997; Levitan 1994). Another possibility is changes in ion channel density or distribution on dendrites by regulation of channel trafficking to and from the membrane that could have an effect on dendritic integration. Further, changes in electrical activity of the cell can regulate the site of action potential initiation, the axon initial segment (AIS). Variations in the location of the AIS have been implicated in information processing capabilities and have been correlated with alterations in current threshold for action potential initiation (Grubb and Burrone 2010). All of

the above mechanisms of intrinsic plasticity could alter firing threshold and regulate the excitability of neurons. What role does intrinsic plasticity play in learning mechanisms? Intrinsic plasticity plays an important role in adaptive plasticity of the vestibulo-ocular reflex and has implications in motor learning (Gittis and du Lac 2006; Matthews et al. 2008; Saar and Barkai 2003).

How may intrinsic plasticity mechanisms bring about changes in cortical receptive fields? One way is to regulate intrinsic excitability by decreasing or increasing the threshold for spiking in response to sensory input and this could lead to broadening or narrowing the receptive field of the neuron. Another way is to change the ion channel composition on certain dendritic regions that subsequently decrease or increase the threshold for synaptic plasticity at those regions, thus shifting neuronal receptive fields. Conversely, when an animal is faced with a destabilizing perturbation, such as visual deprivation, trimmed whisker, or hearing loss, neuronal circuits may act to stabilize neuron and circuit function, presumably by altering excitability. This is known as homeostatic plasticity (Turrigiano 1999).

Neurons in the auditory cortex display several prominent changes in intrinsic properties during development. Between P8 and P29, input resistance, resting membrane potential and membrane time constant have been shown to decrease (Metherate and Aramakis 1999; Oswald and Reyes 2008), glutamate mediated synaptic potentials develop from small amplitude and long duration to large and rapid (Aramakis et al. 2000) and *N*-methyl-D-aspartate (NMDA) receptors rapidly increase to P18 before declining to adult levels (Hsieh et al.

2002). Intrinsic excitability reaches a steady state in adults (P19-P29) (Oswald and Reyes 2008). Changes in these properties could contribute to differences in auditory processing during this plastic developmental epoch.

2.2 Hearing loss in A1

Approximately 17% of adults and 15% of children in the United States have some degree of hearing loss (NIDCD, http://www.nidcd.nih.gov/health/statistics/, CDC survey). Hearing loss results in loss of intensity discrimination, frequency discrimination, and temporal resolution (Halliday and Bishop 2005; Iverson 2003; Wojtczak et al. 2003). Hearing loss during development could affect adversely affect acquisition of auditory skills and lead to impairments in auditory learning. Sensorineural hearing loss (SNHL) is defined as loss of hearing due to an abnormality or damage of cochlear hair cells or auditory nerves. The effects of SNHL are particularly severe in children with the longest periods of auditory deprivation (Sharma et al. 2002a). Recordings from experimentally induced hearing-impaired animals have also revealed many profound changes in auditory processing (reviewed in (Syka 2002)). For example, A1 spiking threshold to electrical stimulation of the cochlea are lower and spatial tuning curves are broader. Long term-deafened animals, with degeneration of auditory nerves, display a tonotopy with no gradient than normal or short-term deafened animals, with partial hair cell survival and complete auditory nerve survival (Raggio and Schreiner 1999; 2003). At the cellular level,

hearing loss increases intrinsic excitability in cochlear nucleus (Francis and Manis 2000) and in gerbil A1 (Kotak et al. 2005; Rao et al. 2010), increases the length of the AIS in avian brain stem neurons (Kuba et al. 2010) and eliminates LTP at cortical synapses (Kotak et al. 2007). Extracellular field potential amplitude and latency defects are restored to normal when congenitally deaf animals are fitted with cochlear implants (Klinke et al. 1999; Kral et al. 2000). Taken together, these results suggest that lack of auditory input produces pronounced changes in intrinsic, synaptic and functional properties in auditory cortex and that plasticity of A1 permits implant-driven inputs to restore normal function and hearing.

3. Neuromodulation in A1

Normal development of the cortex requires a combination of thalamocortical and intracortical connections in cortex in addition to afferent neuromodulatory systems into cortex. For example, auditory cortical neurons receive input from many afferent systems that play a crucial role in circuit formation, beginning in gestation, including cholinergic, serotonergic, adrenergic and dopaminergic innervation (Hasselmo 1995).

In this thesis, the primary focus will be on understanding how signals, that are important for influencing cortical circuits and provide information about the behavioral relevance, can change how that information is stored in the brain. Two signals that provide this information are neuromodulators: serotonin and

acetylcholine. Serotonergic and cholinergic systems play an important role in auditory cortical plasticity according to behavioral demands (Ji and Suga 2007; Weinberger 2003).

3.1 Serotonergic regulation of developmental plasticity

The source of serotonergic afferents to cortex originates from the brainstem raphe nuclei (Lidov and Molliver 1982; Wallace et al. 1982). Serotonin is known to play an important role in neurogenesis, cell migration, dendritic and axonal development, synaptogenesis, and synaptic plasticity (Lauder 1990). Serotonergic afferents enter cortex during development in the first two postnatal weeks (Bennett-Clarke et al. 1996; Wallace and Lauder 1983), but this innervation is transient as serotonin decrease after 3 weeks of age. The serotonergic innervation becomes more uniform in adult neocortex (D'Amato et al. 1987).

3.1.1 Role of serotonin in developmental plasticity

Serotonin or 5-hydroxytryptamine (5-HT) is an important modulator of activity-dependent cortical development. For example, depletion of serotonin in newborn rats results in a significant reduction of thalamocortical afferents to somatosensory cortex (Bennett-Clarke et al. 1995; Bennett-Clarke et al. 1994). Conversely, monoamine oxidase A knockout mice, which have enhanced 5-HT levels in cortex, lack somatosensory receptive field organization (Cases et al.

1996). Depletion of serotonergic afferents to cortex results in a retardation of the maturation of barrel fields in mice with whisker follicle lesions (Osterheld-Haas et al. 1994). The contribution of serotonin to the development of auditory cortex is still unknown. Recent findings from our lab indicate that a subtype of serotonin receptor demonstrates a developmental trend in auditory cortex, increasing at P10 to P17 and decreasing back to P8 level at older ages (Basura et al. 2008). These results suggest that neonatal serotonergic innervation plays an important transient role in the development of auditory cortex circuitry.

3.1.2 Role of serotonin in auditory cortical function

The serotonergic system plays an important role in behaviorally relevant auditory cortex functions. For example, application of 5-HT in bat A1 can suppress or potentiate fear-induced plasticity of acoustic response areas (Ji and Suga 2007). In addition, depletion of the 5-HT precursor tryptophan, in humans, modulates auditory selective attention (Ahveninen et al. 2003) and decreases the intensity dependence of auditory evoked magnetic N1/P2 dipole source activity (Kahkonen et al. 2002a; Kahkonen et al. 2002b). The level of 5-HT released in A1 can adjust the level of sensory processing by regulating loudness growth functions (Hegerl and Juckel 1993). Although 5-HT has been shown to play a crucial role in modulating cortical function, not much is known about the cellular mechanisms involved. Given the lack of knowledge in serotonergic modulation of cellular mechanisms in cortex, we studied the role of 5-HT in modulating intrinsic excitability in normal A1 and in A1 of bilaterally deafened rats.

3.2 Cholinergic regulation of developmental plasticity

The primary source of cholinergic afferents to the cortex in mammals originates from the nucleus basalis of the basal forebrain (Mesulam et al. 1983; Rye et al. 1984). However, in mice and rats, another source of cholinergic innervation arises from intracortical neurons (Consonni et al. 2009; Houser et al. 1985). During the first two postnatal weeks of cortical circuit development, cholinergic innervation of the neocortex increases and reaches mature levels by the third week (Mechawar and Descarries 2001). Cholinergic activity in cortex is highest during cortical maturation and synapse formation (Hohmann and Ebner 1985; Kristt 1979). The presence of the cholinergic innervation suggests it is potentially important for cortical function.

3.2.1 Role of acetylcholine in developmental plasticity

Acetylcholine has recently been found to play an important role in many aspects of cortical development (Hohmann and Berger-Sweeney 1998; Robertson et al. 1998). For example, ablation of the cholinergic innervation of neonatal cortex leads to delays in emergence of differentiated neurons in superficial layers of cortex (Hohmann et al. 1988; Hohmann et al. 1991). These results suggest that changes in cortical morphogenesis could lead to functional deficits in neocortex. Furthermore, mice lacking the M1 subtype of muscarinic cholinergic receptor display frequency tuning curves with multiple peaks, as

compared to the sharply tuned neurons in wild-type A1. Multi-peaked tuning curves implicate abnormal thalamocortical synaptic competition and could lead to disorganized tonotopic organization in M1 knockouts (Zhang et al. 2005). It was found that the disrupted tuning and tonotopy in knockout mice was not inherited from subcortical nuclei but arises in thalamocortical connections in cortex (Zhang et al. 2005). These findings suggest that the refinement and maturation of the tonotopic map depends on functional muscarinic cholinergic receptors.

3.2.2 Role of acetylcholine in auditory cortical function

Acetylcholine plays an important role in auditory cortex function. It is well known that NB neurons are activated as a function of the behavioral significance of stimuli (Testylier and Dykes 1996). NB activation increases thalamocortical synaptic potentials, neuronal spiking and facilitates excitatory synaptic potentials in A1 (Metherate and Ashe 1993). Pairing electrical stimulation of NB with tone presentation produces large shifts in frequency tuning of A1 neurons and corresponding massive reorganization of the tonotopic map specific for the paired frequency (Froemke et al. 2007; Kilgard and Merzenich 1998). However, in mice lacking M1 muscarinic receptors, pairing NB stimulation and tones produces much smaller shifts in tuning in A1 (Zhang et al. 2006). Further, NB induced frequency specific plasticity resembles the plasticity produced by classical conditioning or by long-term behavioral training (Bakin and Weinberger 1996; Pandya et al. 2005). Blockade of cortical cholinergic receptors prevents the receptive field plasticity that would otherwise result from conditioning (Ji et al.

2001) or NB stimulation (Miasnikov et al. 2001). Taken together these results suggest that cholinergic inputs are important for experience-dependent plasticity of the auditory cortex.

4. Mechanisms of neuromodulation in A1

We have seen so far that serotonin and acetylcholine regulate experience dependent plasticity in auditory cortex. How do these neuromodulators regulate plasticity mechanisms? There are many ways by which neuromodulators regulate intrinsic and spike timing-dependent plasticity (STDP). Neuromodulators can activate kinases and by altering the kinetics and density of dendritic ion channels can bring about changes in firing rate or dendritic spine calcium level (Froemke et al. 2006; Magee and Johnston 1997). Neuromodulators can activate intracellular calcium release and can alter polarity and input-specificity of STDP and firing threshold (Nishiyama et al. 2000). Neuromodulators can directly act on NMDA receptors by facilitating or depressing currents and regulating STDP induction (Brocher et al. 1992; Flores-Hernandez et al. 2009; Metherate and Ashe 1995).

Below, I discuss serotonergic modulation of intrinsic plasticity and cholinergic modulation of STDP.

4.1 Serotonergic modulation of plasticity

The effects of 5-HT are mediated by 14 receptor subtypes. These receptor subtypes are linked to multiple signal transduction mechanisms (for review see (Hoyer and Martin 1997)). 5-HT-receptor subtypes are classified into 5-HT1 (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and5-HT 1F), 5-HT2 (5-HT2A, 5-HT2B, and5-HT 2C), 5-HT3, 5-HT4, 5-HT5 (5-HT5A, 5-HT5B), 5-HT6, and 5-HT7 receptors. 5-HT1 receptors are negatively coupled to adenylyl cyclase and inhibit the formation of cAMP. 5-HT2 receptors stimulate the hydrolysis of phosphatidylinositol. 5-HT3 receptors are ligand-gated cation channels. 5-HT4, 5-HT6 and 5-HT 7 receptors all enhance adenylyl cyclase activity, and promote intracellular accumulation of cAMP. 5-HT5A receptors may be negatively coupled to adenylyl cyclase, while no functional coupling has yet been described for 5-HT5B receptors. Almost all of the 5-HT-receptor subtypes are present in the neocortex (reviewed in (Gu 2002)).

In auditory cortex, our lab used subtype specific receptor binding to show that 5-HT2A and 2C receptor number increases from P10 to P17 and decreases back to the P8 level at older ages (Basura et al. 2008). Our lab showed that 5-HT2A receptors are present on layer 2/3 pyramidal soma and apical dendrites in A1 (Basura et al. 2008). The functional role of 5-HT is dependent upon specific receptor subtypes and target ion channels.

5-HT modulates both synaptic and intrinsic plasticity. For example, in visual cortex, 5-HT2C activation facilitates LTP and LTD within the critical period of development [(Kojic et al. 1997) but see (Edagawa et al. 2001)]. 5-HT2A and 5-HT7 receptor activation mediates depolarization, while 5-HT1A activation mediates hyperpolarization in prefrontal cortex (Beique et al. 2004). 5-HT1A activation that signals through potassium channels hyperpolarizes neurons in entorhinal cortex (Grunschlag et al. 1997). Interestingly 5-HT2 receptors increase the excitability, 5-HT1A receptor decreases excitability in pre-frontal cortex (Araneda and Andrade 1991). This regulation of dendritic channels by 5-HT is consistent with the high density of 5-HT2A and 5-HT2C receptors along the apical dendrites of these neurons (Jakab and Goldman-Rakic 1998). Stimulation of 5-HT2 receptors in prefrontal cortex neurons inhibits Ca(v)1.2 L type Ca2+ currents via a PLCbeta/IP3/calcineurin signaling cascade (Day et al. 2002) and could block dendritic depolarization. Serotonergic reduction of persistent Na currents could reduce EPSP enhancement at depolarized potentials (Aghajanian and Marek 1997).

Given the importance of 5-HT receptors in regulating cortical plasticity, my research presented in Chapter 2 evaluates the role of the serotonergic system and specifically 5-HT2 receptors within the developing auditory cortex.

4.2 Cholinergic modulation of plasticity

Cholinergic actions are mediated by two receptor classes, nicotinic acetylcholine receptors (nAChRs) and muscarinic receptors (mAChRs). While nAChRs are ionotropic ACh-gated cation channels, mAChRs are metabotropic members of the GPCR superfamily. Five mAChR genes (M1-M5) are known, which encode receptors M1-M5, respectively (Bonner et al. 1987). All mAChR subtypes act via activation of G-proteins to influence membrane properties via different second messengers; M1, M3 and M5 receptors are associated with G-proteins (Gq/11), which activate phospholipase C (Haley et al. 2000), whereas M2 and M4 receptors are associated with G-proteins (Gi/Go), which inhibit adenylyl cyclase.

Both nAChRs and mAChRs are present in the mammalian cerebral cortex. Hohmann et al found that during the course of forebrain cortical development mRNA levels of most nAChRs are constant, however mAChRs mRNAs vary, having peak periods during morphogenesis and synaptogenesis (Hohmann et al. 1995). These patterns suggest that the presence of mAChRs could play an important role in the establishment of circuits in the auditory cortex.

Muscarinic receptor activation modulates both synaptic and intrinsic plasticity. For example, spontaneous acetylcholine release in auditory cortex

tonically depresses synaptic potentials, an effect mediated by mAChR (Metherate and Ashe 1995). Muscarinic agonists suppress intracortical synaptic potentials while having less suppression or enhancing effects on thalamocortical synapses in A1 (Hsieh et al. 2000). mAChR activation increases intrinsic excitability *in vivo* in A1 (Froemke et al. 2007). Therefore, stimulation of the muscarinic receptor system in auditory cortex could increase postsynaptic excitability, reduce intracortical transmission and simultaneously increase thalamocortical transmission. The significance of the varied circuit actions points to an increase in the signal to noise ratio of incoming (thalamocortically-transmitted) sound information relative to intracortical inputs.

Muscarinic receptors can modulate STDP by several mechanisms (refer Figure 2). Muscarinic receptors can inhibit potassium currents and control dendritic calcium level and the three main channels postulated to participate in this response are Kv4.2/Kv4.3 voltage-gated transient currents, the M-current (Muscarine-activated or Kv7 current) and the calcium activated potassium channels, K(Ca) (Buchanan et al. 2010; Muller and Connor 1991a; Nakamura et al. 1997; Selyanko et al. 2000). Activation of muscarinic receptors on cortical neurons has been shown to reduce activation of Kv4.2 channels through a PKC dependent mechanism thereby increasing calcium influx, amplitude and width of BAPs (Acker and White 2007; Cho et al. 2008; Kampa and Stuart 2006; Muller and Connor 1991a). Two recent studies provide evidence of M1 receptor inhibition of small conductance, calcium activated potassium channels (SK channels) (Buchanan et al. 2010; Giessel and Sabatini 2010). Therefore,

modulation of potassium channels and thus calcium influx at synapses, at which coincident pre and postsynaptic inputs arrive, could affect polarity and magnitude of STDP.

Another mechanism of modulation by mAChRs is the activation of endocannabinoid signaling leading to presynaptic changes in glutamate release. M1 and M3 receptors have been shown to convert tLTP to tLTD at dorsal cochlear nucleus synapses via endocannabinoid signaling (Zhao and Tzounopoulos 2011). Additionally, mAChRs can modulate NMDA receptors via a PKC dependent mechanism (Michailidis et al. 2007). Muscarinic receptor activation has been shown to reduce (Flores-Hernandez et al. 2009; Metherate and Ashe 1995) or enhance (Aramakis et al. 1997) NMDA current in auditory cortex. A cholinergic reduction of NMDARs suggests that activation of muscarinic systems might control induction of tLTP or tLTD. Furthermore, mAChRs can promote tLTD by a PLC induced phosphorylation of AMPA receptor GluR1 at Serine 831 at visual cortex synapses (Seol et al. 2007).

Conclusion

In this chapter I have reviewed evidence that L2/3 auditory cortical neurons and the synapses between them are crucial for tonotopic map plasticity and are subject to modulation by serotonin and acetylcholine. Both these neuromodulators are known to be important for cortical arousal as well as

learning. Although increases or decreases in electrical responsiveness of A1 neurons might be the basis of arousal, the relationship of serotonergic or cholinergic transmission to the intrinsic or synaptic plasticity that might underlie learning is lacking. In chapter 2, I provide evidence of serotonergic modulation of intrinsic electrical excitability and how the modulation changes with auditory experience, i.e. in normal A1 and A1 in bilaterally deafened rats. In chapter 3, I provide evidence of cholinergic modulation of spike-timing dependent plasticity as a possible mechanism for receptive field plasticity.

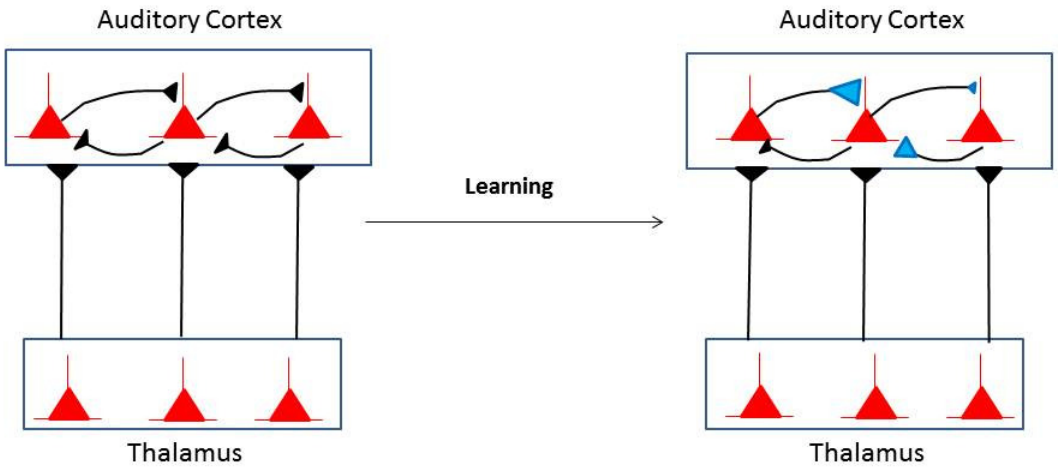

Figure 1: Learning induced changes in synaptic strength in auditory cortex.

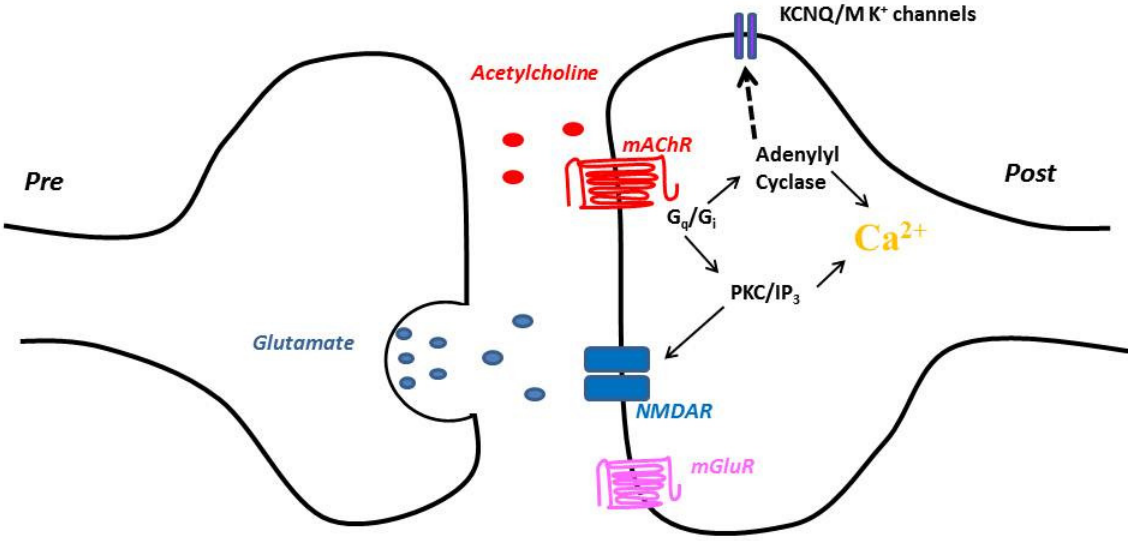

Figure 2: Muscarinic receptor signaling. Sites of muscarinic modulation of STDP mechanism. Pre and Postsynaptic terminals are shown. Under conditions of cholinergic efferent activation, acetylcholine is released and binds to muscarinic receptors. Depending on the subtype of receptor and the G-protein it is coupled to, adenylyl cyclase or PKC pathways are activated ultimately influencing calcium level. Other sites of modulation include, NMDARs and K+ channels.

CHAPTER 2

HEARING LOSS ALTERS SEROTONERGIC MODULATION OF INTRINSIC EXCITABILITY IN AUDITORY CORTEX (Rao et al. 2010)

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1. Abstract

Sensorineural hearing loss during early childhood alters auditory cortical evoked potentials in humans and profoundly changes auditory processing in hearing-impaired animals. Multiple mechanisms underlie the early postnatal establishment of cortical circuits, but one important set of developmental mechanisms relies on the neuromodulator serotonin (5-hydroxytryptamine, 5-HT). On the other hand, early sensory activity may also regulate the establishment of adult-like 5-HT receptor expression and function. We examined the role of 5-HT in auditory cortex by first investigating how 5-HT neurotransmission and 5-HT2 receptors influence the intrinsic excitability of layer II/III pyramidal neurons in brain slices of primary auditory cortex (A1). A brief application of 5-HT (50 μM) transiently and reversibly decreased firing rates, input resistance, and spike rate adaptation in normal P12-21 rats. Compared to sham operated animals, cochlear ablation increased excitability at P12-21, but all of the effects of 5-HT, except for the decrease in adaptation, were eliminated in both sham operated and cochlear ablated rats. At P30-35, cochlear ablation did not increase intrinsic excitability compared to shams, but it did prevent a pronounced decrease in excitability that appeared 10 mins after 5HT application. We also tested whether the effects on excitability were mediated by 5-HT2 receptors. In the presence of the 5-HT2-receptor antagonist, ketanserin, 5-HT significantly decreased excitability compared to 5-HT or ketanserin alone in both sham-operated and cochlear-ablated rats P12-21. However at P30-35, ketanserin had no effect in sham-operated and only a modest effect cochlear-

ablated animals. The 5HT2-specific agonist 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) also had no effect at P12-21. These results suggest that 5-HT likely regulates pyramidal cell excitability via multiple receptor subtypes with opposing effects. These data also show that early sensorineural hearing loss affects the ability of 5-HT receptor activation to modulate A1 pyramidal cell excitability.

2. Introduction

Hearing impairment during development produces significant changes in the acquisition of speech, sound discrimination, and cognitive function that may permanently diminish auditory perceptual skills and compromise language acquisition (Emmorey et al. 2003; Kidd and Bavin 2002; Psarommatis et al. 2001; Sanes and Bao 2009). While hearing loss in animal models of deafness has been associated with numerous alterations in the cell biology and physiology of brainstem and cortical neurons, the mechanisms that underlie the changes in the primary auditory (A1) cortex remain largely unexplored. Understanding which elements of neural plasticity are engaged or perturbed in the CNS following sensorineural hearing loss (SNHL) is important, since such mechanisms may represent sites for novel clinical intervention strategies to improve or restore perceptual skills.

Perinatal bilateral hearing loss has been shown to increase the intrinsic excitability of auditory cortical neurons, decrease the strength of inhibition,

decrease adaptation, and increase the strength of excitation in young gerbils (Kotak et al. 2007; Kotak et al. 2008; Xu et al. 2007). While these results strongly indicate that hearing loss raises overall cortical excitability and they also raise questions as to what other aspects of cortical physiology are influenced by early hearing loss. In particular, the normal development of the auditory cortical circuit depends on both sensory stimulation and modulatory systems that gate synaptic and intrinsic plasticity. For example, it is well documented that the tuning of cortical neurons and the organization of the tonotopic map in A1 depends on cholinergic afferents that arise from the nucleus basalis in the basal forebrain (Bakin and Weinberger 1996; Kilgard and Merzenich 1998; Kilgard et al. 2001) and on dopaminergic afferents from the ventral tegmental area (Bao et al. 2001). Two other neuromodulatory systems known to innervate A1 neurons include serotonin (5-hydroxytryptamine, 5-HT) and noradrenaline (Campbell et al. 1987), but the role of these systems in regulating cortical plasticity, either in the normal or compromised auditory system, have not been actively explored. Ji et al (Ji and Suga 2007) found that application of 5-HT in the bat auditory cortex can suppress or potentiate fear-induced plasticity of acoustic response areas, implicating serotonergic systems in the regulation of cortical plasticity according to behavioral context.

Clear evidence exists regarding the influential role of the serotonergic system during brain development, where it affects cellular proliferation, migration and differentiation, synaptogenesis, and apoptosis (Azmitia 2001; Lauder 1990). However, 5-HT also plays an important role in normal auditory processing in the

adult brain. In humans, depletion of the 5-HT precursor, tryptophan, decreases the intensity dependence of auditory evoked magnetic N1/P2 dipole source activity (Kahkonen et al. 2002a; Kahkonen et al. 2002b) and modulates auditory selective attention (Ahveninen et al. 2003). Serotonin also modulates auditory cortical evoked potentials (Dierks et al. 1999; Hegerl and Juckel 1993; Juckel et al. 1997). While 5-HT plays a crucial role in human auditory processing, the mechanisms by which 5-HT modulates auditory cortical activity at the cellular level are unknown. The effects of 5-HT are mediated by more than fourteen receptor subtypes (Hoyer et al. 2002), many of which can be found in the developing cerebral cortex. The 5-HT2A and 5-HT3 receptors are highly expressed by post mitotic-neurons of the cerebral cortex (Johnson and Heinemann 1995; Vitalis and Parnavelas 2003), whereas 5-HT1A, 5-HT2B and 5-HT3 are localized to the ventricular zones (Johnson and Heinemann 1995). Serotonin has been shown to affect the electrical excitability of neurons in several cortical areas, although the effects seem to be region-specific. In prefrontal cortex, 5-HT2A and 5-HT7 receptors mediate depolarization during first two postnatal weeks whereas 5-HT1A receptors mediate hyperpolarization during the third week (Beique et al. 2004). In the lateral entorhinal cortex, 5-HT reduces input resistance and hyperpolarizes layer II/III neurons through potassium channels coupled to 5-HT1A receptors (Grunschlag et al. 1997). In the medial entorhinal cortex, 5-HT evokes a biphasic response, first hyperpolarizing neurons via 5-HT1A receptors and then depolarizing neurons by an Ih channel-dependent mechanism (Ma et al. 2007). The role of 5-HT in regulating cortical

excitability therefore depends on the developmental trajectory and activity-dependent expression of both specific receptor subtypes and target ion channels.

Existing evidence cited above suggests that 5-HT can developmentally regulate cortical neuron excitability, and that 5-HT may play a role in auditory plasticity in adults. On the other hand, sensory activity may also regulate the developmentally programmed establishment of adult-like 5-HT receptor expression and function. We tested this hypothesis by investigating how 5-HT neurotransmission and 5-HT2 receptors influence the intrinsic excitability of layer II/III pyramidal neurons in primary auditory cortex (A1), and how the effects of 5-HT are modified by pre-hearing bilateral cochlear ablations at P8 in rats.

3. Materials and Methods

All protocols for cochlear ablation, sham surgeries, and brain slice preparation were reviewed and approved by the University of North Carolina, Chapel Hill Institutional Animal Care and Use Committee. These experiments report on results from 14 normal P12-21 rat pups, 12 sham surgery P12-21 pups, 8 cochlear-ablated P12-21 pups, 4 sham surgery P30-35 rats, and 4 cochlear ablated P30-35 rats.

Cochlear ablations

Cochlear ablations were performed in postnatal day 8 (P8) Sprague-Dawley rat pups. The pups were anesthetized with ketamine-xylazine (80 mg/kg, 8 mg/kg, IP, respectively), and after anesthesia was confirmed (no withdrawal induced by tail pinch), the surgical field was cleaned and made sterile. The

incision site was cleaned with chlorhexidine soap scrub and a retro-auricular incision was made in the skin and dissection carried down to the tympanic bulla. The middle ear was entered and ossicles removed, after which a small hole was made in the cochlear wall and the contents removed with small forceps. A small piece of Gelfoam was then placed in the cavity and the wound closed with 5-0 proline suture threads. Ablations were performed bilaterally. Following surgery, the pups were given ketoprofen analgesic (5mg/kg), and warmed on a heating pad until they were ambulatory. They were then returned to their home cage. Pups were then reared with their mothers until they were used for experiments (P12-P21), or were weaned at P21 and raised in groups of 4 or fewer until they were tested at P30-35. Sham surgeries were also performed, in which the anesthesia, skin incision and wound closure were the same as for cochlear ablations, but the bulla was not invaded, the ossicular chain was not removed, and the cochlea was not ablated.

Prior to each slice experiment, the animals were first tested for a Preyer's reflex (Jero et al. 2001), anesthetized with ketamine-xylazine (80 mg/kg, 8 mg/kg, IP, respectively), decapitated and the brain removed. In a subset of experiments, the Preyer's test was supplemented with auditory brainstem evoked response measures to confirm hearing loss. In all experiments, the inner wall of the cochlea was also observed under a dissection microscope to confirm the absence of cochlear tissue and the persistence of the Gelfoam insert. Therefore the recordings were not performed blind. Rats without any surgery (normal), sham-surgery rats, and cochlear-ablated rats were recorded when they were

P12-21 or at P30-35. Recordings from rats with cochlear ablations were then compared to aged-matched sham-operated controls. Following surgery, no vestibular complications were observed, as would be indicated by balance or overall motor activity, suggesting that the vestibular apparatus was not significantly affected by the surgical procedure.

Auditory cortex brain slice recordings

Following decapitation, the brain was immersed in ice-cold (4℃) cutting solution, blocked to a region containing A1, and 400 µm thick slices cut with a tissue slicer (Leica VT1000-S, Leica Microsystems, Bannockburn, IL). All slices were cut along the plane of the auditory thalamocortical fibers (Cruikshank et al. 2002; Metherate and Cruikshank 1999). Two sections, starting at least ~ 400 µm dorsal to the rhinal fissure, were selected for study. Slices were transferred to an incubation chamber, maintained at 34℃ for 30 minutes, thereafter incubated at room temperature (~22℃) until recording. The standard slicing, incubation and recording solution was an artificial cerebrospinal fluid (aCSF) that contained (in mM) 134 NaCl, 3.0 KCl, 2.5 CaCl2, 1.3 MgCl2, 1.25 KH2PO4, 10 glucose and 20 NaHCO3, 0.4 ascorbic acid, 2 sodium pyruvate, and 3 myoinositol. Slicing in P12-21 rats was performed in this standard solution. Slicing in P30-35 rats was done in an NMDG-based solution to increase neuron survival (Tanaka et al. 2008), and afterwards the slices were incubated and recorded in the standard aCSF. All solutions were continually equilibrated with 95% O2-5% CO2, setting the pH to 7.3-7.4. During recording, the slices were bathed at 34℃ in a submersion-type recording chamber on the stage of an upright fixed-stage

microscope (Zeiss FS-2). The preparation was viewed with a 40X, 0.75NA water immersion objective, using video-enhanced differential interference contrast illumination in infrared light. Whole-cell current-clamp recordings were obtained from layer II/III pyramidal neurons in A1 using Multiclamp 700A and 700B amplifiers, (MDS Analytical Technologies, Toronto, Canada). Recording electrodes were pulled (P-2000, Sutter Instruments, Novato, CA) from 1.5 mm dia. KG-33 glass (Garner Glass, Claremont, CA) to a tip diameter of ~1 µm, ends were fire polished and tips were coated with Sylgard 184 (Dow Corning, Midland, MI). The pipettes were backfilled with a solution containing (in mM) 130 K-gluconate, 4 NaCl, 0.2 EGTA, 10 HEPES, 2 Mg2ATP and 0.3 Na3GTP, 10 phosphocreatine, pH 7.2 with KOH. Membrane potentials were corrected for the measured junction potential (-12 mV for gluconate) between the electrode and bathing solutions. Data were acquired from neurons with a resting potential negative to 50mV and with overshooting action potentials. Three separate protocols were tested on each cell. First, complete current-response curves were obtained for 500-msec duration test pulses, to measure both the input resistance and the frequency-current (F-I) curve. Second, action potential threshold was measured in some experiments by injecting a 5 msec current pulse at multiple levels. The brief pulses were alternated with a ¼ -amplitude hyperpolarizing pulse, which was then scaled and added to the action potential during analysis to remove the passive component of the response. Action potential threshold was measured as the point of voltage inflection on the rising phase of the action potential that exhibited the maximum point of curvature (Erisir et al. 1999). In the

experiments in older animals, the brief pulse failed to consistently elicit action potentials during the drug treatment, in which case measurements were taken from trains of action potentials during longer current pulses (as in (Francis and Manis 2000). Third, when drugs were washed onto the slice or washed out, a current pulse that produced an average of 5 action potentials in control conditions was presented every 20 seconds, and changes in excitability monitored. The current-voltage and current-firing relationships were also measured at the end of each solution wash period.

The primary auditory cortex (A1) was first located at low-magnification (4 or 5X) and the recording electrode position was established in layer II/III. Neurons were visually identified using infrared-differential contrast optics at 40X magnification and whole cell current-clamp recordings were then obtained following formation of a tight-seal. Layer II/III pyramidal neurons were selected for recording and identified by their electrophysiological characteristics. Fast-spiking neurons and bursting neurons were excluded from analysis.

5-HT and 5-HT2 receptor electrophysiology

To investigate the effects of 5-HT on intrinsic excitability, serotonin hydrochloride (Sigma-Aldrich, St. Louis, MO) was bath applied at 50µM to each slice, using the protocol in Figure 1. This concentration was chosen to be in the middle of the dose-response curve for modulation of synaptic currents in the brain slice (Tanaka and North 1993). To investigate the role of 5-HT2 receptors, the 5-HT2 receptor antagonist ketanserin tartrate (Sigma-Aldrich) was bath

applied at 1μM, a concentration high enough to ensure nearly complete receptor block (Beique et al. 2004; Shapiro et al. 2000). The excitability of each recorded cell was measured before and during a series of drug applications. After an initial period in control aCSF lasting 5 minutes, 50μM 5-HT was bath applied to each slice for 5 minutes. Following the application of 5-HT, the drug was washed out for 10-15 minutes and ketanserin was then applied alone for 5 minutes and in combination with 5-HT for another 5 minutes. A final aCSF wash lasted 10 min. The same protocol was used for all age and surgery groups. In an independent set of experiments, the 5-HT2 receptor agonist, 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT, Sigma-Aldrich) was bath applied at 5μM, a concentration that is above the Kact for phosphotidyl inositol production from 5HT2A receptors, but which is below saturation (Shapiro et al. 2000). Because 5-HT can initiate long-lasting signal transduction cascades, only one cell was studied from each slice.

Data acquisition and analysis

Electrophysiological data were acquired using custom-written scripts in MATLAB (Version 7.0-7.6, Nantuck, MA) with the Data Acquisition Toolbox and high-speed 12 or 16-bit A/D, D/A boards (National Instruments, Austin TX). Analysis was performed with MATLAB using custom routines and statistical analysis was performed with Prism 5.0 (Graphpad, San Diego, CA). Frequency-current plots were collected in steps of 20pA or 50pA for younger and older rat experiments respectively, and interpolated onto a common scale. Input resistance was measured as the maximum slope in the region of the current-

voltage relationship just below resting potential. Spike rate adaptation ratios were calculated as the mean of the last two interspike intervals divided by the first interspike interval, for firing rates between 8 and 20 Hz (4 to 10 spikes elicited by a 500 msec depolarizing pulse). The sag in the hyperpolarizing direction was measured as the steady-state voltage divided by the peak voltage, for peak voltages between -90 and -110 mV during hyperpolarizing currents, as described by Fujino and Oertel (Fujino and Oertel 2003). Action potential properties such as after-hyperpolarization depth, action potential height, maximum rising slope, width at half-amplitude and threshold were measured from isolated spikes elicited by a brief current pulse, or from trains of spikes elicited by 500 msec depolarizing current steps, as discussed above. For calculations of significance of resting membrane potential and input resistance, paired (where required) and unpaired Student's 2-tailed t-test were used. For calculations of significance of F-I curves a two-way ANOVA was used followed by a Bonferroni post-hoc test (DF=degrees of freedom, F=F ratio). Whenever possible, ANOVA's were computed using repeated measures across treatments in single cells. However, in a few cases cells could not be recorded for the entire duration of the protocol, and so there were unequal numbers of observations across experimental treatments. In these cases, the two-way ANOVA's were computed without matching, and the number of observations for each treatment group is given. Results are presented as mean and standard error.

4. Results

Recordings were made from “normal” animals, in which the cochleae were not surgically manipulated, “sham” animals, which were subject to sham surgery, and “ablated” animals with bilateral cochlear ablations. Neurons were only included in the final analysis if they met criteria for input resistance (at least 40 MOhm), resting potential (negative to -50 mV) and spike height (more than 80 mV, as measured from rest). In this section, we will first discuss the effects of 5-HT on normal A1 neurons. We then present the effects of the sham surgery and cochlear ablations. We conclude with an examination of the effects of cochlear ablations on the responses of A1 neurons to 5-HT.

5-HT modulation of intrinsic excitability in normal A1

Serotonin has been shown to affect the intrinsic properties of neurons in several cortical areas, with specific regional effects (see Introduction). To investigate how 5-HT affects intrinsic firing in A1, responses to intracellular current pulses were collected from neurons in normal A1. Application of 5-HT decreased the number of spikes evoked by depolarizing current injections (Fig. 2AB; 2-way ANOVA, F-I in aCSF vs. F-I in 5-HT, DF=1, F=12.25, P=0.0006 n=17). Serotonin produced a rightward shift in the F-I relationship that was largest for currents just above spike threshold, and was statistically significant for the 80 and 100 pA steps ($p < 0.05$; Bonferroni posttest). Serotonin produced no

difference in firing rates at the highest currents tested. Serotonin did not affect the resting membrane potential (Fig. 2C, aCSF: –65.8 ± 1.3 mV; 5-HT: -64.7 ± 2.6 mV, n=19, P=0.47, paired t-test), or the sag seen with hyperpolarizing pulses (see Supplemental Table 1). However, it did reduce input resistance (Fig. 2D). Immediately after establishing whole-cell recordings, and prior to the application of 5-HT, the mean input resistance was 178.5 ± 22.6 MΩ. At the end of a 5-minute application of 5-HT, the input resistance fell to 132.5 ± 14.7 MΩ (P=0.020, paired t-test; n=19). Serotonin also significantly decreased the adaptation ratio (Fig 2E) from 2.7 ± 0.2 to 2.2 ± 0.2 (P=0.004, paired t-test, n=17), We conclude that 5-HT modulates the excitability of A1 layer II/III neurons in three ways: it decreases the firing rate, the input resistance, and firing rate adaption.

Effects of sham surgery

Since the cochlear ablations were performed in neonatal P8 rat pups, we were concerned that the surgery alone might influence cortical development. Therefore, before testing for the effects of 5-HT in cochlear-ablated animals, we first evaluated the effect of surgery on A1 neurons, by comparing the physiology of cells from normal and sham surgery controls. Surprisingly, the sham surgery significantly decreased the number of spikes (Fig. 3A,B) evoked by depolarizing current injections at all levels (2-way ANOVA, F-I in normal vs. F-I in sham, DF=1, F=15.5, P<0.0001). However, the changes in spike rate were not accompanied by significant changes in the resting membrane potential, input resistance, or the spike adaptation ratio (Fig. 3C-E, also see Table 1 in Supplementary Data). Our results suggest that removal of the pup from the nest,

anesthesia, and recovery from anesthesia during surgery is sufficient to produce an effect on cortical physiology, and specifically, electrical excitability, in these young animals. Thus, in subsequent experiments, comparisons were made between the sham and ablated surgery groups, which differed only in the surgical removal of the cochlea.

Effects of cochlear ablation on excitability in rat A1

Kotak et al. (2005) showed that layer II/III neurons from animals with SNHL had an increased intrinsic excitability when compared to normal animals. The changes in excitability seen in the animals with sham surgeries prompted us to reevaluate the changes due to cochlear ablations, by comparing effects of bilateral cochlear ablation on the excitability of layer II/III A1 neurons to sham surgery controls. Cochlear ablation resulted in significantly increased firing rate of neurons when compared to shams (Fig. 3A,B 2-way ANOVA, F-I in sham n=27 vs. F-I in ablated n=20, DF=1, F=49.1, $P<0.0001$). The mean firing rate was elevated at all current levels, and was significant for all currents >= 100 pA ($P < 0.05$, Bonferroni posttest). Consistent with the results of Kotak et al. (Kotak et al. 2005), the firing rate also was elevated when compared to normal hearing animals (Fig. 3A,B 2-way ANOVA, F-I in 17 cochlear ablated rats vs. F-I in 17 normal rats, DF=1, F=6.44, P=0.012). We conclude that the increase in excitability caused by cochlear ablation is still evident when compared to sham surgery, and thus is not solely the consequence of other aspects of the surgical procedure. However, paradoxically, the amplitude of the action potentials in the sham animals was significantly larger than that in the ablated animals

(Supplemental Table 1, unpaired t-test, P=0.034), while no other aspects of the action potential shape were different.

Effects of cochlear ablation on 5-HT modulation of excitability

We next assessed the effects of SNHL on 5-HT modulation of excitability, by testing the effects of bath-applied 5-HT (50μM) on cells from cochlear ablated and sham operated rats. In contrast to its effects in normal animals (Fig. 2), 5-HT did not alter the number of spikes produced by neurons from sham animals aged P12-21 (Fig. 4A,C, 2-way ANOVA, F-I in aCSF vs. F-I in 5-HT, DF=1, F=0.34, P=0.56, n=21). These results in sham animals were obtained in two separate experimental series performed over a year apart, on different setups and by different individuals. Since both series showed the same lack of effect of 5-HT compared to contemporaneous controls and were not different from each other, the data from the two series have been combined. In addition, 5-HT had no effect on the intrinsic excitability of cells from animals with cochlear ablations (Fig. 4B,D, 2-way ANOVA, F-I in aCSF vs. F-I in 5-HT, DF=1, F=2.23, P=0.13 n=15).

Figure 5 summarizes the measurements of resting potential, input resistance, and adaptation ratio through the protocol shown in Figure 1 for each of the experimental conditions for both age groups. While the effects of 5-HT in the sham and ablated P12-21 animals do not always reach statistical significance, the overall pattern of membrane potential and input resistance changes resembles that of the normal group, suggesting that there may be an

attenuated response to 5-HT. Similar to its effect in normal hearing animals, 5-HT reduced the input resistance of neurons from both sham (P = 0.023, two-tailed t-test) and ablated animals (P = 0.020, two-tailed t-test; Fig 5B, left). Serotonin significantly decreased the adaptation ratio of neurons in ablated animals (Fig 5C, left, Supplementary Table 1, P = 0.0002, two tailed t-test), but not in sham animals.

Previous work has shown that activation of different 5-HT receptor subtypes can trigger depolarizing or hyperpolarizing membrane potential responses in various CNS neurons (Andrade and Chaput 1991; Andrade and Nicoll 1987; Araneda and Andrade 1991; Chapin and Andrade 2001; Tanaka and North 1993). Previously, we found that 5HT2 receptors are highly expressed in layer II/III neurons of the auditory cortex (Basura et al. 2008). We therefore used ketanserin (a 5-HT2 receptor antagonist, 1µM) to block the effects of 5-HT on 5-HT2 receptors. In the absence of exogenous 5-HT, blocking 5-HT2 receptors with ketanserin did not affect firing of neurons from sham (Fig. 4A,C 2-way ANOVA, F-I in aCSF vs. F-I in Ket, DF=1, F=0.58, P=0.45 n=11) or cochlear ablated animals (Fig. 4B,D 2-way ANOVA, F-I in aCSF vs. F-I in Ket, DF=1, F=0.67, P=0.42 n=8). This suggests that the basal tone of 5-HT in the slice is not sufficient to drive changes in excitability or firing. Interestingly, when ketanserin was applied to the bath concurrently with 5-HT (50µM), the number of spikes in neurons from sham animals decreased relative to ketanserin (Fig 4A,C 2-way ANOVA, F-I in Ket vs. F-I in Ket+5HT, DF=1, F=45.3, P<0.0001 n=11), and was significantly reduced for currents between 100 and 140 pA (P < 0.05, Bonferroni

posttest). Moreover, in the presence of ketanserin, 5-HT markedly reduced the firing rates in cochlear-ablated animals below the rates evoked in ketanserin alone (Fig. 4B,D 2-way ANOVA, F-I in Ket vs. F-I in Ket+5HT, DF=1, F=30.0, P=0.0001 n=8). The rate reduction was significant for currents between 80 and 120 pA ($P < 0.05$, Bonferroni posttest). We conclude that blocking 5-HT2 receptors with ketanserin unmasks an action of 5-HT on other 5-HT receptor subtypes, and that this effect is not changed by cochlear ablation in animals tested at P12-21.

To test the hypothesis that specific activation of 5-HT2 receptors affects excitability, we bath applied a 5-HT2 receptor agonist 5-MeO-DMT (5μM) in 0.1% DMSO. DMSO alone had no effect on the firing rate. Similarly, 5-MeO-DMT did not affect the firing rate in normal neurons (P16-18) (Fig 6, 2-way ANOVA, F-I in DMSO vs. F-I in 5-MeO-DMT, DF=1, F=0.023, P=0.88; N = 5 cells). Although the spike rate was not changed by 5-MeO-DMT, the input resistance decreased (97.5 ± 11.0 Mohm in DMSO vs 79.1 ± 11.6 Mohm 5-MeO-DMT, two-tailed t-test, P = 0.0007). However, the adaptation ratio did not significantly change. These results suggest that stimulation of 5-HT2 receptors does not influence the firing rate of A1 neurons.

Developmental effects of hearing loss on 5-HT2 modulation of excitability

Neurons in the auditory cortex exhibit several prominent changes during development. In rat, the tonotopic map undergoes significant refinement between P14 and P22 (Chang and Merzenich 2003; de Villers-Sidani et al. 2007; Zhang et

al. 2001). The bandwidths of excitatory receptive fields continue to decrease and the ability of neurons to follow repetitive stimuli improves until at least P35 (Chang et al. 2005; Chang and Merzenich 2003). At the cellular level, there is an overall decrease in neural excitability and a rightward shift in the F/I curves of auditory cortical neurons at P19–P29 compared to P10-P18 (Oswald and Reyes 2008). The data we have presented so far (up to P21) was collected prior to an important developmental turning point. Consequently, we next examined an older group of P8 sham-operated and cochlear-ablated animals using the same paradigm used for the P12-21 group.

First we tested if the increased firing rate of neurons following hearing loss persists to P30-35. However, we could find no difference in excitability due to hearing loss between sham and cochlear-ablated animals at P30-35 (Supplementary Figure 1; 2-way ANOVA, F-I in sham vs. ablated, DF=1, F=0.83, P=0.37, n=3 sham and 6 ablated cells). Futhermore, the difference in action potential height seen in P12-21 animals was absent in the older animals (Supplementary Table 1, unpaired t-test, P=0.49). We next asked whether 5-HT2 modulation of excitability is present only transiently during P12-21 or persists to P30. Serotonin did decrease the firing rates of neurons from sham animals 5 minutes after application (Fig. 7C, 2-way ANOVA , F-I in aCSF vs. 5-HT, DF=1, F=8.56, P=0.0062, n=4). However, in contrast to younger animals, there was an even stronger suppression of firing that appeared 10-15 minutes after 5-HT was washed out (Fig. 7C, 2-way ANOVA , F-I in aCSF vs Wash1, DF=1, F=7.00, P=0.011, n=4 aCSF and n=3, Wash1). In cochlear ablated P30-35 animals

however, 5-HT did not suppress firing either during application, or 10-15 minutes later (Fig. 7B,D 5-minutes: 2-way ANOVA, F-I in aCSF vs. F-I in 5-HT, DF=1, F=0.5, P=0.48; 10-15 minutes, FI in aCSF vs. Wash 1, DF-1, F=1.87, P = 0.17). In contrast to these effects on the F-I relationship, the effects of 5HT on resting potential, input resistance, and the adaptation ratio were small and not significant (Fig. 5, right). Thus, at this later time point, 5-HT was ineffective in depressing the excitability of cells after cochlear ablation.

In these same cells, we also tested if age affected 5-HT2 receptor modulation of excitability following cochlear ablation using the paradigm shown in Figure 1, with simultaneous application of ketanserin (1µM) and 5-HT (50 µM). Application of ketanserin alone did not alter firing in sham animals (Fig. 7A,C 2-way ANOVA, F-I in Wash1 vs. F-I in Ket, DF=1, F=1.75, P=0.19). Simultaneous application of ketanserin and 5-HT also did not alter firing (Fig. 7A,C 2-way ANOVA, F-I in Wash1 vs. F-I in Ket, DF=1, F=0.14, P=0.71). In contrast to our findings in younger neurons (Fig. 4), application of ketanserin alone significantly decreased neuronal firing in cochlear-ablated animals (Fig. 7B,D 2-way ANOVA, F-I in Wash1 vs. F-I in Ket, DF=1, F=5.75, P=0.021). This result is surprising, given that 5-HT alone had no effect in these animals. A subsequent 5-minute application of 5-HT in the presence of ketanserin however produced an additional small but significant decrease in firing (Fig. 7B,D 2-way ANOVA, F-I in Ket vs. F-I in Ket+5HT, DF=1, F=5.19, P=0.028). These results suggest that non-5HT2 receptors are present and can regulate the intrinsic excitability. They are also suggest, in comparison with the P12-21 cells, that there is a developmental shift

in the expression of 5-HT receptor subtypes or their signaling mechanisms that is further affected by cochlear ablation.

5. Discussion

The results of these experiments may be summarized as follows. First, we have shown that a sham surgery has an effect on the excitability of auditory cortical neurons. Second, we have confirmed that rat auditory cortical layer II/III pyramidal cells show increased excitability with hearing loss, even when compared to sham controls. Third, we show that 5-HT decreases excitability in P12-21 normal auditory cortex. Fourth, we found that both sham surgery and cochlear ablation occlude the ability of 5-HT to decrease excitability. However, in the presence of ketanserin to block 5-HT2 receptors, 5-HT can still further decrease excitability, suggesting that 5-HT likely operates through two receptor systems with opposing actions on excitability. Finally, electrical excitability is the same in sham and ablated animals at P30-35, or 21-27 days after the cochlear ablation. However, the modulation of excitability by 5-HT is blunted in the animals with hearing loss. Overall, our results suggest that 5-HT plays a functional role in regulating cellular excitability in A1 and that this role is both developmentally regulated. In addition, the ability of 5-HT to modulate the intrinsic excitability of auditory cortical neurons depends on the hearing status of the animal. These experiments also raise a cautionary note regarding comparisons between cochlear ablated and non-operated experimental groups.

Effects of SNHL on intrinsic properties

The present data demonstrate increased pyramidal cell excitability in A1 layer II/III neurons following bilateral cochlear ablation (Fig. 3), as a model of early-onset SNHL, confirming similar findings in gerbils (Kotak et al. 2005). Kotak et al. (2005) reported cochlear ablation resulted in a decrease in adapting-type neurons and an increase in sustained-type neurons. Our results indicate that cochlear ablation increased the excitability of adapting-type neurons.

The underlying mechanisms contributing to increased excitability after cochlear ablation are unclear. In cochlear-ablated gerbils, A1 neurons display a depolarized resting potential, increased input resistance, and a higher incidence of sustained firing (Kotak et al. 2005). However, in the present study, resting potential, input resistance and spike rate adaptation were not affected either by sham surgery or cochlear ablations; only the relationship between injected current and the firing rate was altered. This is consistent with previous reports that deprivation of afferent input can result in changes in intrinsic excitability in cerebral cortex ((Desai et al. 1999; Maffei et al. 2004) but see (Maravall et al. 2004)) and in the cochlear nucleus (Francis and Manis 2000; Wang and Manis 2006). Such changes likely reflect sensory activity-dependent homeostatic mechanisms, perhaps driven by downregulation of BDNF and pCREB after hearing loss (Tan et al. 2008). Tan et al. (2008) also observed a reduction in sodium channel immunoreactivity, which should reflect channel availability, and might indicate reduced excitability. Our finding that cochlear ablation caused a significant decrease in the action potential height (and a trend towards a

decrease in the rising slope; Supplemental Table 1), which is primarily controlled by sodium channel density, is consistent with a reduction in sodium channel availability. The increased excitability, measured as the number of spikes for a given injection current, which we and Kotak et al (2005) have both observed, could in turn be due to a decrease in the activation of conductances that control the interspike interval of cortical pyramidal cells. Our data in older animals is also consistent with this overall argument, in that there was no change in action potential amplitude or in the firing rate for a given current between sham and ablated animals. We would expect that smaller action potentials could lead to less calcium influx, could decrease the engagement of calcium-activated potassium currents that regulate the slow afterhyperpolarization (Lorenzon and Foehring 1993) in cortical pyramidal neurons, and could lead to higher firing rates. Interestingly, and consistent with this argument, calcium-activated potassium currents have also been shown to be modulated by sensory experience (Maravall et al. 2004) in the somatosensory cortex during the early critical period between P12 and 17.

The role of homeostatic mechanisms in regulating excitability may be more complex than with simple deprivation, because it is unlikely that A1 is completely electrically silent after cochlear ablation. In the absence of auditory inputs, A1 has been shown to become responsive over time to both somatosensory and visual stimuli (Hunt et al. 2006; Kral 2007). This raises the possibility that part of the difference between the P12-P21 neurons and the P30-35 neurons, where the F-I curves were not different between sham and ablated

animals, is that the latter are engaged in a crossmodal sensory processing. This sensory activity may be sufficient to return the excitability of the neurons to their "normal" operating point.

On the other hand, the coupling between 5-HT receptors and modulation of neuronal firing is decreased in the ablated animals at P30-35, suggesting that the receptors failed to become engaged, either due to inadequate expression, dysfunctional coupling to their second messengers, or a mislocalization with respect to their target proteins. Thus, it appears that early hearing loss disrupts the serotonergic signaling system in auditory cortex, and in turn likely limits the serotonergic modulation of cortical function later in life. The manner in which these changes in the 5-HT systems affect subsequent cortical function and plasticity, for example with reintroduction of auditory activity with cochlear implants, remains to be studied.

Role of 5-HT and 5-HT2 Receptors in A1 neurons

Our observation that serotonin decreased neuronal firing in the normal auditory cortex suggests that one role of 5-HT is to suppress neural activity under conditions when serotonergic neurons are activated (Ji and Suga 2007). In contrast to the clear effects in normal animals, activation of 5-HT receptors did not alter firing rate or action potential shape in sham-operated P12-21 animals, although it did affect firing rate adaptation in cochlear-ablated animals. In both shams and cochlear-ablated animals, 5-HT reduced firing even in the presence

of the 5-HT2 receptor antagonist ketanserin, when compared to the effects of ketanserin alone. This suggests that the reduction in firing depends on non-5-HT2 receptors, and is consistent with our observation that the specific 5-HT2 agonist 5MeO-DMT did not affect the firing rate in normal cortex. It would appear that 5-HT2 receptors do not directly couple to ion channels in auditory cortex. One interpretation of these results is that there are two different serotonergic receptor systems that can regulate excitability in A1. This idea is supported by observations in pyramidal cells of the adult prefrontal cortex, which co-express 5-HT1A and 5-HT2A receptors (Martin-Ruiz et al. 2001). For example, parallel electrophysiological studies have shown that 5-HT1A receptors can mediate hyperpolarization while 5-HT2A receptors can cause depolarization (Araneda and Andrade 1991; Davies et al. 1987; Tanaka and North 1993). These systems affect excitability in opposite directions, such that when the 5-HT2 receptors are blocked by ketanserin, a separate class of 5-HT receptors drives a decrease in excitability. A similar regulatory interaction of 5-HT2A/C receptors on modulation by 5-HT1A receptors has been shown for N-methyl-D-aspartate receptors in prefrontal cortex (Yuen et al. 2008).

The decrease in input resistance and firing rate in auditory cortex might be driven by 5-HT1A receptors (Gurevich et al. 1990), which have been shown to activate outward currents mediated by GIRK channels (Luscher et al. 1997). 5-HT1A receptors are poorly expressed in the cerebral cortex immediately after birth, but increase in expression during the early postnatal period (Daval et al. 1987; Miquel et al. 1994). While perinatal layer V prefrontal cortical neurons can

be depolarized through the activation of 5-HT2A and 5-HT7 receptors, by the beginning of the third week of age in rats, the depolarization is replaced by a hyperpolarization mediated by 5-HT1A receptors (Beique et al. 2004). In our experiments, changes in membrane potential in the normal cells (Fig. 5A, left) are somewhat consistent with this pattern in that before the 3rd week of life, 5-HT produces a depolarization. In this respect, it is interesting that in the P30-35 sham group, there is a very strong effect of 5-HT alone, although this effect is delayed by many minutes as if it might be mediated through a slow second messenger cascade. However, the changes in firing with current injection, which were independent of membrane potential, suggest that an additional set of target mechanisms is involved in auditory cortex.

The acute decrease in firing rate adaptation in normal A1 (Fig. 5C, left) suggests that 5-HT receptors can modulate cortical information processing by modifying spike timing patterns. In particular, we observed a decrease in spike rate adaptation acutely in the presence of 5-HT in normal and cochlear-ablated animals at P12-21, and a similar (but not signficicant) trend in ablated animals at P30-35. A decrease in the adaptation ratio implies that the neuron may be signaling the steady-state component of its response to stimuli more than its transient response to the onset of the stimulus. That this occurs acutely and appears to be reversible (Fig. 5C), and is dissociated from the mean firing rate patterns, suggests that adaptation may be regulated by a separate mechanism that is coupled to the 5-HT receptors. Modification of rate adaption could be related to attentive or aroused states, where the presence of sustained discharge

patterns, as opposed to rapidly adapting firing, might be critical for auditory signal detection or discrimination (Ahveninen et al. 2003; Oranje et al. 2008).

Serotonin has been shown to play an important role in the developing mammalian brain (Hoyer et al. 2002; Lauder 1990). Consequently, a shift in the expression of these receptors during critical periods of development, as we have shown to be produced by both the sham surgery and by cochlear ablation in neonatal rats, could have long-lasting effects on cortical wiring and sensory processing. While many 5-HT receptor subtypes may be also be present and play roles in regulating excitability and synaptic strength (e.g., 5-HT1A, 5-HT3, and 5-HT7), the current data provide a foundation for focused pharmacological experiments that specifically isolate 5-HT receptor pathways and their contributions to A1 pyramidal cell activity in this model of SNHL. Such studies could provide understanding of the electrophysiological changes observed following bilateral cochlear ablation, and provide important pharmacological clues for methods to help restore auditory function and plasticity in hearing impaired children.

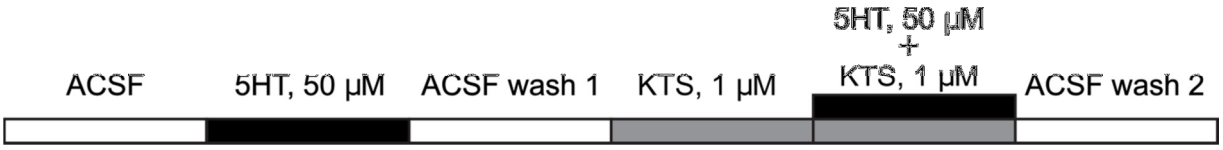


Figure 1. Experimental protocol for measuring neuronal excitability and the actions of 5-HT. ACSF: artificial cerebrospinal fluid, 5HT: Serotonin, KTS: Ketanserin. Each condition lasted 5 minutes except ACSF wash 1 and wash 2, which lasted 15 minutes and 10 minutes respectively.

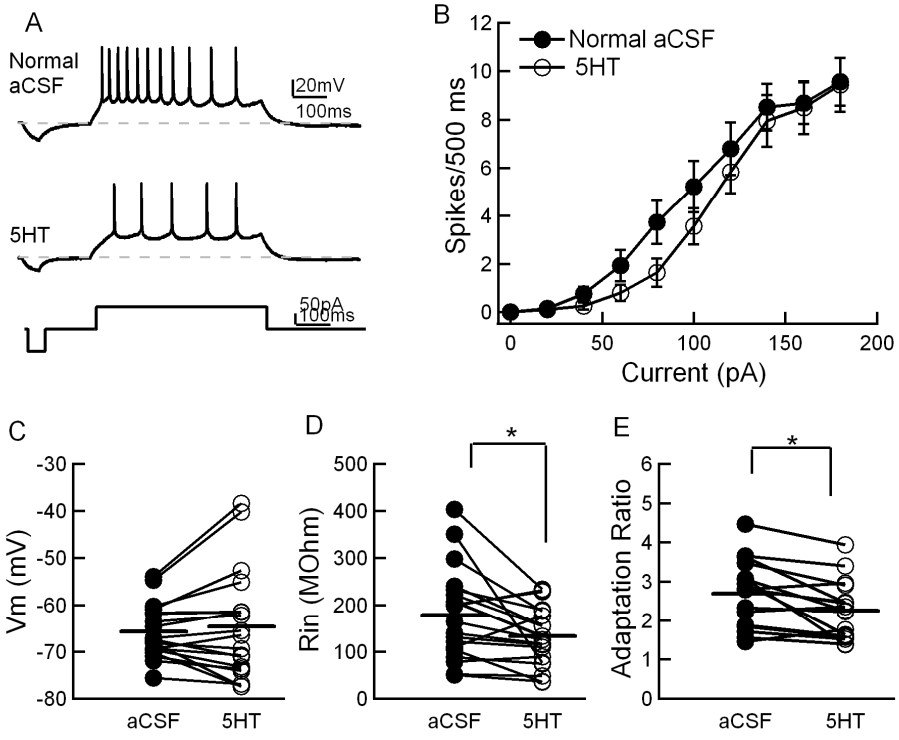


Figure 2. Serotonin decreases excitability of A1 neurons in normal rats. A. Voltage responses of an example neuron to 100pA depolarizing current steps. The current protocol is shown below the voltage traces. Dashed grey lines indicate resting membrane potential. B. Number of spikes evoked by depolarizing current injections for a population of neurons in aCSF (closed circles) and in presence of 50µM 5-HT (open circles). Serotonin decreased the number of spikes for currents just above threshold. Error bars are 1 SEM. C. Serotonin did not affect the mean resting membrane potential of neurons. D. Serotonin significantly reduced the mean input resistance of neurons. E. Serotonin

significantly decreased the adaptation ratio of neurons. Horizontal lines in C,D,E show the mean of each group. Statistical significance * = $P < 0.05$.

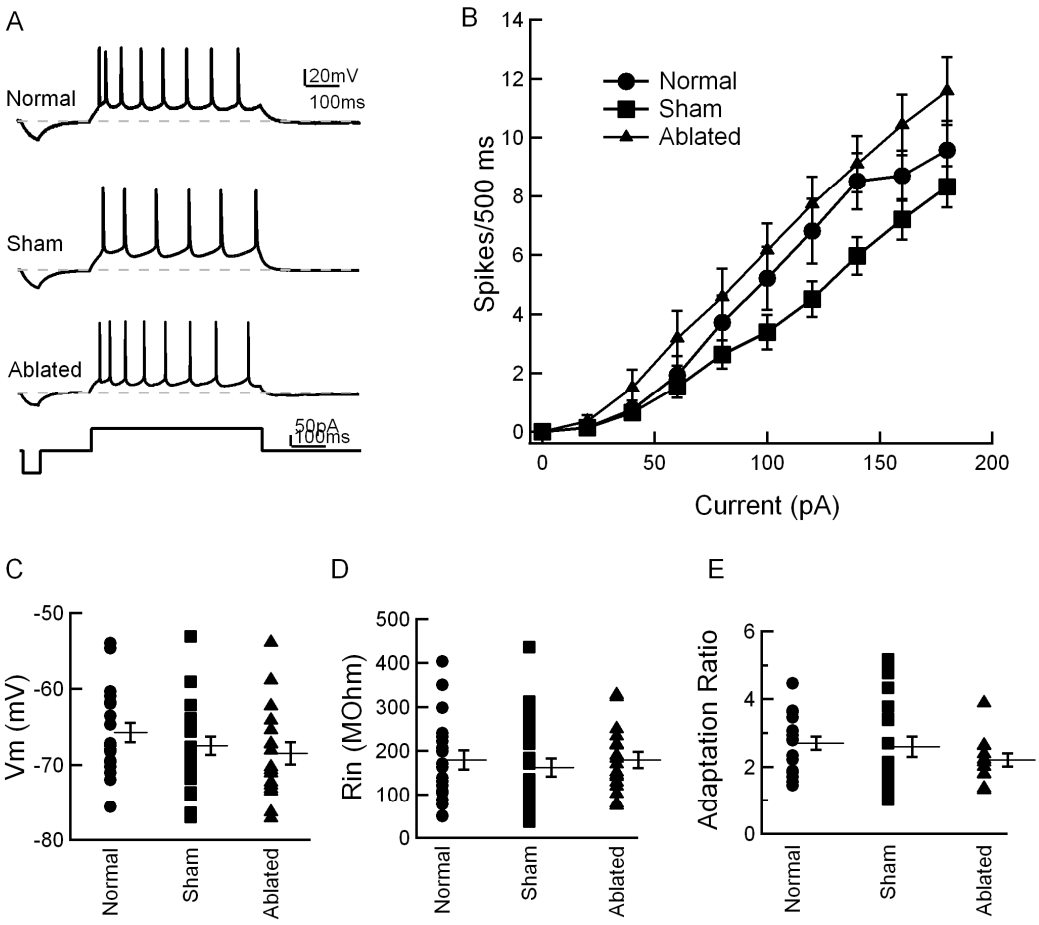


Figure 3. Sham surgery decreases, while cochlear ablation increases, excitability in P12-21 rat A1. A. Voltage responses of neurons in response to 100pA depolarizing current steps. Dashed grey lines indicate resting membrane potential; the current protocol is shown below the voltage traces. B. Spike count as a function of depolarizing current amplitude in neurons from normal (closed circles), sham (closed squares) and cochlear ablated animals (closed triangles). Sham surgery significantly decreased the firing rate as compared to normal A1. Cochlear ablation increased the firing rate compared to both shams and normals. C-E: Sham surgery and cochlear ablation did not affect mean resting membrane

potential,(C), input resistance (D) or adaptation ratio (E). Horizontal lines in C,D,E represent the mean of each group and vertical lines represent 1 SEM.

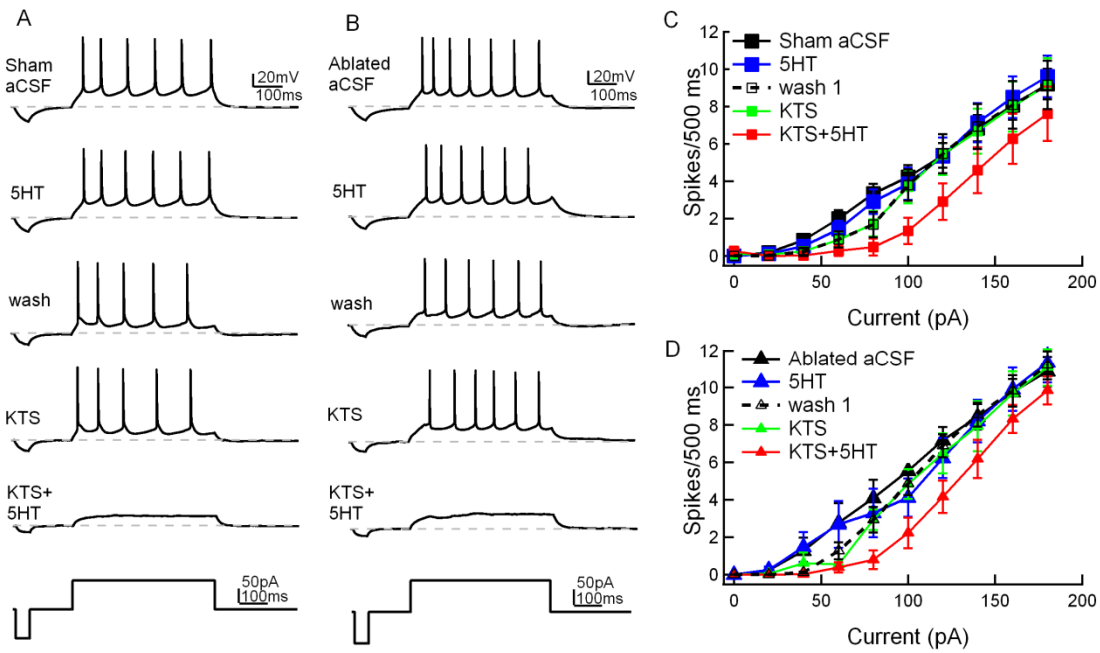


Figure 4. Cochlear ablation does not affect 5HT modulation of excitability in P12-21 rat A1. A. Voltage traces from two example neurons from sham animals in response to a 100pA depolarizing current step under different drug conditions (as in Fig. 1). Dashed grey lines indicate resting membrane potential; current injection is shown below the voltage traces. Traces in aCSF and 5HT are taken from a different neuron than those in wash, KTS and KTS+5HT. B. Voltage traces as in A from two example neurons from cochlear ablated animals. C. Mean firing rates evoked by depolarizing current injections inform a population of sham rats: aCSF (squares), in presence of 50µM 5-HT (blue squares), after wash 1 (open squares with dashed line), in 1 µM ketanserin (green squares) and in ketanserin+5HT (red squares). Neither serotonin nor ketanserin changed the

firing rate. In the presence of ketanserin, serotonin significantly decreased firing rate. D. Data for cochlear ablated rats, in the same format as panel C.

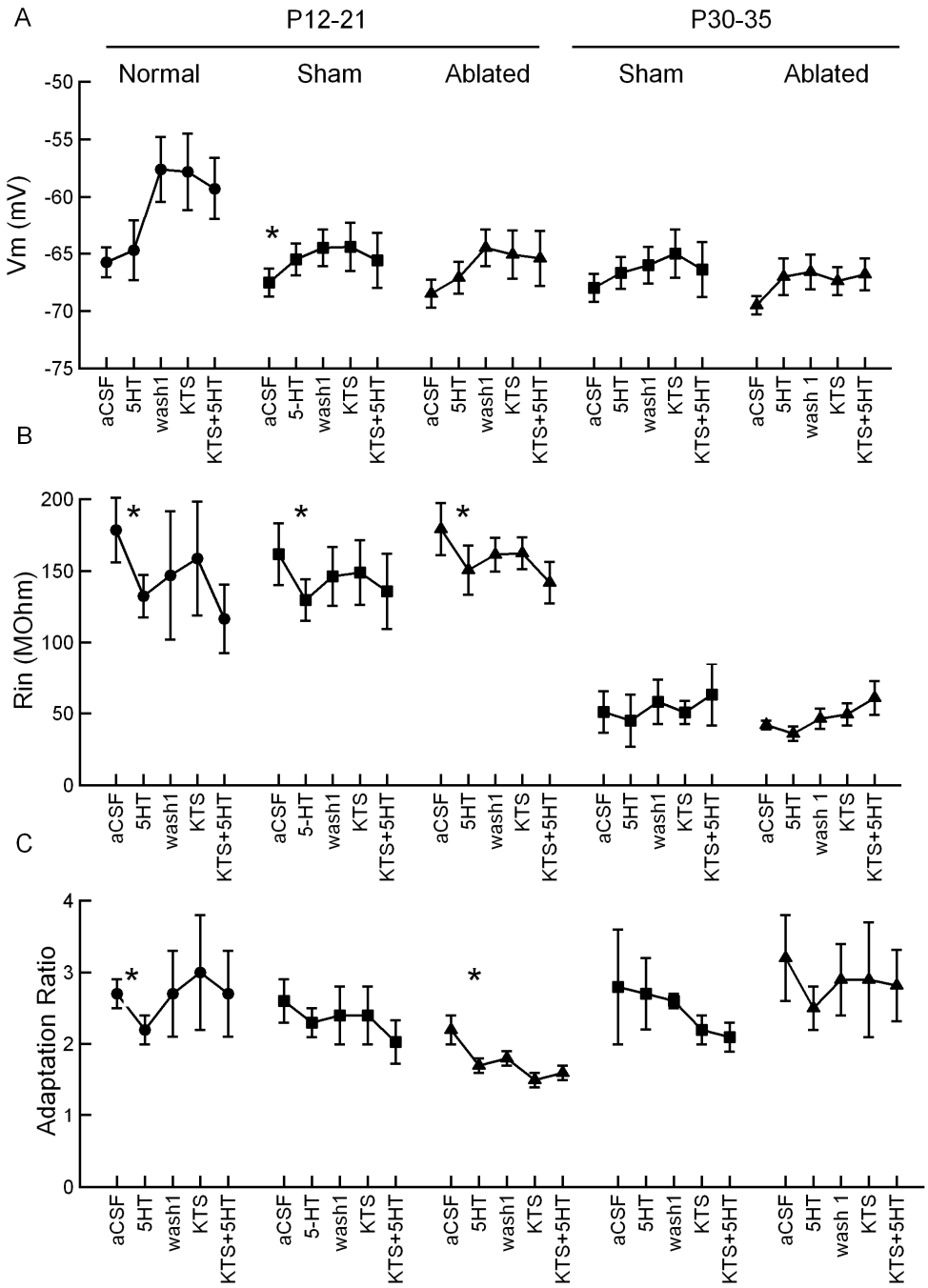

A
P12-21
P30-35
Normal
Sham
Ablated
Sham
Ablated
Vm (mV)
-50
-55
-60
-65
-70
-75
*
aCSF
5HT
wash1
KTS
KTS+5HT
B
Rin (MOhm)
200
150
100
50
0
C
Adaptation Ratio
4
3
2
1
0
wash 1

Figure 5. Summary of changes in resting potential, input resistance and adaptation ratios for each experimental group. In each row, data from normal (unoperated) cells are shown in filled circles, data from shams with squares, and data from ablated animals with triangles. B. Effects of 5HT pharmacology on input resistance. C. Effects of 5HT pharmacology on adaptation ratio.

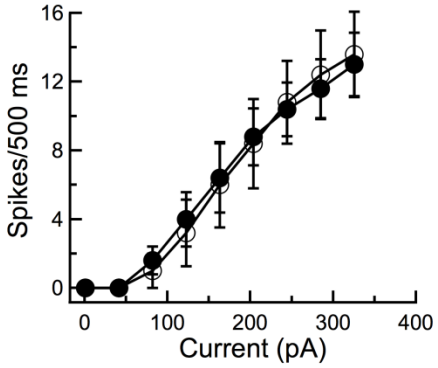


Figure 6. Activation of 5-HT_2 receptors does not affect the firing rate to current pulses. A. Firing rate as a function of current level from cells in normal rats P16-18 measured in 0.1% DMSO (filled circles) and subsequently in the presence of 5µM 5-MeO-DMT (open circles). N = 5 cells.

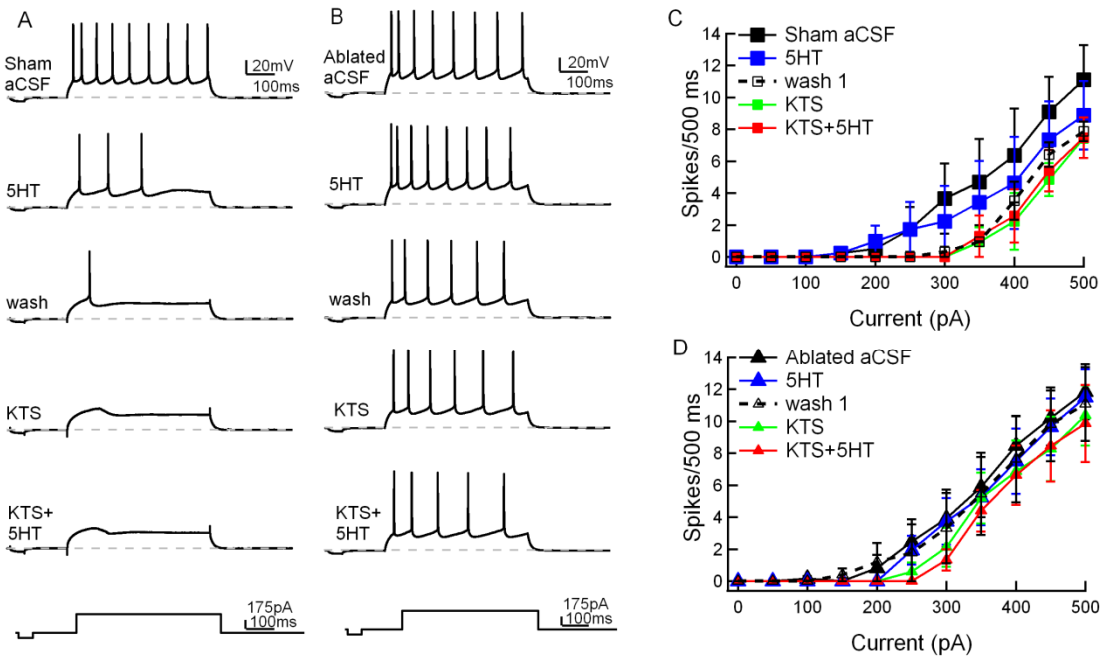


Figure 7. Cochlear ablation decreases the ability of 5HT to modulate excitability in P30-35 rat A1. A. Voltage traces from an example neuron from a sham animal in response to a 100pA depolarizing current step under different drug conditions (as in Fig. 1). Dashed grey lines indicate resting membrane potential; current injection is shown below the voltage traces. B. Voltage traces as in A from an example neuron from a cochlear ablated animal. C. Mean firing rates evoked by depolarizing current injections inform a population of sham rats: aCSF (squares), in presence of 50μM 5-HT (blue squares), after wash 1 (open squares with dashed line), in 1 μM ketanserin (green squares) and in ketanserin+5HT (red squares). Serotonin acutely decreased the firing rate, but also had a strong

delayed effect after 15 minutes of aCSF wash, greatly raising threshold and decreasing the firing rage. Subsequent challenges with ketanserin and 5-HT had no further effect. D. Data for cochlear ablated rats, in the same format as for panel C. 5-HT had no acute or delayed effect. Ketanserin decreased firing slightly, while the subsequent addition of 5-HT decreased firing further.

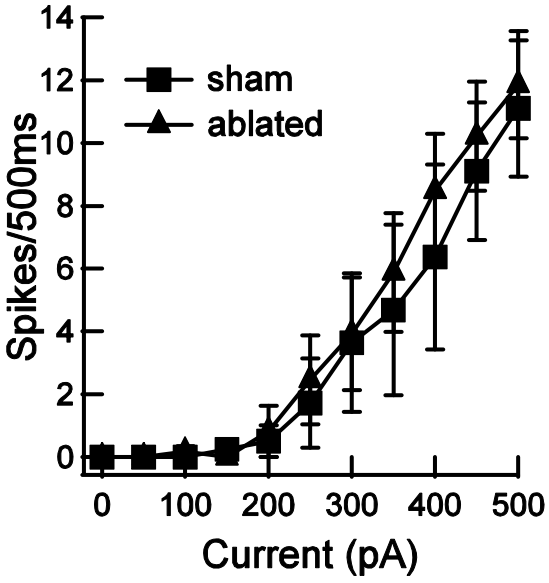
Supplementary Table 1

Table 1: Membrane and action potential measurements.

Normal (P12-21)					
	ACSF	5-HT	WASH	KET	KET+5-HT
Vm (mV)	-65.8±1.3(19)	-64.7±2.6(19)	-57.6±2.8(5)	-57.8±3.3(5)	-59.3±2.7(5)
Rin (MΩ)	178.5±22.6(19)	132.5±14.7(19)	146.8±44.6(5)	158.8±39.8(5)	116.5±24.0(5)
Adaptation Ratio	2.69±0.20(17)	2.25±0.17(17)	2.78±0.58(5)	3.08±0.80(5)	2.76±0.58(5)
Sag Ratio	0.977±0.002(13)	0.974±0.002(13)	0.969±0.002(5)	0.967±0.003(5)	0.969±0.005(5)
AHP Depth (mV)	-2.3±0.4(16)	-2.5±0.4(16)	-1.3±0.4(4)	-1.5±0.5(4)	-1.5±0.5(4)
AP Height (mV)	107.7±4.2 (16)	103.8±4.5 (16)	122.3±8.5 (4)	123.2±8.9 (4)	123.8±9.2(4)
dV/dt Max (mV/ms)	275.3±17.4(16)	260.1±21.2(16)	321.5±40.9(4)	332.3±44.0(4)	333.0±39.5(4)
Half-width (mV)	1.06±0.05(11)	1.08±0.07(11)	1.24±0.11 (4)	1.22±0.09(4)	1.21±0.09(4)
Threshold (mV)	-53.0±2.0(16)	-50.7±2.6(16)	-46.5±4.2(4)	-46.3±5.7(4)	-45.1±5.0(4)
Sham (P12-21)					
	ACSF	5-HT	WASH	KET	KET+5-HT
Vm (mV)	-67.5±1.2(23)	-65.5±1.4(23)	-64.5±1.6(14)	-64.4±2.1(14)	-65.6±2.4(14)
Rin (MΩ)	161.7±21.4(24)	129.8±14.6(24)	146.3±20.6(14)	148.8±22.6(14)	135.8±26.2(14)
Adaptation Ratio	2.60±0.29(21)	2.30±0.24(20)	2.44±0.45(12)	2.37±0.40(12)	2.03±0.32(12)
Sag Ratio	0.950±0.011(21)	0.943±0.017(21)	0.966±0.006(12)	0.966±0.006(12)	0.969±0.006(12)
AHP Depth (mV)	-1.1±0.1(21)	-1.3±0.3(21)	-1.4±0.2(13)	-3.1±1.6(13)	-2.7±1.4(10)
AP Height (mV)	130.9±10.2(21)	113.4±4.3 (21)	119.5±3.1(13)	119.4±3.5 (13)	120.2±4.9 (10)
dV/dt Max (mV/ms)	380.3±33.2(21)	326.0±28.3(21)	335.0±25.5(13)	350.6±30.0(13)	369.4±41.2(10)
Half-width (mV)	1.20±0.09(21)	1.19±0.09(21)	1.18±0.08(13)	1.14±0.07(13)	1.24±0.13(10)
Threshold (mV)	-51.7±2.4(21)	-50.2±1.7(21)	-47.5±2.7(13)	-47.4±3.0(13)	-41.8±3.5(10)
Ablated (P12-21)					
	ACSF	5-HT	WASH	KET	KET+5-HT
Vm (mV)	-68.5±1.5(17)	-67.1±1.6(17)	-64.5±1.6(14)	-65.1±2.2(14)	-65.4±2.5(14)
Rin (MΩ)	179.2±18.2(17)	150.5±17.0(17)	161.5±11.8(14)	162.3±11.1(14)	142.0±14.4(14)
Adaptation Ratio	2.23±0.19(12)	1.74±0.15(12)	1.79±0.17(9)	1.50±0.09(10)	1.55±0.10(8)
Sag Ratio	0.984±0.003(14)	0.984±0.002(14)	0.975±0.006(12)	0.982±0.002(12)	0.986±0.002(12)
AHP Depth (mV)	-0.8±0.1(14)	-1.2±0.3(10)	-1.6±0.2(13)	-1.0±0.1(13)	-0.9±0.1(11)
AP Height (mV)	102. 6±3.0(14)	107.2±3.5(10)	105.7±3.6(13)	103.9±3.9(13)	97.2±5.4(11)
dV/dt Max (mV/ms)	301.8±21.5(14)	324.8±25.5(10)	325.9±26.8(13)	308.9±28.4(13)	267.7±36.0(11)
Half-width (mV)	0.99±0.02(14)	0.98±0.03(10)	0.99±0.04(13)	0.99±0.04(13)	1.07±0.07(10)
Threshold (mV)	-57.0±1.5(14)	-53.8±1.7(10)	-51.7±2.0(13)	-51.8±2.0(13)	-52.2±1.7(10)
Sham (P30-35)					
	ACSF	5-HT	WASH	KET	KET+5-HT
Vm (mV)	-68.0±3.1(4)	-66.7±4.3(4)	-66.0±3.6(4)	-65.0±4.3(3)	-66.4±3.0(3)
Rin (MΩ)	51.02±14.3(4)	45.01±18.0(4)	58.3±15.4(4)	50.17±8.0(3)	63.25±21.4(3)
Adaptation Ratio	2.83±0.84(4)	2.69±0.58(4)	2.60±0.15(3)	2.16±0.27(3)	2.07±0.23(3)
Sag Ratio	0.988±0.006(4)	0.983±0.003(4)	0.989±0.0007(4)	0.990±0.004(3)	0.991±0.0008(3)
AHP Depth (mV)	-4.9±2.9(4)	-4.0±2.4(4)	-1.4±1.1(3)	-0.3±0.1(3)	-1.1±0.9(3)
AP Height (mV)	113.3±6.1(4)	111.0±7.8(4)	112.7±7.1(3)	109.7±3.5(3)	112.1±6.0(3)
dV/dt Max (mV/ms)	249.0±26.1(4)	237.5±28.2(4)	226.4±32.9(3)	208.9±37.0(3)	210.7±43.3(3)
Half-width (mV)	1.09±0.18(4)	1.11±0.21(4)	1.02±0.06(3)	1.07±0.08(3)	1.05±0.10(3)

Threshold (mV)	-33.5±3.7(4)	-32.3±3.7(4)	-31.7±0.6(3)	-24.9±3.8(3)	-23.8±4.4(3)
			Ablated (P30-35)		
	ACSF	5-HT	WASH	KET	KET+5-HT
Vm (mV)	-69.5±0.8(7)	-67.0±1.6(7)	-66.6±1.5(6)	-67.4±1.2(6)	-66.8±1.4(6)
Rin (MΩ)	41.9±3.0(7)	35.9±5.0(7)	46.3±7.0(6)	49.3±7.7(6)	60.7±11.6(6)
Adaptation Ratio	3.21±0.67(6)	2.45±0.30(6)	2.85±0.48(5)	2.93±0.59(5)	2.82±0.49(4)
Sag Ratio	0.980±0.004(7)	0.981±0.004(7)	0.980±0.005(6)	0.988±0.003(6)	0.985±0.002(6)
AHP Depth (mV)	-7.1±1.9(7)	-6.3±1.4(6)	-5.0±2.1(6)	-4.2±1.4(6)	-3.7±1.3(6)
AP Height (mV)	117.2±2.3(7)	111.9±2.9(6)	110.6±3.5(6)	109.3±3.8(6)	109.2±4.4(6)
dV/dt Max (mV/ms)	254.9±7.2(7)	236.2±8.5(6)	234.3±11.4(6)	219.0±16.1(6)	218.9±19.0(6)
Half-width (mV)	1.13±0.09(7)	1.16±0.09(6)	1.14±0.06(6)	1.17±0.07(6)	1.16±0.07(6)
Threshold (mV)	-34.8±0.9(7)	-32.0±1.0(6)	-32.8±1.5(6)	-31.8±2.1(6)	-31.1±2.3(6)

Membrane properties and action potential measurements grouped by surgery type, drug condition and age. V_m; resting membrane potential. R_{in}: input resistance. AHP Depth: after-hyperpolarization depth, measured from rest for single spikes, and from spike threshold for current pulse trains. AP Height: distance from the resting potential to the peak of the action potential. dV/dt Max: maximum slope of the rising phase of action potential. Half-width: width of the spike at half-height. Threshold: voltage at which the rising phase of the action potential has maximum curvature (see Methods). Values show means ± SE and number of neurons recorded in (n). Note: AHP Depth, AP Height, dV/dt Max, Half-width and Threshold for Normals, P12-21 Shams and Ablated were measured from single action potentials in response to a pulse injection of supra-threshold current (see Methods). In P30-35 animals, the same AP properties were calculated from trains of action potentials elicited during 500 msec current pulses.



Supplementary Figure 1: Comparison of firing rate as a function of current injection in sham (filled squares) and cochlear ablated (filled triangles) neurons from P30-35 rats. At P30-35, hearing loss does not have a significant effect on neuronal excitability.

CHAPTER 3

MUSCARINIC MODULATION OF STDP AT RECURRENT SYNAPSES IN AUDITORY CORTEX

1. Abstract

Acetylcholine refines cortical receptive fields by activating muscarinic acetylcholine receptors (mAChRs). However, the specific cellular and synaptic mechanisms underlying acetylcholine's effects on cortical circuits remain elusive. In this study we investigated the effects of muscarinic receptor modulation of long-term synaptic plasticity. We show that recurrent synapses in layer 2/3 of primary auditory cortex (A1) follow unique spike timing-dependent plasticity (STDP) rules. mAChR activation at these synapses regulates tLTP induction. During coincident presynaptic and postsynaptic activity, mAChR activation prevents an increase in calcium in dendrites by decreasing postsynaptic NMDA receptor conductance without affecting transmitter release. Thus, activating mAChRs in intracortical neurons affects A1 information processing and storage by decreasing spike-timing dependent amplification of recurrent information.

2. Introduction

Experience-dependent plasticity contributes to organizing the representation of sensory information in maps in auditory, visual and somatosensory cortices (Buonomano and Merzenich 1998). Neuromodulators are critical for experience-dependent plasticity as they provide information about the behavioral significance of sensory information. Representational plasticity is hypothesized to be driven by correlations between pre and postsynaptic activity requires LTP and LTD of synapses (Buonomano and Merzenich 1998). Despite several demonstrations that neuromodulation can engage or prevent map plasticity in cortex, very little is known of the cellular and synaptic mechanisms involved in this modulation.

Intracortical and thalamic inputs contribute to the tonal response map of A1 neurons (Kaur et al. 2005; Liu et al. 2007). L2/3 neurons extend their axons laterally and are, on average, aligned along the tonotopic axis, linking columns of neurons with different frequency tuning (Clarke et al. 1993; Matsubara and Phillips 1988; Ojima et al. 1991; Read et al. 2002; Song et al. 2006). L2/3 pyramidal neurons have broad sub-threshold receptive fields (Kaur et al. 2004; Liu et al. 2007; Ojima and Murakami 2002). Notably, subthreshold receptive fields could play an important role in integrating responses to spectrotemporally complex stimuli, such as frequency modulated sounds, and offer a substrate for plasticity of the tonotopic map.

Plasticity of sensory representations has recently demonstrated to be mediated by spike timing-dependent plasticity (STDP) *in vivo* in auditory, visual and somatosensory cortices (Dahmen et al. 2008; Jacob et al. 2007; Yao and Dan 2001). STDP involves changes in strength of synapses that is dependent upon the precise timing of pre- and postsynaptic activity (Bi and Poo 1998; Markram et al. 1997). Presynaptic activity that precedes postsynaptic firing, by up to tens of milliseconds, causes strengthening of synapses (tLTP), whereas reversing this temporal order causes synaptic weakening (tLTD) (Debanne et al. 1994; Levy and Steward 1983). STDP seems to depend on the interplay between NMDA receptor activation and the timing of back-propagating action potentials in dendrites of the postsynaptic neuron (Linden 1999; Magee and Johnston 1997; Sourdet and Debanne 1999). The critical time window for STDP induction varies broadly with brain region, cell and synapse type (reviewed in (Abbott and Nelson 2000; Larsen et al. 2010)).

The cholinergic system has been implicated in the modulation of map plasticity in auditory cortex (Froemke et al. 2007; Kilgard and Merzenich 1998; Weinberger 2003). Muscarinic cholinergic receptors play a crucial role in the development and function of the normal auditory cortex (Zhang et al. 2005; Zhang et al. 2006). Even though the cholinergic system plays an important role in auditory cortex, it remains unclear how acetylcholine influences LTP and LTD at cortical synapses.

In the auditory system, the dorsal cochlear nucleus (DCN) follows Hebbian and anti-Hebbian patterns of STDP in a cell-specific manner (Tzounopoulos et al.

2004). In principal neurons of the DCN, activation of muscarinic receptors converts postsynaptic tLTP to presynaptic tLTD (Zhao and Tzounopoulos 2011). Evidence that recurrent synapses in auditory cortex also display STDP has been presented (Karmarkar et al. 2002). Given these results, not much is known about the timing rules of STDP in the auditory cortex and its mechanism or regulation by neuromodulators.

We investigated the timing rules of STDP and modulation by muscarinic receptor activation at recurrent synapses in auditory cortex. We find that the STDP in auditory cortex follows unique timing rules, in which tLTP occurs at +10 ms, while tLTD occurs at -10 and +50 ms. Activation of mAChRs modulates the timing rules: carbachol regulates tLTP induction and at some synapses converts tLTD to tLTP. During repetitive pairing of pre and postsynaptic activity with a 10 ms delay, carbachol prevented an increase in calcium influx, likely caused by a reduction in NMDAR currents.

3. **Materials and Methods**

Thalamocortical brain slices were made from CBA mice (P12–P16). The preparation and use of thalamocortical slices containing A1 has been described in detail (Rao et al. 2010). Animals were sacrificed according to methods approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill. Single cells were visualized with IR interference contrast optics and recorded using patch pipettes in current-clamp, and in some

experiments, in voltage-clamp. Cortical pyramidal cells in A1 layer 2/3 were identified on the basis of morphological and electrophysiological criteria (for more details, see (Rao et al. 2010)). The standard slicing, incubation, and recording solution was an artificial cerebrospinal fluid (aCSF) that contained (in mM) 134 NaCl, 3.0 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 KH_2PO_4, 10 glucose, 20 $NaHCO_3$, 0.4 ascorbic acid, 2 sodium pyruvate, and 3 myoinositol; saturated with 95%O_2/5%CO_2. Slicing was performed with ice-cold aCSF, and during a 1-hour recovery period, the slices were incubated at 34℃. Slices then were maintained at room temperature until used. All recordings were performed using whole-cell tight seal methods at 34℃.

Current clamp recordings

The pipettes were backfilled with a solution containing (in mM) 130 K-gluconate, 4 NaCl, 0.2 EGTA, 10 HEPES, 2 Mg_2ATP, 0.3 Tris GTP, and 10 phosphocreatine (pH 7.2 with KOH). All the internal solutions were adjusted to pH 7.2, 290 mOsmol. A concentric bipolar stimulating electrode was placed in L2/3 in A1, approximately 500μm from the recording site, and whole-cell recordings performed from pyramidal neurons in L2/3. Single minimal excitatory postsynaptic potentials (EPSPs), 2-5 mV, were evoked every 10 seconds by stimulating L2/3 cells to activate presynaptic fibers. Postsynaptic activation was achieved with a train of 3-5 ms duration depolarizing current pulses that produced 5 action potentials with an 8 ms inter-pulse-interval. The slope of the initial 2-3 ms of the EPSP was analyzed to ensure that the data reflected only the monosynaptic component of each experiment. EPSP slope ratio was measured

as the change in average EPSP slope when comparing a 20 min period, 20–40 min post-conditioning to the baseline EPSP slope measured during the 5 mins of baseline recording. Cells that were retained for analysis had resting membrane potentials less than -60 mV, less than 8 mV shifts in membrane potential during the protocol and survived completion of the protocol. The measured EPSP was averaged in one-minute blocks, and then normalized to baseline. During the induction protocol, spike-timing was measured from the onset of the evoked EPSP to the peak of the first postsynaptic action potential (for pre→post pairs), and from the peak of the 5th action potential to the onset of the EPSP (for post→pre pairs). To assess the effect of mAChR activation in these experiments, the cholinergic agonist carbachol was applied during the pairing protocol (2 minutes before through 1 min after start of the 100 second pairing protocol). To measure the frequency-current (F-I) curve, complete current-response curves were obtained for 500-msec duration test pulses.

Voltage clamp recordings

Slices were placed in a submersion chamber and perfused with oxygenated modified aCSF (above) containing 4 mM $MgCl_2$, 4 mM $CaCl_2$, 50 μM picrotoxin, and 10 μM CNQX. These conditions are sufficient to pharmacologically isolate NMDAR-mediated responses (Philpot et al. 2001a; Philpot et al. 2001b). Pipettes were filled with an internal solution containing (in mM): 120 cesium methane sulfonate, 8 TEA-chloride, 10 Hepes, 0.2 EGTA, 4

TRIS adenosine triphosphate, 0.3 TRIS guanosine triphosphate, 10 Creatinine phosphate, and 3 QX-314 chloride with pH adjusted to 7.2 and osmolarity adjusted to ~300 mOsmol with sucrose. The internal solution also contained Alexa 488 for post-hoc identification of pyramidal neurons. Pipette capacitive transients were minimized prior to breakthrough and after break in whole cell capacitance and series resistance compensation (65-80%) was applied. Cells were stepped to +40 mV when measuring NMDA currents, and traces with and without stimulation were subtracted to isolate the synaptic current from residual outward potassium current. Data were filtered at 2 kHz. Excitatory postsynaptic currents (EPSCs) were evoked from a stimulating electrode (concentric bipolar stimulating) placed in L2/3, and stimulation intensity was adjusted to evoke ~100-500 pA response. Stimulation was given every 8 sec. In some cells, the paired-pulse ratio was measured by evoking 2 EPSCs, 50 ms apart. Ten traces were averaged for each measurement.

Data acquisition and analysis

Data were acquired and analyzed using MATLAB R2008-R2010 (The Mathworks, Natick, MA), Igor Pro (6.2 Wavemetrics, Oswego, OR) and Prism 5.0 (Graphpad, San Diego, CA). Data are reported as means and SEM. Statistical comparisons were made using with two-way ANOVA (with Bonferroni post hoc-test), paired or unpaired two-tailed Student's *t* tests, as appropriate. Statistical significance was based on p values <0.05.

Calcium imaging

Pipettes were filled with intracellular solution containing Alexa 568 (50 µM) to reveal neuronal morphology and the low-affinity calcium-indicator Fluo-5f (100 µM). Subsequent to break-in, cells were monitored for a minimum of 15 min to allow stabilization of the dye before fluorescence measurements were taken. Responses were measured to a burst of 5 action potentials elicited by current injection. To stimulate EPSPs, an extracellular stimulation pipette filled with aCSF was placed within 20 µm of the apical dendrite in L2/3 neurons. Fluorescence measurements were made for 5 min either in aCSF, during and following bath application of 20 µM carbachol. Imaging took place on a Zeiss FS-2Plus microscope under a 40X 0.75NA water immersion objective, using a 100W halogen light source and a Sutter Lambda-2 filter wheel to select excitation wavelengths. Photometrics QuantEM 512-SC was used to image the cells. Imaging of soma and dendrites were carried out at 93 frames/second, using 8X8 binning. Fluorescence imaging and electrophysiological recordings were synchronized and all image acquisition was controlled by a custom-written program written in Python. Each trace consisted of 50 ms (or longer) baseline, followed by stimulation and continued recording for 3 seconds. The pre-stimulus baseline was used to compute “green/red” ratio to obtain the relative resting calcium level. A region of interest (ROI) outside of any indicator-filled ROI was used to measure background fluorescence. Ratio-metric imaging was used to normalize for changes in fluorescence intensities by calculating green/red (G/R) ratios, the ratio of the Fluo-5f to Alexa 568 fluorescence (a single Alexa 568

image was taken before each run). Changes in fluorescence (F-F0/F0), or DF/F, and DG/R signals were measured by computing the area under the curve of the calcium response. ROIs were selected at or near the synaptic site for measurements of calcium changes. Fluorescence traces for bursts of action potentials, with or without preceding EPSPs, are averages of 10 traces. Off-line data analysis was carried out using in-house-written procedures in Igor Pro software. Differences between groups were tested using t tests (paired or two-tailed independent samples) in Prism statistical software, with $p < 0.05$ indicating significance. For F-I curves, 2-way ANOVA with Bonferroni posttest was used (DF=degrees of freedom, F=F ratio).

Drugs

Carbachol, Eserine, Oxotremorine-M, Pirenzepine, 4-DAMP, APV, CNQX, Picrotoxin, and BAPTA were purchased from TOCRIS. Alexa 568, Alexa 488 and Fluo-5f were purchased from Invitrogen. All other salts or chemicals were purchased from Sigma-Aldrich.

4. Results

Spike timing-dependent plasticity at layer 2/3 synapses in auditory cortex

STDP was induced by pairing EPSPs with postsynaptic action potentials evoked by direct current injection through the recording electrode. Baseline EPSPs of 2-5 mV were monitored while stimulating at 0.1Hz. After 5 minutes of

baseline stimulation, a pairing protocol was presented, consisting of a single pulse activating L2/3 parallel fibers, paired with a postsynaptic burst of 5 action potentials at defined times before or after each EPSP. Pairings were repeated at 100 times at 1-second intervals. At synapses between L2/3 neurons pairing of EPSPs with postsynaptic spikes resulted in bidirectional plasticity. When the onset of EPSPs preceded spikes by 10 ms tLTP was induced (Figure 1, B1,B2, EPSP slope ratio=Post-pairing/Pre-pairing: 1.44 ± 0.12, n=9, P=0.01). When the spikes preceded the EPSP by 10 ms, tLTD was observed (Figure 1, A1,A2, 0.66 ± 0.07, n=7, P=0.04). This synaptic plasticity is associative, as induction requires both specific timing and temporal order between pre- and postsynaptic activity. Measures of the STDP window revealed interesting results (Figure 1, D). EPSPs preceding spikes by 50 ms resulted in tLTD (Figure 1, C1,C2, 0.60 ± 0.10, n=5, P=0.02). No synaptic plasticity resulted when the interval between EPSP and spikes were 20 ms (Figure 1, D, At +20ms: 0.97 ± 0.13, n=7 P=0.74 and at -20ms: 0.88 ± 0.09, n=6, P=0.38).

mAChR activation induces LTD of synaptic potentials at L2/3→L2/3 synapses in A1

Previously, it was shown that activation of muscarinic cholinergic receptors (mAChRs) at L6→L3 synapses in auditory cortex induces LTD of synaptic potentials (Metherate and Ashe 1995). To test if mAChR activation at L2/3→L2/3 also causes LTD, we bath applied the cholinergic receptor agonist,

carbachol (20 µM, 10 minutes) while measuring synaptic potentials in L2/3 neurons. Carbachol induced a large transient depression during drug application followed by LTD (Figue 2, A1, 0.66 ± 0.04, P=0.0009), followed by LTD (0.77 ± 0.04, P=0.005, n=6) after drug washout that lasted for the duration of the experiment. To determine if the transient depression and the long-lasting depression is induced pre- or postsynaptically, paired pulse ratio (PPR) was analyzed. A change in PPR suggests a presynaptic locus of expression, whereas no change is an indicator of a postsynaptic locus (Dobrunz and Stevens 1997). A change in PPR was not observed during carbachol application (Figure 2, B3, control: 1.17 ± 0.22, carbachol: 1.14 ± 0.17 n = 5, *P* =0.5), suggesting that a postsynaptic expression mechanism underlies the LTD. To confirm that the carbachol-induced LTD measured at synapses in L2/3 requires activation of mAChRs rather than nicotinic acetylcholine receptors, the nonselective mAChR antagonist atropine was applied at 10 µM, a concentration that blocks all mAChR subtypes. The transient depression, but not the LTD was prevented (Figure 2, A2, with atropine, transient: 0.96 ± 0.02, P=0.0006, LTD: 0.87 ± 0.10, n = 4, P= 0.41, compared to without atropine). To investigate the muscarinic receptor subtype involved in the LTD, pirenzepine (75nm), an M1 receptor antagonist was used. Neither the transient depression nor LTD was prevented. Pirenzepine at 75nM, a concentration that blocks M1 receptors was used that did not prevent the transient depression or LTD (Figure 2, A3, with pirenzepine, transient: 0.58 ± 0.09, P=0.45, LTD: 0.82 ± 0.08, n=4, P=0.62, compared to without pirenzepine). Endogenous activation of AChRs with Eserine, a cholinesterase inhibitor (1µM)

also induced a transient depression of synaptic potentials (Figure 2, A4, transient: 0.81 ± 0.06, P=0.08, long term: 1.04 ± 0.05, P=0.70, n=6). These results suggest that exogenous or endogenous activation of cholinergic receptors causes synaptic depression at L2/3→L2/3 synapses.

Spike-timing dependent plasticity can be affected by changes in intrinsic excitability. Carbachol caused a reversible increase in excitability measured as an enhancement in firing rate in response to current steps (Figure 2, B1, 2-way ANOVA, control F-I vs. F-I in carbachol, DF=2, F=5.35, P=0.005 n=10). The enhanced excitability was not significantly affected by M1 receptor blockade using pirenzepine (75nM) (Figure 2, B2, 2-way ANOVA, pirenzepine F-I vs. F-I in carbachol, DF=1.9, F=2, P=0.15 n=4). We conclude that activation of mAChRs by carbachol produced both changes in intrinsic excitability and in synaptic transmission, mediated by postsynaptic mechanisms, but not by M1 receptors.

mAChR activation regulates tLTP and in some neurons converts tLTD to tLTP

We next tested the hypothesis that cholinergic neuromodulation by mAChRs can change STDP timing rules by modulating the relative strength of tLTP and tLTD. To test this hypothesis, we activated cholinergic receptors during our STDP pairing protocol. To examine the effect of mAChRs activation on STDP, carbachol was applied during LTP induction (pre→post pairing at 10 ms). Surprisingly, mAChR activation prevented LTP induction (Figure 3, A1,A3 carbachol: 0.92 ± 0.17, n=6, p=0.03 compared to control +10ms). To determine

the effects of cholinergic modulation on pre-post LTD and post-pre LTD, we applied carbachol during EPSP-spikes pairing at 50 ms and -10 ms respectively. This protocol resulted in heterogenous changes in synaptic strength. While at some synapses tLTD was converted to tLTP, others remained unaffected. However on average post→pre tLTD was blocked (Figure 3, A2,A3 0.92 ± 0.20, n=9, P=0.26 compared to control -10ms) and pre→post tLTD was converted to tLTP (Figure 3, A3, 1.22 ± 0.47, n=7, P=0.24 compared to control +50ms). These results suggest that activation of cholinergic receptors modulates the polarity and magnitude of LTP depending on timing of pre- and postsynaptic activity. In order to further explore the ability of intrinsic cortical acetylcholine to modulate STDP in auditory cortical neurons, we attempted to examine the effects of endogenous acetylcholine using the anticholinesterase, eserine. Application of eserine during pre→post pairing prevented tLTP induction (Figure 3, B1, B3 0.95 ± 0.10, n=7, P=0.009 compared to control +10ms), thus mimicking carbachol, but did not have an effect on tLTD induction at -10 ms (Figure 3, B2, B3 0.74 ± 0.11, n=6, P=0.59 compared to control -10ms). These results indicate that endogenous acetylcholine prevents tLTP at L2/3→L2/3 synapses.

Activation of mAChRs reduces NMDA current

To test whether mAChR activation blocked tLTP by directly acting on NMDA receptors, we recorded pharmacologically isolated NMDA receptor mediated EPSCs with and without mAChR activation. We detected a reduction in the evoked NMDAR currents in the presence of carbachol (Figure 4, A1,A2, control: 263.2 ± 34.15, carbachol: 148.2 ± 28.84, n=13, P=0.0008), which was

partially restored after washout. We verified that the current was mediated by NMDA receptors, and found that it was completely blocked following the application of the NMDAR antagonist aminophosphonovaleric acid (APV, 50 μM, data not shown). In a subset of cells, the EPSC paired-pulse ratio was calculated to test for presynaptic effects of carbachol. The paired-pulse ratio of the isolated NMDA current was not altered by carbachol application (Figure 4, A3, control: 0.97 ± 0.08, carbachol: 0.93 ± 0.08, n=7, P=0.66) consistent with the postsynaptic effect of carbachol on EPSPs described above.

Dendritic Calcium Signaling Is Reduced by mAChR activation

Back propagating action potentials are crucial for induction of STDP. Postsynaptic calcium transients provide an associative link between synapse activation, postsynaptic cell firing, and synaptic plasticity (Koester and Sakmann 1998; Malenka et al. 1988). Since carbachol reduced the NMDA current, it is possible that it also could reduce subsequent calcium influx in dendrites of auditory cortical pyramidal neurons. We first tested whether changes in intracellular calcium concentration are necessary for STDP in auditory cortical layer 2/3 pyramidal neurons. When exogenous calcium chelators such as BAPTA are present in the intracellular solution, incoming calcium ions are rapidly buffered and free calcium concentration changes are strongly reduced (Tsien 1980). In the presence of BAPTA (20 mM), pairing pre→post at +10 ms prevented tLTP induction but revealed an underlying LTD (Figure 5, A1, 0.57 ± 0.09, n=5, P=0.0001, compared to +10ms control). Thus, changes in calcium concentration during STDP induction are necessary for the tLTP to occur. To

investigate whether carbachol reduced calcium transients produced by dendritic action potential propagation, we monitored postsynaptic calcium signaling in apical dendrites of L2/3 pyramidal neurons during timed pre and postsynaptic activity. Pyramidal neurons were filled with Alexa 568 and the calcium indicator Fluo-5f through patch pipettes (Figure 5, A2). The Alexa 568 image was visualized and used to select a region on the apical dendrite for placement of the extracellular stimulation. Somatically-evoked action potentials invaded apical dendrites and induce calcium changes throughout the dendritic tree of neurons. A burst of action potentials (APs) were preceded by extracellular stimulation of synaptic input by 10 ms, as was used for the induction of STDP. Calcium signals were analyzed on apical dendritic region closest to extracellular stimulation (about 10-20 µm from electrode). Pairing EPSP-APs at +10ms resulted in an increase in calcium compared to APs alone (Figure 5, C2,D2, EPSP-APs: 1.23 ± 0.29; APs: 0.74 ± 0.28, n=5, P=0.004). After 5 minutes of baseline measurements, carbachol (20 µM) was bath applied for 5 min. Carbachol did not induce changes in the baseline calcium signal. However, carbachol increased the AP-mediated calcium influx that occurred both during and after the AP train (Figure 5, C1,D1, control 0.74 ± 0.28; carbachol: 1.70 ± 0.53, n=5, P=0.05). The increase in AP-mediated calcium influx was blocked by atropine, implicating mAChRs (data not shown). However, pairing EPSP-APs at +10ms in the presence of carbachol did not significantly affect the calcium influx (Figure 5, C3,D3, control: 1.23 ± 0.29; carbachol: 1.40 ± 0.4, n=5, P=0.35). Thus, in the presence of carbachol, postsynaptic calcium signals associated with coincident

pre- and postsynaptic activity were not affected in apical dendrites of auditory cortical neurons. We conclude that mAChRs enhance AP-mediated calcium influx but when APs are preceded with EPSPs, the increase in calcium influx is prevented.

Cholinergic receptor activation by OxoM also prevents tLTP

In the DCN, it was shown that, muscarinic receptor activation with Oxotremorine-M (Oxo-M) converted postsynaptic tLTP to presynaptic tLTD by acting on M1/M3 receptors. We tested the hypothesis that carbachol's effects on tLTP induction in auditory cortex were mediated by M1/M3 receptors. However, carbachol (20 µM) application during tLTP in the presence of M1 antagonist, pirenzepine (10µM) and M3 antagonist, 4-DAMP (1µM), did not restore tLTP (Figure 6, A1, 0.94 ± 0.07, n=6, P=0.89 compared to carbachol at +10 ms). As Oxo-M switched tLTP to tLTD in DCN (Zhao and Tzounopoulos 2011), we therefore tested our hypothesis using Oxo-M. Application of Oxo-M (3 µM, 10 mins) induced depression, that was not significant, of synaptic transmission at L2/3→L2/3 synapses (Figure 6, A2, 0.61 ± 0.11, n=3, P=0.21). Application of Oxo-M during induction prevented tLTP (Figure 6, A3, 0.72 ± 0.31, n=3, P=0.02 compared to control +10ms). Application of Oxo-M during induction in the presence of M1/M3 antagonists partially restored tLTP, but was not significant (Fig6 A4 1.4 ± 0.57, n=4, P=0.33 compared to Oxo-M at +10 ms).

5. Discussion

We found that recurrent synapses in A1 follow unique STDP rules. Although tLTP and tLTD were observed in the expected positive and negative intervals, respectively, our results reveal tLTD at long positive intervals. Our results indicate that mAChRs modulate tLTP and tLTD in a manner that is dependent on spike timing. Activation of mAChRs during short positive intervals prevented tLTP induction and in some cells converted tLTD to tLTP at negative and long positive intervals. We found that mAChR activation reduced NMDA current at recurrent synapses. Additionally, mAChR activation decreased dendritic calcium influx when EPSPs were paired with bAPs at positive intervals.

Recurrent synapses in A1 follow unique STDP rules

STDP varies with brain area, cell and synapse type (reviewed in (Abbott and Nelson 2000; Larsen et al. 2010)). At recurrent synapses in rat A1 slices, tLTP was observed at +10 ms intervals and tLTD at -40 ms at L2/3→L2/3 synapses (Karmarkar et al. 2002). However, the complete STDP window was not examined in this study. Recurrent synapses in rat V1 (Froemke et al. 2006) and in rat S1 (Nevian and Sakmann 2006) show pre→post tLTP and post→pre tLTD at 10 ms intervals. Our results with pre→post tLTP and post→pre tLTD at 10 ms are consistent with the above studies. In most cortical areas, the magnitude of tLTP falls off approximately exponentially as a function of the difference between pre- and postsynaptic spike times. However, we also observed a pre→post tLTD at +50 ms, and on average, no tLTP or tLTD at +20 ms intervals, suggesting that

not all regions of cortex follow the same STDP rules. Computational models have suggested that STDP curves could exhibit tLTD at longer positive pre→post intervals (Karmarkar et al. 2002; Shouval and Kalantzis 2005). This prediction is based on three observations: calcium influx through NMDARs is a necessary and sufficient signal to induce bidirectional plasticity (Lisman et al. 1998), the sign and magnitude of synaptic plasticity is determined by the calcium concentration in postsynaptic spines (Cormier et al. 2001; Yang et al. 1999), and peak calcium level varies with time interval between pre- and postsynaptic spiking (Karmarkar et al. 2002). Although these theoretical predictions of pre→post tLTD are consistent with experimental evidence in hippocampal slices (Nishiyama et al. 2000; Wittenberg and Wang 2006), to our knowledge this is the first demonstration of similar tLTD in auditory cortex. Pre→post tLTD could result from several mechanisms. First, as predicted in a model, the magnitude of calcium influx through NMDA receptors increase from negative to positive intervals and then decreases for long positive intervals (Karmarkar et al. 2002). Thus, at long positive intervals, the calcium level should fall below the tLTP induction threshold, but be sufficient to induce tLTD. A different model predicts that with higher levels of NMDA receptors, it is likely that pre→post tLTD will be observed (Shouval and Kalantzis 2005).

What could be the advantage of unique STDP rules in auditory cortex? STDP has been implicated as a mechanism for receptive field plasticity in V1, S1 and A1 (Dahmen et al. 2008; Jacob et al. 2007; Yao and Dan 2001). In the visual system, the receptive field of xenopus tectal neurons can become direction-

sensitive by repeated exposure to stimuli in a particular direction in a manner consistent STDP (Engert et al. 2002). In the auditory system, repetitive pairing of two different frequencies produces shifts in tuning of A1 neurons. The magnitude and direction of the shift depends on the time delay and temporal order between the two frequencies (Dahmen et al. 2008), and is broadly consistent with general STDP rules. The tuning plasticity was most prominent in L4 and L2/3 neurons, suggesting that receptive field plasticity might occur through a STDP-like mechanism at recurrent synapses. L2/3 neurons extend their axons laterally and are aligned along the tonotopic axis, linking columns of neurons with different frequency tuning (Ojima et al. 1991; Song et al. 2006). L2/3 neurons are responsible for subthreshold receptive fields (Kaur et al. 2004; Liu et al. 2007). Further, a recent study measured sensory evoked-calcium transients in individual dendritic spines in L2/3 of A1 *in vivo* and found that sounds played at different frequencies revealed spines on the same dendrite are heterogeneously tuned (Chen et al. 2011). The tLTD windows flanking the tLTP window could serve to enhance temporal selection for the timing of synaptic inputs. This proposal is consistent with the idea that recurrent connections allow positive feedback that can help to amplify selected afferent signals, and modeling studies that suggest that local amplification is important in enhancing the sensory selectivity of cortical neurons (Douglas et al. 1995; Sompolinsky and Shapley 1997). Frequency modulated sounds are a common feature of natural sounds, including, speech and could produce neural activity required for STDP. For example, modulations of frequency can activate differentially tuned neurons in A1 and the arrival times

of the different frequencies could be sufficient to can induce coincident pre and post synaptic activity which could engage tLTP. Unfavorable timings could lead to tLTD. This suggests that only when arrival times of synaptic events produced by frequency modulated sounds are short, recurrent synapses could potentiate.

Muscarinic modulation of A1 activity

Acetylcholine has recently been found to play an important role in many aspects of cortical development (Hohmann and Berger-Sweeney 1998; Robertson et al. 1998). These effects are mediated by mAChRs. Mice lacking muscarinic M1 receptors display multi-peak frequency tuning curves compared to sharply tuned neurons in wild-type A1. The deficit in the tuning curves is associated with a disorganized tonotopic map (Zhang et al. 2005). Cholinergic receptor function thus appears to be a critical factor for establishing the normal tonotopic organization of the auditory cortex. Pairing electrical stimulation of NB with tones produces large shifts in frequency tuning of A1 neurons (Weinberger and Bakin 1998) and a corresponding reorganization of the tonotopic map that results in an over-representation of the paired tone frequency (Froemke et al. 2007; Kilgard and Merzenich 1998; Weinberger and Bakin 1998). However, in M1 receptor knockout mice, pairing NB stimulation and tones produces much smaller shifts in frequency tuning in A1 (Zhang et al. 2006).

At the cellular level, acetylcholine acting on mAChRs can affect intrinsic excitability, synaptic potentials, neurotransmitter release and calcium influx (Cho et al. 2008; Froemke et al. 2007; Metherate and Ashe 1995; Salgado et al. 2007).

Consistent with findings in auditory and visual cortices (McCoy and McMahon 2007; Metherate and Ashe 1995) we found that the cholinergic agonist, carbachol depresses glutamatergic synaptic transmission. Additionally, the mAChR specific agonist, Oxo-M induced synaptic depression, although this effect was not significant. Endogenous activation of mAChRs with an anticholinesterase also depressed synaptic potentials suggesting that acetylcholine tonically depresses synaptic transmission in A1. Carbachol's effects on synaptic depression were blocked by atropine implicating mAChRs, however, they were not blocked by pirenzepine, an M1 receptor antagonist, consistent with results in prefrontal cortex (Vidal and Changeux 1993) suggesting that receptors other than the M1 subtype are responsible. Other muscarinic receptor subtypes that are expressed in auditory cortex are M2 and M3 (Salgado et al. 2007). In auditory cortex M2 receptors are localized to both excitatory terminals from white matter inputs and L2/3 GABAergic axon terminals and modulate neurotransmitter release (Salgado et al. 2007). However, we found that the effects of mAChR activation with carbachol were postsynaptic confirmed by unchanged paired-pulse ratio of the synaptic responses, suggesting that M2 receptors did not mediate carbachol's effects. Our results showed that Oxo-M application during pre→post pairing in the presence of M1/M3 blockers partially restored tLTP. As M1 receptors are not involved in carbachol's effects on synaptic depression, it likely that M3 receptors might mediate the prevention of tLTP. Consistent with a postsynaptic effect, we found that carbachol increased intrinsic excitability and enhanced bAP-mediated calcium influx. The increase in

intrinsic excitability was not prevented by the M1 blocker, pirenzenpine. The increase in excitability and calcium influx are consistent with others in auditory and visual cortices (Cho et al. 2008; Metherate and Ashe 1995), but not mediated by M1 receptors. This inconsistency in muscarinic receptor subtype that mediates excitability and calcium affects could be attributed to the differences in age or species of the animals used in our study. Activation of mAChRs is suggested to involve inhibition of dendritic voltage gated potassium channels (Cho et al. 2008). Carbachol-mediated increase in excitability and bAP-calcium influx could be attributed to modulations of potassium channels on dendrites.

Taken together, these results suggest that activation of mAChRs would tend to increase postsynaptic excitability while decreasing intracortical transmission and may serve to reduce the local spread of cortical excitation during heightened sensory activity and enhance incoming auditory information via thalamocortical input (Hsieh et al. 2000). Suppression of recurrent excitatory connections by mAChR activation could alter the frequency tuning of A1 neurons. The muscarinic pathway would act to reduce the lateral spread of recurrent excitation, and amplify cortical sensory input.

mAChR modulation of STDP

There is growing evidence that neuromodulators, including acetylcholine control STDP rules by regulating polarity, magnitude and temporal requirements

for plasticity. For example, mAChR activation during pre-post pairings induces tLTP (Wespatat et al. 2004), gates tLTD in V1 (Seol et al. 2007), enhances tLTP while blocking tLTD in hippocampus (Sugisaki et al. 2011). β-adrenergic receptor activation controls the gating of tLTP in V1 (Seol et al. 2007) and broadens the tLTP window in hippocampus (Lin et al. 2003). Nicotinic receptor activation prevents tLTP induction in prefrontal cortex (Couey et al. 2007). Dopamine activation extends the tLTP window and converts tLTD to tLTP in hippocampus (Zhang et al. 2009). Our results show that mAChR activation during pre→post pairings prevents tLTP induction and instead causes a weak tLTD. This result was also confirmed during endogenous activation of mAChR during anticholinesterase application. The mAChR-mediated prevention of tLTP in A1 is consistent with the recent finding that increasing acetylcholine levels with eserine in CA1, during activation of the cholinergic medial septal inputs prevents tLTP induction (Sugisaki et al. 2011). Interestingly, we found that mAChR during post→pre tLTD, on average had no effect, but in some cells, converted tLTD to tLTP, while during pre→post tLTD, mAChR activation converted tLTD to tLTP. However when eserine was applied during post→pre pairings, tLTD remained unaffected and we did not observe a conversion of tLTD to tLTP in the presence of eserine. As eserine is an anticholinesterase it is unknown what the synaptic concentration of free acetylcholine would be when eserine inhibits cholinesterase. It is possible that eserine at 1 µM might result in an elevation of acetylcholine sufficient to prevent conversion of tLTD to tLTP. In hippocampus,

0.6 µM eserine during post→pre pairing prevented tLTD induction, while increasing eserine to 2 µM, converted tLTD to tLTP (Sugisaki et al. 2011).

mAChR activation regulation of NMDA receptor current

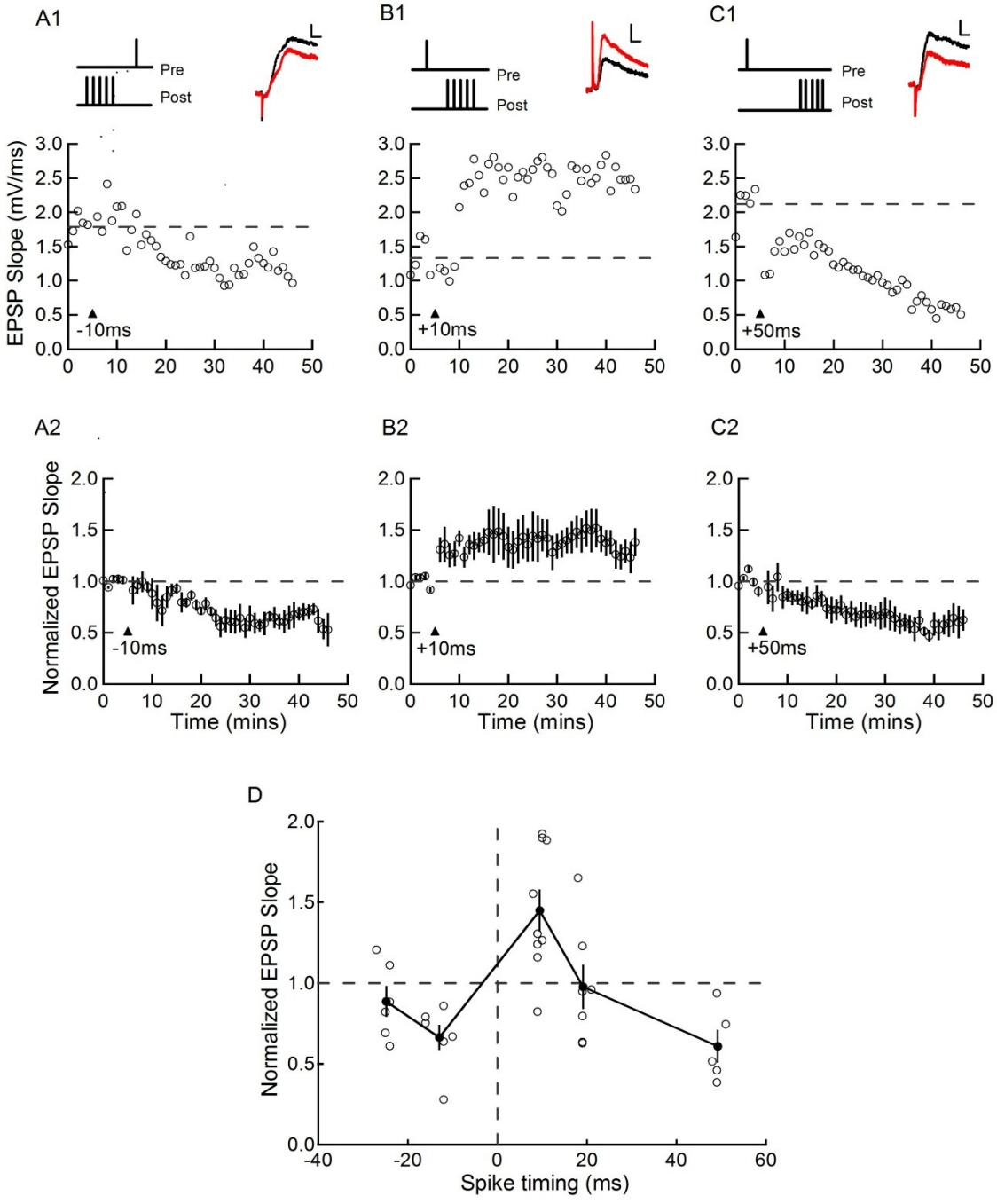
mAChR activation reduced NMDA current at L2/3 synapses and this result is consistent with observations in juvenile rat A1 slices (Flores-Hernandez et al. 2009). The decrease in NMDA current we observed with mAChR activation might be due to activation of PKC or PLC. Activation of mAChR, with carbachol triggers PLC activation and PIP2 hydrolysis; thus, these receptors may suppress NMDAR responses via a PIP2-dependent mechanism (Mandal and Yan 2009). The suppression of NMDA currents in that study was caused by internalization of NMDARs from the plasma membrane consequent to a reduction in PIP2 levels. PIP2 hydrolysis, activated by PLC coupled receptors, inhibits NR1/NR2A currents in neurons pretreated with thapsigargin that depletes calcium from intracellular stores, and suppresses NR1/2C currents, which are insensitive to regulation by PKC (Michailidis et al. 2007 228). Thus, carbachol mediated reduction of NMDAR currents is probably due to PIP2 hydrolysis. A decrease in number of NMDA receptors is shown to underlie LTD in hippocampus (Morishita et al. 2005) and could underlie the carbachol-induced depression of transmission we observed. A reduction in NMDAR current might also play a pivotal role in tLTD induction in A1. Thus, a reduction of NMDAR currents could provide a crucial mechanism by which cholinergic input to the cortex modulates learning and information storage.

mAChR modulation of pre→post calcium influx

We also found that tLTP induction is dependent on postsynaptic calcium presumably via NMDARs. This result could be explained by our finding that mAChR activation reduces NMDAR current thus blocking tLTP induction. The overall calcium transient induced by the postsynaptic action potential burst increased in the presence of carbachol. This result is consistent with similar findings in V1 (Cho et al. 2008). When an EPSP was paired with the action potential burst, the calcium transients at the synapse were larger than with action potentials alone. The increase in calcium influx, when the action potentials followed the EPSP, could be caused by a transient removal of the Mg2+ block of NMDARs (Nowak et al. 1984) resulting in an amplification of calcium influx through NMDAR receptors (Schiller et al. 1998) and might account for the tLTP we observed in the STDP experiment. However, carbachol did not significantly change the calcium influx when EPSPs were paired with action potentials. During coincident EPSPs and action potentials, NMDARs are opened and mediate calcium influx, which may be limited to single dendritic spines and the adjoining dendritic shaft (Muller and Connor 1991b) or could expand into more of the shaft area (Eilers et al. 1995). We measured synaptic calcium influx on dendritic shafts and could observe the increase in calcium during coincident inputs yet we did not observe a significant change in this calcium increase when mAChRs were activated. However, mAChR activation did increase AP-mediated calcium influx. Taken together, these results suggest that mAChR activation prevented the

increase in calcium required for tLTP induction. The prevention of increase in calcium influx could be due to mAChR-induced reduction in NMDA current, suggesting that tLTP could be reduced or prevented.

The results of our study suggest that A1 follows unique STDP rules and these rules might have developed to process auditory-specific information. We also show that mAChRs modulate STDP rules in A1 suggesting that behavioral states that activate the cholinergic system change learning rules to process auditory information differently. Further investigation of the STDP rules at thalamocortical synapses in A1 and modulation by mAChRs are expected to reveal whether incoming auditory information is processed differently.


A1
B1
C1
Pre
Post
L
EPSP Slope (mV/ms)
-10ms
+10ms
+50ms
A2
B2
C2
Normalized EPSP Slope
Time (mins)
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Spike timing (ms)

Figure 1: Synaptic modification of L2/3 auditory cortical synapses induced by repetitive pre-postsynaptic pairing. A1, Example of cell with post→pre induction at a negative interval (10 ms). Post→pre pairing at 10 ms resulted in tLTD. Left inset, pairing paradigm. Right inset, trace of EPSPs. Black trace, baseline; grey trace, post pairing. Arrowhead, induction time. A2, Summary of effects of post→pre pairing at 10 ms. B1, Example of cell with pre→post pairing at a positive interval (10 ms). Pre→post pairing at 10 ms resulted in tLTP. B2, Summary of effects of pre→post pairing at 10 ms. C1, Example of cell with pre→post pairing at a positive interval (48 ms). Pre→post spiking at 50 ms resulted in tLTD. C2, Summary of effects of pre→post pairing at 50 ms. D, Synaptic plasticity depends on pre and postsynaptic interval. Each open circle represents one cell. Solid circles represent averages for each timing interval. Error bars are SEMs.

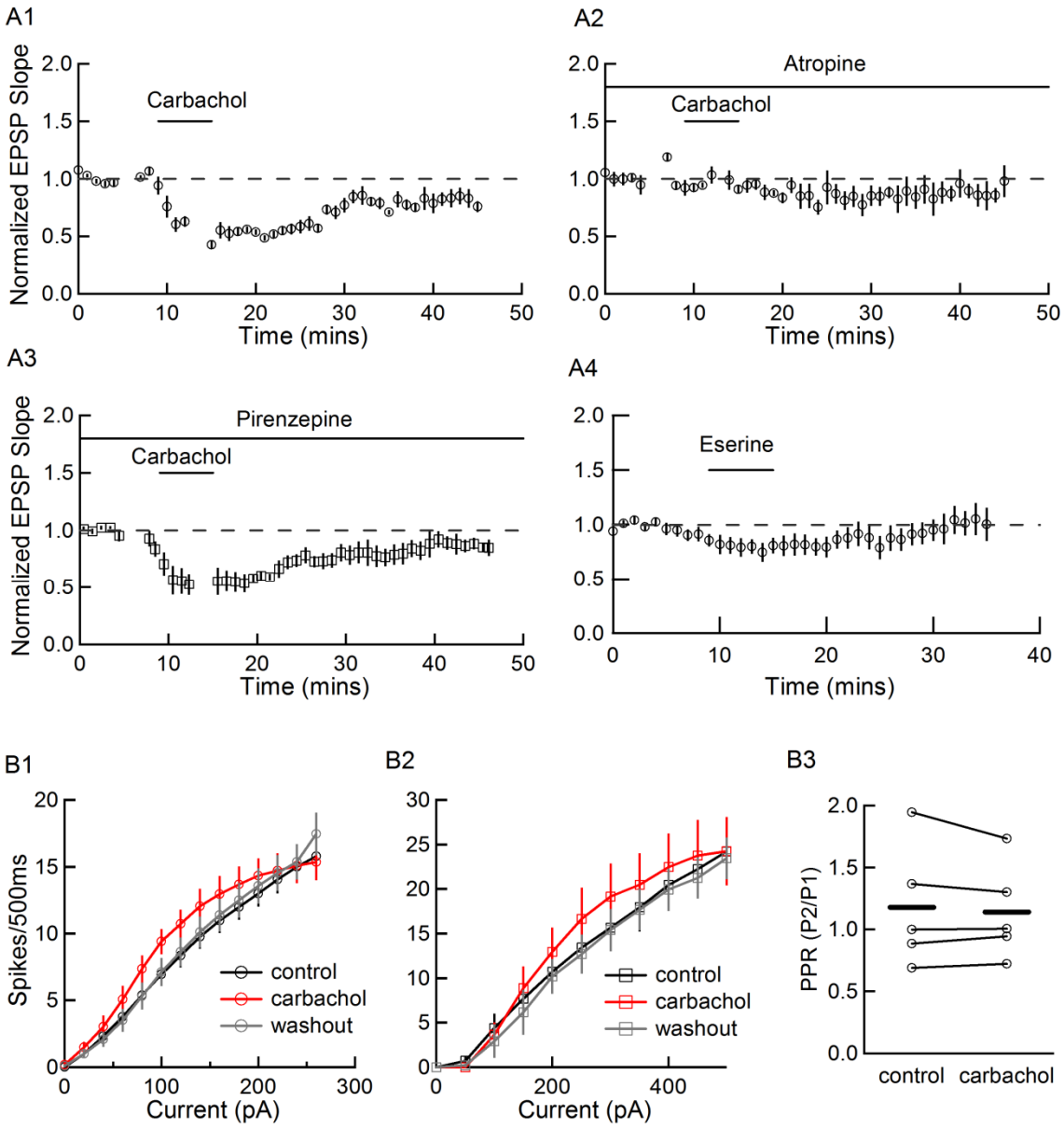

Figure 2: Muscarinic receptor activation induces LTD and increases excitability in auditory cortex. A1, Cholinergic agonist carbachol (20 μM, 5 mins) elicits a transient depression during agonist application followed by LTD after agonist washout that lasts the duration of the recording. A2, carbachol-induced transient depression is prevented but not LTD by 10 μM atropine, a nonselective mAChR antagonist. A3, Muscarinic LTD is not inhibited by pirenzepine (75 nM), an M1

receptor antagonist. A4, Application of a anticholinesterase eserine (1 µM, 5mins) induces a reversible transient depression. B1, Carbachol induces a reversible increase in firing rate. Black open circles are controls, black solid circles are carbachol, grey circles are washout. B2, Muscarinic increase in firing is not inhibited by pirenzepine. Black open squares are controls, black solid squares are carbachol, grey open squares are washout. B3, Carbachol does not affect paired pulse ratio (PPR). Error bars are SEMs.

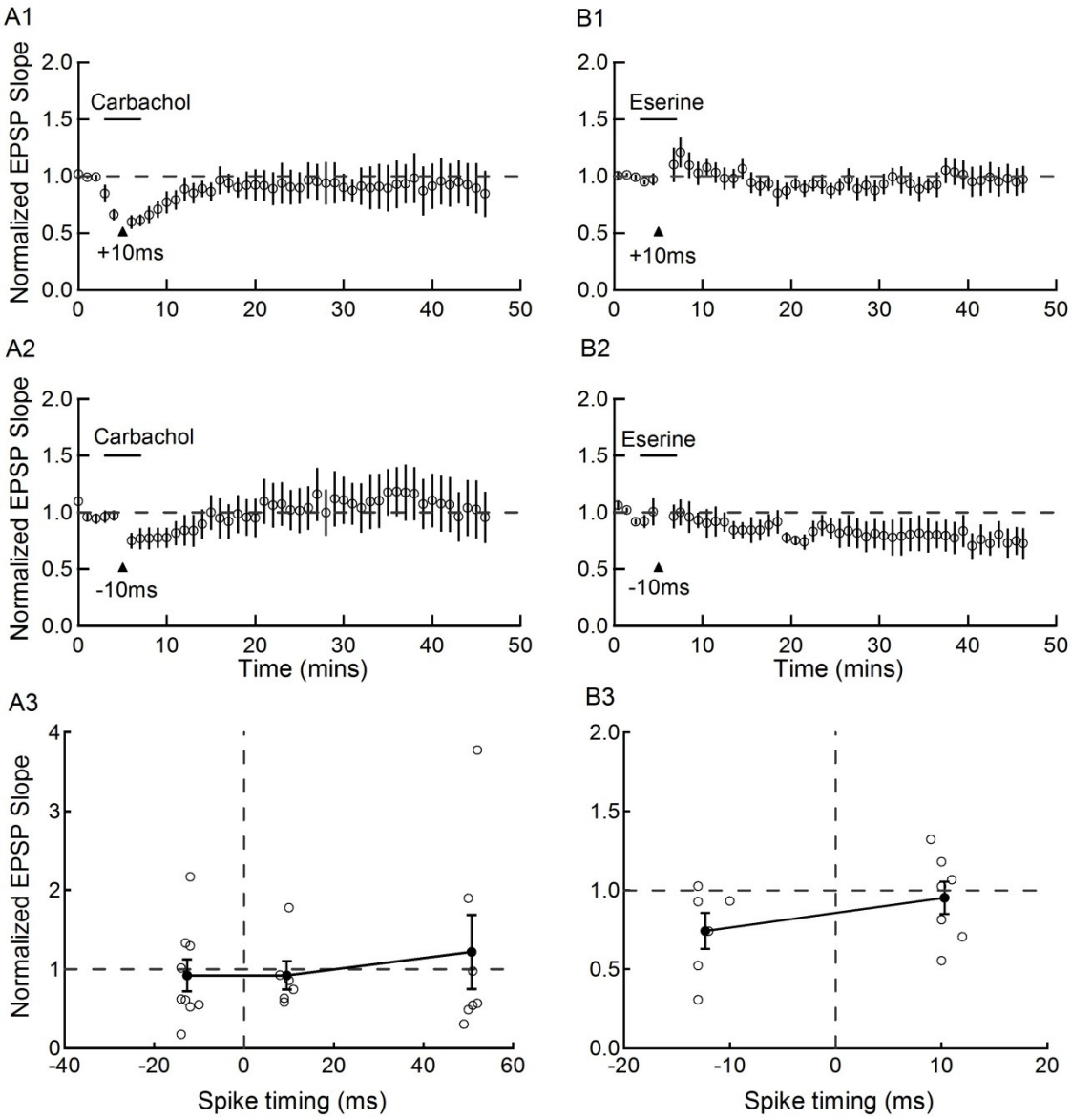

Figure 3: mAChR activation modulates STDP. A1, Summary of effects of carbachol (20μM) on pre→post pairing at +10ms as in Figure1 B1 (STDP protocol). Carbachol prevents tLTP induction. A2, Summary of effects of carbachol on post→pre pairing at -10ms as in Figure1 A1 (STDP protocol). A3, Carbachol modulation of STDP window. Carbachol prevents pre→post tLTP, but at some synapses converts post→pre and pre→post tLTD to tLTP (refer Fig 1D). B1, Summary of effects of eserine (1μM) after pre→post pairing at +10ms. B2

Summary of effects of eserine on post→pre pairing at -10ms. A3, Carbachol modulation of STDP window. Carbachol prevents pre→post tLTP, but at some synapses converts post→pre and pre→post tLTD to tLTP. B3, Eserine prevents tLTP induction but leaves post→pre tLTD unaffected. Each open circle represents one cell. Solid circles represent averages for each timing interval. Error bars are SEMs.

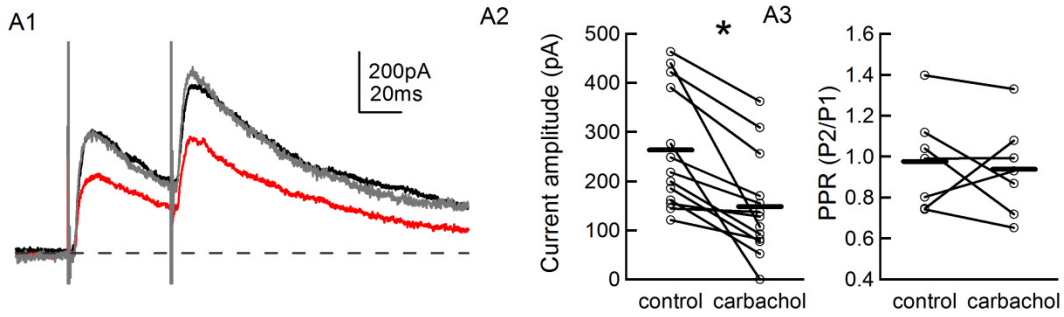

Figure 4: Activation of mAChRs reduced NMDA current. A1, NMDA mediated synaptic currents isolated with CNQX (10µM) and picrotoxin (50µM) at Vh= +40mV. A paired pulse protocol was used to measure PPR. Carbachol (20µM, 5mins) reversibly reduced the amplitude of the isolated NMDA current. Black trace is control, red is carbachol, grey is washout. A2, Carbachol reduced the amplitude of the NMDA current. * indicates $P<0.05$. A3, Paired pulse ratio (PPR) was unaffected by carbachol. Horizontal black bars represent averages of populations.

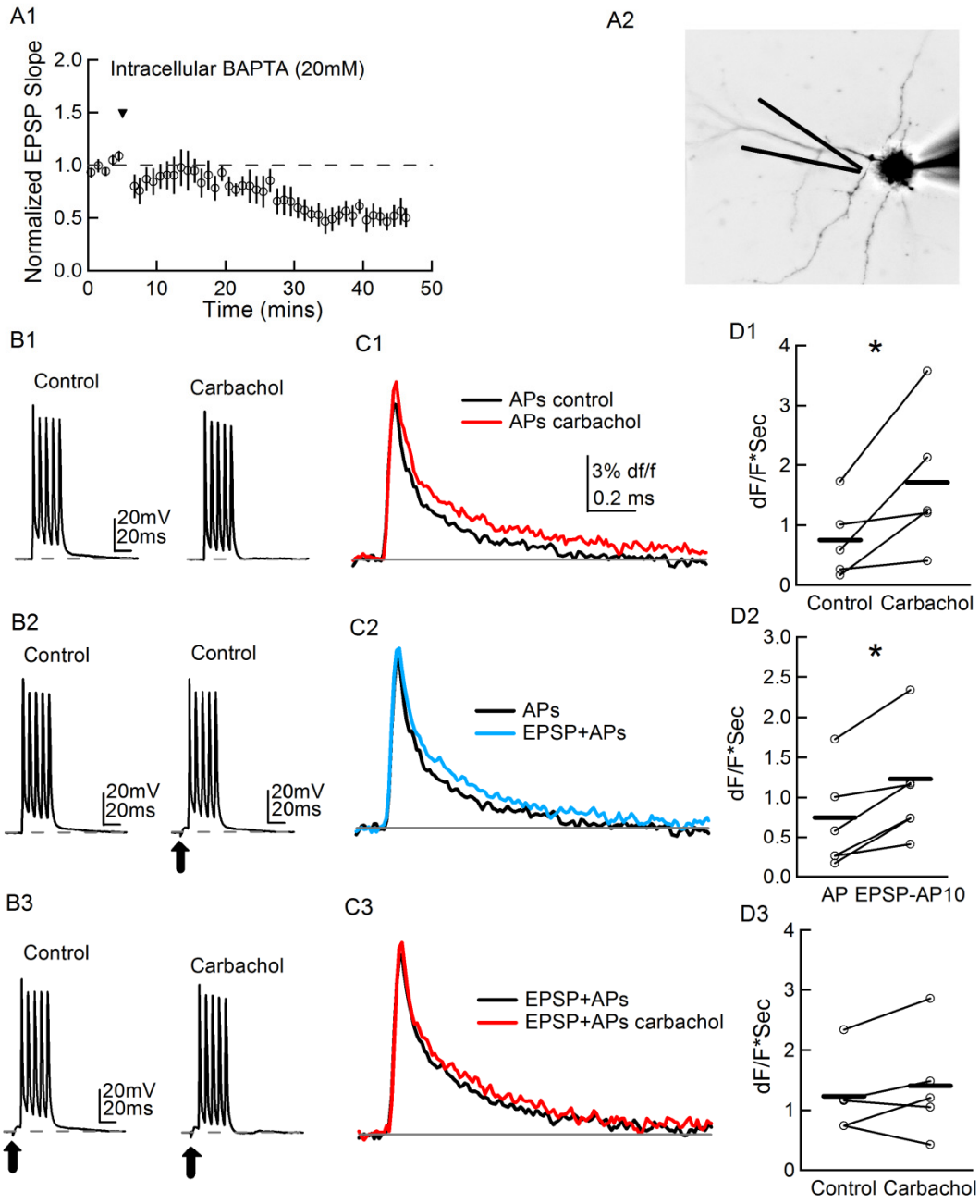

Figure 5: mAChR modulation of dendritic calcium influx. A1, Pre→post tLTP induction is dependent on postsynaptic calcium. Inclusion of BAPTA 20 mM in intracellular pipette prevented tLTP induction and revealed tLTD. A2, Image of L2/3 A1 neuron filled with Alexa 488. Stimulating electrode is placed next to proximal dendrite. B1, Trace of AP burst, in the absence and presence of carbachol. C1, Fluorescence measurement of calcium in response to AP burst in

the presence and absence of carbachol. D1, Carbachol enhanced AP burst mediated calcium influx, measured by area under the curve of calcium response. Horizontal lines represents the average of the populations. B2, Trace of AP burst and EPSP+AP paired with +10 ms interval. The arrow indicates the onset of the synaptic stimulation. C2, Fluorescence measurement of calcium in response to AP burst and EPSP+APs. D2, Pairing EPSP+APs at +10 ms enhanced calcium influx when compared to APs alone. Horizontal lines represents the average of the populations. B3, Trace of EPSP+APs paired with +10 ms interval, in the absence and presence of carbachol. The arrow indicates the onset of the synaptic stimulation. C3, Fluorescence measurement of calcium in response to EPSP+APs in the absence and presence of carbachol. D3, Carbachol did not affect calcium influx during paired EPSP+APs. Horizontal lines represents the average of the populations. * indicates $P<0.05$. Grey lines under traces represent baseline.

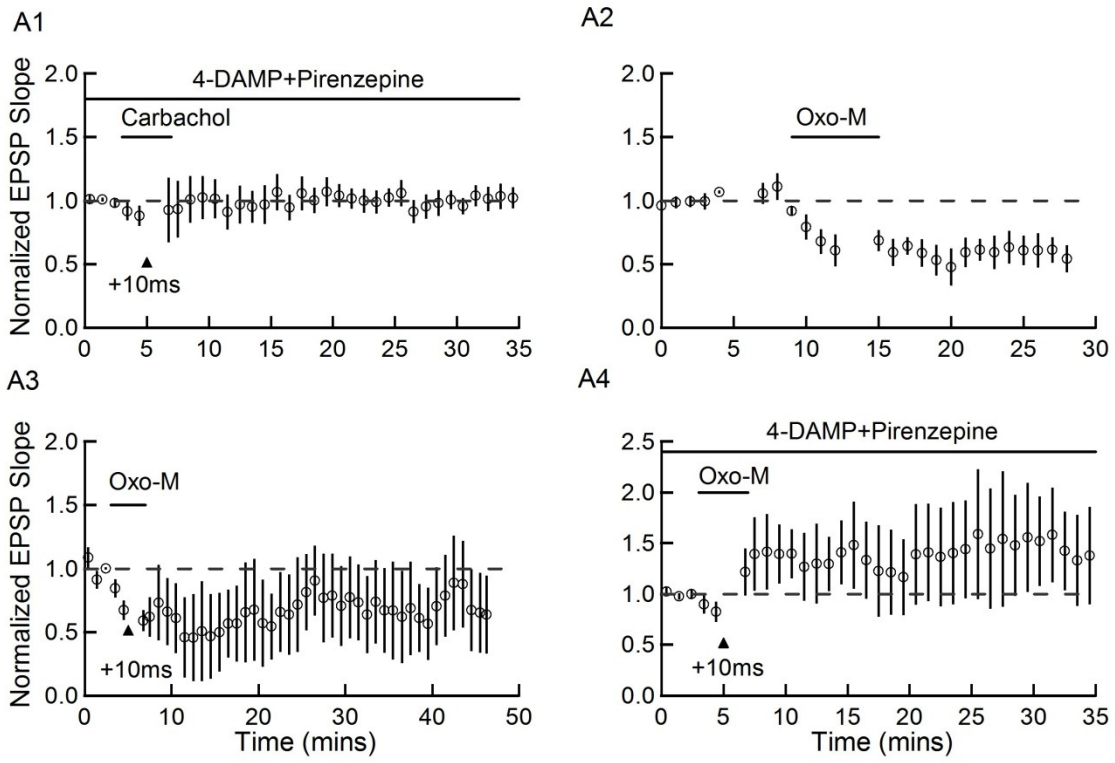

Figure 6: Oxo-M prevents tLTP induction. A1, Blocking M1 and M3 receptors did not prevent carbachol's effect on blocking tLTP. A2, Oxo-M (3 μM) induced LTD of basal synaptic transmission. A3, Oxo-M application during pre→post pairing at +10ms prevented tLTP induction. A4. Blocking M1 and M3 receptors restored tLTP that Oxo-M prevented.

CHAPTER 4

DISCUSSION

In this thesis, I have discussed two studies related to developmental plasticity in auditory cortex.

In the first study, I investigated how sensorineural hearing loss affects serotonergic modulation of intrinsic excitability. First, this study reveals that sensorineural hearing loss increases auditory cortical excitability, and that this effect does not persist at P30 in the rat. Second, this study suggests that 5-HT modulates excitability via multiple receptor subtypes and this modulation is altered by hearing loss.

In the second study, I showed that auditory cortical synapses can undergo long-term plasticity in a manner that is dependent on spike timing and that the plasticity timing rules are modulated by postsynaptic muscarinic receptor activation. The results of this study indicate that spike timing dependent plasticity at auditory cortical synapses follow unique rules. Further, this study revealed that muscarinic receptor activation modulates STDP rules by regulating NMDAR current and postsynaptic calcium influx.

In this section I will discuss in further detail the results of the two studies and speculate on the functional significance of serotonergic and cholinergic modulation of auditory cortical plasticity and function.

1. Auditory development and plasticity

Auditory perception, in humans, begins in the prenatal fetus and persists through adolescence. The human fetus responds to spectro-temporal features of sounds at post conception 25-40 weeks (Groome et al. 2000). Frequency discrimination develops until postnatal 9 years (Moore et al. 2008), and detection of frequency modulated sounds develops until 8 years (Dawes and Bishop 2008). Children can learn to recognize speech sounds easier than adults, signifying a sensitive period for the auditory system. This sensitive period is especially important when considering studies of profoundly deaf children who receive a cochlear implant. The importance of early auditory experience prior to deafness is emphasized in speech perception abilities of adult cochlear implant patients. Speech perception of pre-linguistically deaf adult implant users is much poorer than that of post-linguistically deaf adults (Busby and Clark 1999). Interestingly, children who are implanted display better language acquisition and could reach performance levels similar to their normal hearing peers, if implanted before 12 months (Sharma et al. 2002b; Svirsky et al. 2004). These functional studies support the existence of a critical period and emphasize the importance of early auditory experience. As the young pre-language developmental brain is much more plastic than adult brains, cochlear implants would be more effective in preventing degenerative effects associated with hearing loss (Eggermont 1986). There are now increasing numbers of hearing impaired children – including congenitally deaf children – receiving cochlear implants at an early age (NIH Consensus Statement, 1995). It is therefore important to evaluate the response

of the central auditory pathways to early auditory experience and early onset SNHL, and response to reinstating electrical stimulation of the auditory nerve to improve treatment strategies and restore normal hearing and speech.

2. SNHL and A1 plasticity

The plasticity of the auditory system enables the reorganization of its structure and function after the loss of hearing. Hair cells in the cochlea are especially vulnerable: overstimulation by intense sound can damage components of the delicate stereocilia, as well as induce excitotoxicity of the hair-cell to auditory nerve synapses, leading hearing threshold shifts. Hair cells and spiral ganglion cell degeneration starts a cascade of deleterious effects on central auditory nuclei, affecting the central pathways all the way up to the auditory cortex. For example, surgical removal of the cochlea in neonatal animals, as a model of early onset SNHL, results in a reduction in the number of neurons in the cochlear nuclei (Hashisaki and Rubel 1989), a loss of potassium-dependent chloride transport function in the inferior colliculus (Vale et al. 2003), larger arborizations of MNTB neurons in LSO (Sanes and Takacs 1993), and decrease in inhibition and increase in excitation in auditory cortex (Kotak et al. 2005; Takesian et al. 2009). An understanding of SNHL–induced changes is important considering the compensatory changes that are in conjunction with peripheral injury (Syka 2002) and the ability to restore stimulation with cochlear implants (Klinke et al. 1999; Kral et al. 2000). For example, in congenitally deaf cats with cochlear implants, field potentials of cortical neurons had higher amplitudes, long

latency responses and larger synaptic efficacy than in un-stimulated deaf cats, suggesting that implants can restore function in impaired A1 (Klinke et al. 1999). Cochlear implants also restore a rudimentary level of tonotopy in A1 (Fallon et al. 2009).

Understanding how electrical properties of neurons in the auditory cortex change with auditory experience during the highly plastic developmental epoch and how they would be affected by hearing loss provides sites that can be targeted to improve function. The plasticity of intrinsic electrical properties due to hearing loss may affect how neurons respond to restored electrical input with cochlear implants and other hearing devices. Intrinsic electrical properties play an important role in determining how neurons integrate and represent auditory information (Manis and Marx 1991). SNHL increases intrinsic excitability in the cochlear nucleus (Francis and Manis 2000) and in gerbil auditory cortex (Kotak et al. 2005). I have shown that L2/3 pyramidal neurons in rat A1 display increased excitability with hearing loss. My study extends our understanding of the long-term effects of hearing loss in cortex. I found that deafness induced increase in excitability was transient appearing only during P12-P21 and disappeared 21–27 days after the cochlear ablation. This result suggests that activity is normalized over time. How does the activity return to a normal functioning level? Studies have shown that A1, in deaf patients or animals, is available for functional recruitment and may be involved in cross-modal plasticity (Bavelier et al. 2006; Hauser et al. 2007). For example, increased visual cognition is measured in some hearing loss patients (Stivalet et al. 1998). Given these results, we suggest

experiments to test if hearing loss initiates cross modal plasticity in the adult brain. If cross modal plasticity develops (in the rat) at P30, we should be able to measure visual or somatosensory evoked potentials in A1 *in vivo* in P30 rats that had neonatal cochlear ablations, while finding that such cross-modal potentials would be absent in P12 rats.

Present day cochlear implant and hearing loss treatments do not completely restore normal hearing in individuals. Given that neuromodulators play a crucial role in developmental plasticity and function in auditory cortex, we hypothesized that neuromodulation might be supplemental to cochlear implants in restoring normal hearing. To this end, we sought to understand how neuromodulators, specifically serotonin, changes intrinsic electrical properties in the normal auditory cortex and how serotonergic input regulates plasticity after SNHL. We found that serotonin decreases intrinsic excitability in normal developing auditory cortex. These results suggest that when 5-HT is released in A1, it acts to suppress neural activity. 5-HT is released in auditory cortex under stress or fearful conditions and suppresses frequency tuning plasticity (Ji and Suga 2007). Thus the role of 5HT in A1 might be to suppress of auditory learning in strongly stressful situations by decreasing neuronal excitability and frequency tuning plasticity. I also found that cochlear ablation occludes the effects of serotonin on excitability in developing A1. This suggests that 5-HT may not suppress A1 activity during stress or fear situations. However, in the presence of a 5-HT2 antagonist, serotonin can decrease excitability further, suggesting that

serotonin acts through two receptor mechanisms with opposing actions on excitability.

How may the results of the hearing loss study further our efforts in improving treatment strategies for SNHL? Cochlear implants applied early in a child's development could send electrical impulses through the auditory pathway up to cortex to allow normal development of ion channel composition, density and function and lead to normal intrinsic plasticity and thus could prevent the enhancement of excitability we observed after hearing loss. The long term use of implants could be monitored in these children: as excitability normalizes, the implants might provide overstimulation that isn't required further. How do these results help improve cochlear implant outcomes? For example, supplementing cochlear implants with 5-HT2 antagonists in A1 might restore the normal decrease in excitability seen with 5-HT in compromised A1. I also found that the modulation of excitability by serotonin is diminished in the adult animals with SNHL. These results suggest that 5-HT receptor composition and activation in A1 neurons determines excitability of neurons during development but not in adults. Additional studies are required to understand the 5-HT receptor subtypes that bring about regulation of intrinsic plasticity in A1 plasticity.

3. STDP in the human brain

Although STDP has been studied extensively in animal models, is STDP a relevant mechanism for information storage in the human brain? The human cortex displays STDP suggesting timing dependent plasticity as a relevant

mechanism of learning in the human brain (reviewed (Muller-Dahlhaus et al. 2010)). For example, “STDP-like” pairing of electrical wrist-nerve stimulation (NS) and transcranial magnetic stimulation (TMS) over motor cortex (M1) can induce plasticity in M1 (Stefan et al. 2000). The efficacy of neuronal firing with TMS was assessed by the presence of motor evoked potentials (MEP) in the hand muscle. The interval between NS and TMS is in milliseconds. Repetitive pairing of NS and TMS enhances MEP in the hand muscle and is dependent on the time interval between the two. The STDP-like plasticity could play a role in creating a kinematic memory trace in repetitive thumb movements after training suggesting that repeated movements reinforce network connectional patterns, but those patterns weaken if the movements have not been recently performed (Classen et al. 1998). Taken together these results suggest STDP-like rules induce plasticity in cortical networks and may underlie learning of sensory information in a manner that is dependent on how often the sensory inputs occur in conjunction.

STDP rules and its neuromodulation by dopamine has been recently been employed for the treatment of Parkinson’s disease (PD) in humans. Dopaminergic modulation of TMS intervention in PD was studied using a TMS protocol that induces STDP in PD patients on and off on levodopa. MEP amplitude was larger in PD patients on levodopa than off. This finding suggests that STDP in motor cortex is preserved in PD and the magnitude of the effect is enhanced by dopamine (Rodrigues et al. 2008). This finding highlights the importance of understanding neuromodulation of STDP in the brain.

STDP has even been used as a model to explain functional connectivity changes observed in humans with a neurological syndrome. For example, patients with spastic diplegia, a subtype of cerebral palsy, have motor deficits with diminished tactile sense and kinesthesia called spastic diplegia (Burton et al. 2009). In spastic diplegia, somatosensory and motor cortical networks are disordered: intracortical connections dominate through successful competition with reduced thalamocortical inputs based on an STDP mechanism (Burton et al. 2009). Given these results, STDP serves as a model for understanding cortical network connectivity and cortical dysfunction. Therefore it is important to understand the normal cellular mechanisms of STDP to be able to identify disorders of cortical processing.

4. Functional relevance of STDP rules in A1

STDP has been implicated as a mechanism for receptive field plasticity in visual, somatosensory and auditory cortices (Dahmen et al. 2008; Jacob et al. 2007; Yao and Dan 2001). My results show that recurrent synapses in A1 display unique STDP rules. Pairing pre→post between L2/3 neurons and with a +10 ms delay produces tLTP and if the temporal order is reversed, tLTD is induced. These results are consistent with timing rules observed *in vivo* in A1. Therefore my results suggest that receptive field development and plasticity in A1 might occur through synaptic strengthening (tLTP) and synaptic weakening (tLTD) of recurrent synapses under the guidance of spike timing rules. A unique aspect of

my results is that pairing of pre→post with a +50 ms delay produced a tLTD. The double tLTD window flanking the tLTP could serve to enhance potentiation and decrease ongoing cortical activity, or act as a temporal filter to restrict tLTP to sensory events that are closer together in time.

Given these results, how does A1 process sound information using STDP rules? Frequency modulated (FM) sounds is a common component of species specific vocalizations, including human speech. In many species, including song birds, neurons are selective for vocalizations. A1 can over-represent species-specific vocalizations in a manner that is dependent upon temporal rates (Kim and Bao 2009). In young bats that do not hear their own emitted FM signals, cortical neurons are less selective for the temporal rate of FM sounds (Razak et al. 2008). On the other hand, exposure of young rats to FM sounds results in cortical neurons more selective for the temporal rate of the FM sound (Insanally et al. 2009). How can STDP underlie the selectivity for FM sounds? STDP mechanisms could support the pre and post synaptic activity generated when onset times of differentially tuned inputs are presented. Therefore, repeated exposure to FM sounds would induce STDP in A1. The tLTD windows flanking the tLTP window could serve to enhance temporal selection for the timing of synaptic inputs and this local amplification is important in enhancing the sensory selectivity of cortical neurons (Douglas et al. 1995; Sompolinsky and Shapley 1997). Interestingly, the plasticity of sideband inhibition, in cortex, is shown as a mechanism by which experience influences development of cortical FM

selectivity (Razak et al. 2008), suggesting that the tLTD windows could underlie sideband inhibition.

Is STDP a relevant mechanism when incoming auditory information is behaviorally relevant? Young zebra finches that are involved in a behavioral task during exposure to a father's song require only few stimuli for learning (Tchernichovski et al. 1999). Activation of cholinergic neurons and release of acetylcholine is shown to provide the behavioral relevance for sensory information (Weinberger and Bakin 1998). My results using cholinergic agonists during STDP induction show that pre→post pairing at +10 ms with agonist prevents tLTP induction. However, pre→post at +50 ms or post→pre at -10 ms in the presence of agonist, in some cells, converts tLTD to tLTP. The prevention of tLTP could be relevant for enhancing signal to noise of incoming auditory inputs while stabilizing intracortical auditory processing (see Figure 1). The tLTP windows flanking the tLTD window (the reversal of STDP, in absence of cholinergic activation) could serve to suppress the FM selectivity of cortical neurons. The results suggest that behavioral states that stimulate cholinergic efferents, that activate muscarinic receptors, modulate STDP rules in A1.

5. Closing remarks

The common theme in my two thesis projects understands how signals that provide information about the behavioral significance of information can

change how that information is stored or processed in the brain. Two signals that provide information about the behavioral importance are neuromodulators 5-HT and acetylcholine. In the first project, I showed that 5-HT decreases intrinsic excitability of auditory cortical neurons and hearing loss increases intrinsic excitability, but that this excitability normalizes in older rodents. I also showed that activating a non-5-HT2 receptor restores 5-HT's effect on excitability in compromised A1. In the second project, I showed that auditory cortical neurons follow unique STDP rules. I also showed that muscarinic receptor activation modulates the STDP rules, and that the modulation could serve to select synapses for plasticity, or to suppress the plasticity of other inputs, stabilizing the existing network structure. Therefore these two studies show that neuromodulators associate with auditory neural activity differently to store relevant information.

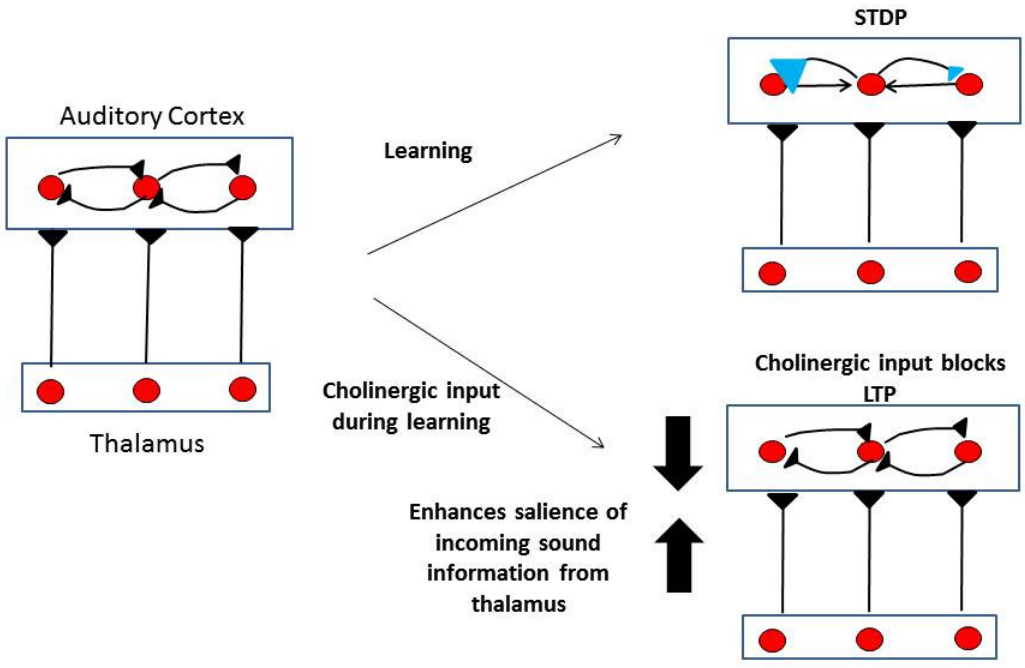

Figure 1: Functional significance of cholinergic modulation of STDP in auditory cortex. Learning induced changes in synaptic strength can be modulated by cholinergic activation. Cholinergic input blocks LTP. The suppression of excitation within cortex could suggest that incoming sound information from thalamic inputs are given salience.

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