Developmental Plasticity
of Tonotopic Maps
in Chinchilla Auditory Midbrain

by

Lisa Margaret D’Alessandro

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Physiology
collaborative with the
Institute of Biomaterials & Biomedical Engineering
University of Toronto

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Abstract

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2015

Sensory areas of the brain have the remarkable ability to reorganize following changes in peripheral input, especially during early development. Cortex of altricial species (post-natal hearing onset) is often investigated. It remains unclear whether neural connections in auditory midbrain reorganize when precocious subjects (hearing onset in utero; e.g., *chinchilla laniger*) are reared in an enhanced acoustic environment.

Neonatal chinchillas were chronically exposed to a moderately-intense (70 dB SPL) narrowband (2 ± 0.25 kHz) sound stimulus for 4 weeks. No difference in tone-specific auditory brainstem response thresholds, hair cell morphology, neural thresholds, and bandwidth 10 dB above threshold were observed around 2 kHz, suggesting the exposure stimulus was non-traumatic. Sound frequency maps in central nucleus of inferior colliculus (IC) were defined with microelectrode recordings. We observed a significant decrease in the proportion of neurons dedicated to octave bands centered at 2- and 8- kHz. Changes were not limited to sound-exposure frequencies: increases in low-frequency representation (below 1 kHz) were observed.
We describe a c-fos immunolabelling protocol for chinchilla. Bands of neurons observed following 90-min, 6-kHz stimulation lay ventro-medial to those present following 90-min, 2-kHz stimulation, consistent with the known tonotopic organization of IC, and verified herein by electrophysiological recordings. Interestingly, we observed decreased labeling adjacent to these bands, which we suggest represent inhibitory regions. Following sound exposure, then 90-min, 2-kHz sound stimulation, the number of labeled cells both in the 2-kHz region and throughout IC was increased. Sound-exposed subjects who received no further sound stimulation had c-fos expression patterns similar to silence controls, suggesting sound-exposure does not alter basal levels of c-fos expression, but changes the way neurons respond to prolonged (90-min) tone stimulation.

This study contributes to a growing body of literature that suggests that sound frequency representation in auditory midbrain is altered following passive peripheral input during development.
Acknowledgements

I would like to thank my thesis advisor, Dr. Bob Harrison, for the opportunity to study in the Auditory Science Lab, and for his generosity and support over the years. My thanks as well to the members of my supervisory committee: Drs. Bill Hutchison, Karen Gordon, and Willy Wong, who offered many helpful suggestions at all stages of the research.

I am indebted to my colleagues and fellow members of the Auditory Science Lab at the Hospital for Sick Children, past and present, for their helpful advice, and to Marvin Estrada in LAS for assistance developing the surgical technique. I would also like to thank my Master’s thesis advisor, Dr. Ken Norwich. Many of the valuable lessons I learned while completing the MSc under his mentorship continued to guide me during the PhD.

I am grateful for financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC) and from Ontario Graduate Scholarships (OGS). The lab is supported by grants from the Canadian Institutes for Health Research (CIHR) and the Masonic Foundation.

Finally, I would like to thank my family and friends for their enduring support.
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<th>Description</th>
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<tr>
<td>A1</td>
<td>primary auditory cortex</td>
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<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
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<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid</td>
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<tr>
<td>AMPAR</td>
<td>AMPA receptor</td>
</tr>
<tr>
<td>arc</td>
<td>activity-regulated cytoskeleton</td>
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<tr>
<td>AVCN</td>
<td>anteroventral cochlear nucleus</td>
</tr>
<tr>
<td>CF</td>
<td>characteristic frequency</td>
</tr>
<tr>
<td>CN</td>
<td>cochlear nucleus</td>
</tr>
<tr>
<td>DCN</td>
<td>dorsal cochlear nucleus</td>
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<tr>
<td>DNLL</td>
<td>dorsal nucleus of the lateral lemniscus</td>
</tr>
<tr>
<td>IC</td>
<td>inferior colliculus</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>IHC</td>
<td>inner hair cell</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>LSO</td>
<td>lateral superior olive</td>
</tr>
<tr>
<td>MET</td>
<td>mechanically-gated electrical transduction (channels)</td>
</tr>
<tr>
<td>MGB</td>
<td>medial geniculate body</td>
</tr>
<tr>
<td>MNTB</td>
<td>medial nucleus of the trapezoid body</td>
</tr>
<tr>
<td>mEPSCs</td>
<td>miniature excitatory post-synaptic currents</td>
</tr>
<tr>
<td>MSO</td>
<td>medial superior olive</td>
</tr>
<tr>
<td>MTB</td>
<td>medial nucleus of the trapezoid body</td>
</tr>
<tr>
<td>NGFI-A</td>
<td>nerve growth factor induced-A</td>
</tr>
<tr>
<td>NLL</td>
<td>nuclei of the lateral lemniscus</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>OHC</td>
<td>outer hair cell</td>
</tr>
<tr>
<td>Px</td>
<td>post-natal day x; e.g., P28 = post-natal day 28</td>
</tr>
<tr>
<td>PVCN</td>
<td>posteroverentral cochlear nucleus</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy; standard error of the mean</td>
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<tr>
<td>SOC</td>
<td>superior olivary complex</td>
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<tr>
<td>VNLL</td>
<td>ventral nucleus of the lateral lemniscus</td>
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Chapter 1

Introduction

In this chapter, we outline the reasoning that led to the undertaking of the current research. In subsequent chapters, we elaborate on many of the concepts introduced in the present chapter.

1.1 Thesis Overview

Sensation involves the ability to transduce, encode, and perceive information from the external environment. A sizeable portion of the brain is devoted to these processes. Historically, the representation of sensory information in central brain centers was considered static. However, during the latter half of the 20th century, theoretical and experimental results began to emerge that provides evidence for a more ‘plastic’ brain. Plasticity can be broadly defined as the ability for neural connections and function to be modified depending on sensory input and previous experience.
Acoustic stimuli from the external environment are represented in the brain by patterns of neural activity established along the sensory epithelium of the cochlea. Beginning in the cochlea, the representation of sound frequency is organized systematically (from low- to high-frequency). The resulting neural activity patterns are transmitted to cortex, maintaining this tonotopic organization. While the representation of sound within the auditory brain is similar among vertebrates, the maturity of the auditory system at birth differs between species. Many common laboratory species, such as the mouse or rat, are altricial species: born with relatively immature auditory systems and that undergo hearing onset post-natally. Humans, on the other hand, are a relatively precocious species. At birth, the cochlea is well-developed, and there is some evidence for hearing responses in utero (e.g., sound-evoked blink startle reflex measured at 24-25 week gestational age by ultrasound imaging, Birnholz and Benacerraf 1983). An appropriate animal model for studies relating to human auditory development is a precocious one, such as the chinchilla.

The degree of neural plasticity differs across the lifespan. Following lesions of the cochlear sensory epithelium, sound frequency representation of the adult auditory cortex and thalamus are altered. Plasticity of sub-thalamic regions (also in an adult model) are less pronounced, suggesting that in a mature model, reorganization occurs mainly at the level of auditory cortex/thalamus in response to peripheral deficits. During early development, there is evidence for considerable plasticity at cortical as well as sub-thalamic auditory regions following cochlear lesions. While studies of sensory deficits provide a useful model of auditory pathway development in a hearing-impaired subject, they are less instructive as to how auditory pathways develop in a normal-hearing subject.
In contrast to sensory deficits, do acoustically-augmented environments alter neural representation of sound frequency in a normal hearing subject?

Studies from the Harrison lab suggest that in cat auditory cortex, sound frequency maps are altered following rearing in an enhanced acoustic environment (Stanton and Harrison 1996). Later work, also from the Harrison lab, provides evidence that thalamo-cortical projections are relatively normal in the cat following neonatal cochlear lesions (Stanton and Harrison 2000), suggesting that reorganization of sound frequency maps occurs at sub-thalamic auditory nuclei. It is unknown to what extent such changes occur, if at all, in the inferior colliculus of auditory midbrain (the first sub-thalamic auditory region) in a precocious animal model following neonatal rearing in an augmented sound environment.

Our working hypothesis is that the development of neural connections within the ascending auditory pathway is influenced, in large part, by patterns of sensory activity elicited by environmental sound stimulation during an early post-natal period. Specifically, we hypothesize that passive exposure to an enhanced sound environment (in our case, a chronic, moderate-level narrowband signal centered at 2 kHz) changes the neural representation of sound frequency in central nucleus of inferior colliculus (IC) of developing subjects compared with age-matched controls.

To test this hypothesis, neonatal chinchillas (Chinchilla laniger) were exposed for 4 weeks to a moderately-intense (70 dB SPL), narrowband-signal-enriched (2 ± 0.25 kHz) sound environment. We were interested in the structural and functional abilities of hair cells of the cochlea, following this 4-week period of neonatal sound exposure. The sound-exposure stimulus was designed to elicit neuronal activation, but not to damage
cochlear hair cells. We conducted pure-tone ABR threshold assessments at frequencies around the sound-exposure stimulus, and assessed hair cell morphology and distribution with scanning electron microscopy, also near the 2 kHz region, to examine the functional and structural integrity of hair cells post-sound-exposure.

We used two techniques to quantify changes in sound frequency representation in inferior colliculus: 1) *in vivo* micro-electrode recordings through central nucleus of inferior colliculus, a direct measure of neural function and a classic technique considered a “gold standard”; and 2) immunolabeling of the c-fos protein, which is expressed by auditory neurons after they have been recently “activated” by sound. This technique provides an indirect histological measure of global neural activation patterns with single-cell resolution, and makes use of advances in molecular biology. To the best of our knowledge, there was no previously reported technique for c-fos immunohistochemistry for the precocious chinchilla, thus our first step here involved developing a c-fos protocol for the chinchilla. Together, these experiments are designed to provide some of the first experimental evidence of possible effects of sound exposure on midbrain reorganization in a precocious species.

Studying the effect of sound on the developmental plasticity of the auditory system is important for our general understanding of how the auditory system develops. This work will add to our understanding of subcortical plasticity in an animal model whose auditory system, in an early developmental epoch, relates closely to that of humans. While not a direct model, a better understanding of how sound influences the normal development of auditory pathways may have some relevance for hearing-impaired children who receive rehabilitative treatment, such as hearing aids or cochlear
implants. These children receive auditory stimulation during a period in which their auditory structures have significant plasticity.

In the next chapter, we expand on many of the concepts introduced in the present chapter. We summarize relevant findings from the literature, as it relates to the research herein: we review the structure and function of the cochlea, and the transmission and processing of auditory information as it passes to higher auditory nuclei; we review sensory plasticity in developing and adult models, and introduce mechanisms by which neural plasticity can occur.
Chapter 2

Background

In this chapter, we review anatomy and physiology of the auditory periphery and central auditory structures, including inferior colliculus: a “hub” of the auditory system. We describe the effects of post-natal experience on the development of brain circuitry, and introduce mechanisms by which neural plasticity can occur. We then discuss the motivation for undertaking the work, including our choice of animal model and the c-fos immunolabeling method. The chapter concludes with our research hypothesis and objectives.

2.1 The Mammalian Auditory System

2.1.1 Auditory periphery: External, middle, inner ear

The three divisions of the ear (external, middle, inner; Figure 2.1) each have specialized functions. The labyrinth of the inner ear is the organ of hearing and balance. We focus here on hearing, considering each division of the ear in turn.
In all species, the external ear modifies the acoustic spectrum of environmental sounds before they reach the tympanic membrane. The pinna and concha filter sound frequencies, providing cues about the elevation of the sound source. The external auditory meatus forms a resonant cavity, with a resonant frequency of 3 kHz in humans; thus, sound frequencies near 3 kHz see the greatest gains. Frequencies in the speech range (2 - 5 kHz) see gains of 10 - 20 dB (Evans 1982; Moller 1983). The primary functions of the external ear are to gather, transmit, and localize sounds from the external environment.

Figure 2.1: Anatomy of the auditory periphery (adapted from Silverthorn, 2004).
The middle ear transforms airborne sound waves into pressure waves in the fluid-filled cochlea of the inner ear. When sound waves traveling in air (a low-impedance medium) strike fluid (a higher-impedance medium), almost all of the acoustical energy (99.7%) is reflected. Two middle ear mechanisms mitigate this impedance mismatch. The first, and most important, is the concentration of sound energy from the tympanic membrane, which has a larger surface area, onto the oval window, which has a smaller surface area (20:1 in humans). The second is the mechanical advantage of the lever action of the smallest bones in the human body, the middle ear ossicles, resulting from the length of the malleus being greater than that of the incus (1.4:1 in humans). With these impedance-matching mechanisms, approximately 60% of sound energy is transmitted to the cochlea.

The main function of the inner ear is to convert mechanical energy to electrical energy (nerve impulses). The cochlea (Figure 2.2) is the structure critical to this process: it houses hair cells, the non-neural auditory receptor cells, within the organ of Corti (named after the 19th century Italian anatomist Alfonso Corti). The organ of Corti is attached to the basilar membrane, partially covered by the tectorial membrane (Figure 2.2C). The organ of Corti houses two types of hair cell: a single row of inner hair cells (IHCs; Figure 2.3C), the sensory receptors that transduce mechanical energy to electrical energy, and three rows of outer hair cells (OHCs, sometimes referred to as cochlear amplifiers, Figure 2.3A), which function to amplify low-intensity sounds and to provide increased frequency resolution and sensitivity. The tallest stereocilia of OHCs are imbedded in the overlying tectorial membrane (Figure 2.3B), whereas the stereocilia of IHCs are either entirely free-standing or only loosely attached to the tectorial membrane.
(Figure 2.3D). Unlike IHCs, OHCs contain actin filaments, which suggest an innate ability to contract. One way OHCs are thought to improve frequency discrimination is by voltage-dependent somatic motility, which locally alters the movement of the basilar membrane thus giving a more sensitive and sharper frequency response (review: Fettiplace and Hackney 2006).

Figure 2.2: Anatomy of cochlear structures. (A) Intact cochlea; (B) Cross-section of the cochlea; (C) Organ of Corti. Adapted from Silverthorn, 2004.
Figure 2.3: Hair cell stereocilia and their imprints on the underside of the tectorial membrane. (A) Outer hair cell (OHC) stereocilia of a young (post-natal day 29, P29), normal-hearing chinchilla, seen with SEM. (B) Imprints of individual stereocilia from the tallest row of OHCs are seen on the underside of the tectorial membrane: OHCs are attached to the tectorial membrane. (C) Inner hair cells (IHCs) in a young (P29), normal-hearing chinchilla. (D) “Imprints” from the tallest row of IHC stereocilia are much less pronounced than those of OHCs (and may in fact be procedural artifacts): IHCs are free-standing or only loosely connected to the overlying tectorial membrane. (D’Alessandro 2015, unpublished data.)

The shape and stiffness of outer hair cell stereocilia and size of the bundles also differ depending on their location along the basilar membrane: shorter, stiffer stereocilia with broader bundles are observed near the cochlear base (Figure 2.4B); at the apex, stereocilia are more than twice as tall and more flexible with narrower bundles (Figure
2.4A). These variations become important in the present study when we examine mid-frequency regions of the cochlea that correspond approximately to the frequency of the sound exposure stimulus.

Figure 2.4: Shape and height of outer hair cell stereocilia differ along the length of the cochlea. (A) Apical, (B) basal outer hair cell stereocilia from young (4 - 6 week old), normal-hearing chinchillas. (D’Alessandro 2015, unpublished data.)

Cochlear function is similar to that of a spectrum analyzer, decomposing complex acoustical waveforms into simpler frequency components. This occurs, in part, due to the characteristics of the basilar membrane, which change along its length. It is stiff and narrow near the oval and round windows near the cochlear base; it is more flexible and wider near the helicotrema at the cochlear apex, as illustrated diagrammatically in Figure 2.5 (p. 14). Pressure waves initiated by the movement of the stapes footplate in the oval window establish a traveling wave, the envelope of which reaches a maximum at a position along the basilar membrane that is determined by the frequency of the sound stimulus (von Békésy, 1960; Figure 2.5, p. 14).
As fluid waves travel through the cochlea, they displace the flexible basilar and tectorial membranes. Because the pivot points for these membranes differ, displacement of the basilar membrane causes the tectorial membrane to move across hair cell stereocilia, creating a shearing force that bends hair cell stereocilia. The tips of hair cell stereocilia are linked by filamentous structures called tip links that, when stretched, open mechanically-gated electrical transduction (MET) channels. Deflection of hair cell stereocilia towards the tallest stereocilia increases the probability that MET channels are open. When open, MET channels allow K\(^+\) and Ca\(^{2+}\) influx, depolarizing the cell, and creating a receptor potential. The receptor potential opens voltage-gated Ca\(^{2+}\) channels on the hair cell soma. Ca\(^{2+}\) enters the cell, triggering the release of neurotransmitter (primarily glutamate) from vesicles at the basal end of the hair cell. Neurotransmitter diffuses across the relatively narrow space between the hair cell and nerve terminals, binds to receptors on the nerve terminals, and triggers an action potential in the afferent nerve.

### 2.1.2 Topographic representation along sensory pathways

The senses are an essential way in which organisms interact with their environment. A shared feature of most mammalian sensory systems is topographical representation from sensory cell epithelium to cortex. These sensory cortices are made up

---

1. To faithfully transduce high-frequency sounds (that have short waveforms), resolution on the order of microseconds is needed. Since the relatively slow second messenger pathways used in vision and olfaction would not suffice, a mechanically-gated electrical transduction (MET) channel has evolved.

2. Although hair cells have a high internal concentration of K\(^+\), the large electrical gradient (125 mV) between hair cells and the K\(^+\)-rich endolymph, the fluid of the scala media, drives K\(^+\) ions into the hair cell through open MET channels.
of orderly representations, or maps, of receptor surfaces. These maps are organized
topographically. That is, neighbouring points of the sensory epithelium are represented
by adjacent locations in the central nervous system. In the visual system, spatial
relationships among retinal ganglion cells are maintained in their central targets, giving
rise to ordered retinotopic maps. Similarly, somatotopic maps exist in the somatosensory
system. In the auditory system, hair cells along the length of the cochlea respond
preferentially to specific sound frequencies. High frequency sounds are detected by hair
cells at the basal end of the cochlea. Low frequency sounds excite hair cells at the apical
end (Figure 2.5). Pure tones stimulate a narrow range of cells along the cochlea; sounds
of increased spectral complexity stimulate a broader region, and the resulting neural
activation patterns are transmitted to central auditory regions of the brain up to cortex.
Given this place coding of sound frequency, we tend to describe neuronal organization
within the auditory system as tonotopic. This tonotopic organization is maintained at all
successive nuclei within the ascending pathway up to cortex, including in the inferior
colliculus, which is the region of interest in the present thesis. Topographic maps are an
efficient way to represent stimulus patterns from the periphery at higher levels in the
central nervous system.
Figure 2.5: Tonotopic organization of the cochlea. High-frequency tones preferentially activate hair cells at the cochlear base; low-frequency tones, the cochlear apex. (Image adapted from Purves et al. 1997, after data from von Békésy, 1960)

### 2.1.3 The auditory nerve

Hair cells are innervated by bipolar neurons of the spiral ganglion, the axons of which become the auditory portion of the VIII\textsuperscript{th} cranial nerve, the vestibulocochlear nerve. Innervation patterns of hair cells differ. Afferent innervation of inner hair cells is denser than that of outer hair cells: the majority of afferent fibres (90 - 95\%), termed inner radial fibres, innervate inner hair cells. The remaining 5 - 10\% (called outer spiral fibers) innervate outer hair cells. Inner radial fibers become Type 1 (myelinated) cells in the spiral ganglion; outer spiral fibers become Type 2 (unmyelinated) cells. These cells are kept separate in the spiral ganglion to preserve cochleotopic organization. Each afferent innervates a single inner hair cell while each inner hair cell is innervated by multiple (10 - 20) afferents. Efferents to inner hair cells arise from the uncrossed
olivocochlear bundle, while those to outer hair cells arise from the crossed olivocochlear bundle. Outer hair cells are predominantly innervated by efferent fibres, suggesting these cells are mainly under control of descending pathways.

Spiral ganglion cells innervate a single inner hair cell, thus each auditory nerve fiber responds only to a narrow range of frequencies. To determine auditory nerve fibre responsiveness, recordings are made from individual fibres in response to tone bursts of varying frequency and intensity. The tip of the resulting isoresponse curve, or (frequency) tuning curve, indicates characteristic frequency (CF). Frequency tuning curves from eight neurons originating at different cochlear frequency positions are illustrated in Figure 2.6A. The sharp tuning corresponds to the tuning of the inner hair cell that the auditory nerve fibre innervates: fibres with low characteristic frequency innervate inner hair cells at the cochlear apex; high characteristic frequency fibres innervate inner hair cells at the base of the cochlea. There are many overlapping tuning curves in the auditory nerve corresponding to the approximately 30,000 (in humans) auditory nerve fibres.

The sharp frequency tuning is vulnerable to cochlear insults such as noise exposure, ototoxic drugs, and hypoxia. Following partial injury to the stereocilia of outer hair cells, the sharp tip of the tuning curve remains (depicted as the solid line in the right panel of Figure 2.6B), yet threshold is elevated by more than 30 dB. The low-frequency tail becomes hypersensitive, which, together with the threshold elevation, broadens neural bandwidth (Liberman and Dodds 1984).
Figure 2.6: (A) Frequency tuning curves obtained from individual auditory nerve fibres in the guinea pig. Adapted from Evans 1972. (B) Following partial damage to the stereocilia of outer hair cells (depicted semi-schematically in the left panel), tuning curve shape and sharpness of tuning (solid line, right panel) is altered from normal (dashed line, right panel). Adapted from Liberman and Dodds, 1984.
2.1.4 The central auditory system

The primary nuclei of the ascending central auditory system are shown schematically in Figure 2.7. This figure represents ascending pathways from one ear only. The cochlear nuclei are the first auditory brainstem nuclei. It is at this level of the auditory pathway that basic neural response patterns originate, and parallel pathways emerge (a hallmark of the ascending auditory system). Individual fibers of the cochlear nerve branch and terminate in one of three divisions of the cochlear nucleus as they enter the brainstem: the ascending branch innervates the anteroventral cochlear nuclei (AVCN); the descending branch innervates the posteroventral and dorsal cochlear nuclei (PVCN and DCN). Tonotopic organization is maintained in all three divisions of the cochlear nucleus.

Types of neurons and auditory processing vary in the differing divisions of the cochlear nucleus (Figure 2.8). The ventral divisions of the cochlear nucleus contain primarily bushy cells (spherical cells, listed in Figure 2.8, are a form of bushy cell), which signal sound onset, and stellate cells (also called multipolar cells, Figure 2.8), which signal sustained sound (Oertel et al., 1988). The AVCN functions primarily as a relay of information from the auditory nerve; neural responses here are similar to those of the auditory nerve. The cells of the AVCN preserve timing and intensity cues, and project to both the contra- and ipsi-lateral superior olivary complexes (SOC). Information transmitted is largely concerned with sound localization mechanisms where interaural intensity and timing cue differences are compared. The DCN is the first region of the auditory pathway where lateral inhibition is present (Figure 2.8; bottom 3 categories). The many cell types here and the complex intrinsic circuitry allows for more complex
processing of auditory signals, thus neural responses in DCN are different from those seen in the auditory nerve. Cells of the PVCN and the DCN project to the contralateral nucleus of the lateral lemniscus and inferior colliculus.

Figure 2.7: Major nuclei of the ascending auditory pathway. AVCN: anteroventral cochlear nucleus; PVCN: posteroven tral cochlear nucleus; DCN: dorsal cochlear nucleus; LSO: lateral superior olive; MSO medial superior olive; MTB: medial nucleus of the trapezoid body; NLL: nucleus of the lateral lemniscus; IC: inferior colliculus; MGB: medial geniculate body.
Figure 2.8: Frequency-response areas, spike-time histograms (from which the various categories are derived), and their correlated neuronal types and locations in the cochlear nucleus. Solid shading indicates excitation; cross-hatching: inhibition. Note: alternate cell-type nomenclature, where applicable, is given in parenthesis. Adapted from Evans 1982.
The superior olivary complex is the first auditory nucleus where information from both ears meet, thus the emergence of binaural pathways begins here. The main function of this nuclear complex is sound localization, which is accomplished by different mechanisms depending on the frequency components of the sound being localized. For low-frequencies (below approximately 2 kHz in humans), interaural timing differences are used. Interaural timing differences are computed in the medial superior olive (MSO; Figure 2.7), which receives binaural excitatory impulses from both ipsi- and contralateral AVCN. These axons vary in length, creating a delay line system. MSO neurons respond when input from both ears arrives at the same time. This coincidence detection model (Jeffress 1948) is widely accepted for the localization of low-frequency sounds. In humans, neural action potentials can phase-lock to frequencies below about 3 kHz (by contrast, in owls, auditory neurons can phase-lock to sound stimuli up to 9 kHz). This phase-locking is required for localizing sound based on interaural timing differences. For sound frequencies above 2 kHz, where phase-locking is not possible, at least in humans, a different mechanism is used to localize sound: interaural level differences.

In humans, at frequencies greater than about 2 kHz, the head shadow effect becomes important. When the wavelength of sound frequency is less than the diameter of the object in its path, the wavelengths are too short to bend around the object, thus creating an acoustic “shadow”, or region of lower intensity at the far ear. These intensity differences give an indication of the location of a sound source. Interaural level differences are computed by neurons of the lateral superior olive (LSO; Figure 2.7) which receives excitatory projections from the ipsilateral AVCN, and inhibitory (glycinergic) input from the contralateral ear via interneurons originating in the medial nucleus of the
trapezoid body (MTB; Figure 2.7). Since each LSO encodes sound from the ipsilateral sound field, input from both LSOs are required to represent the entire sound field in the horizontal plane. The parallel pathways originating in the cochlear nucleus facilitate these different mechanisms of sound localization (interaural timing and level differences). The two pathways for localizing sound are merged in auditory midbrain; both LSO and MSO project to the inferior colliculus: LSO to the contralateral IC, MSO to the ipsilateral IC.

A distinct set of (monaural) pathways from the cochlear nucleus projects directly onto contralateral nuclei of the lateral lemniscus (NLL; Figure 2.7), the next nuclear complex along the ascending auditory pathway. NLL also receives input from ipsilateral superior olives. Neurons of the NLL have properties and connections similar to those of IC (Aitkin et al., 1970). It consists of three divisions: dorsal, intermediate, and ventral (Figure 2.9C; p. 24). The dorsal nucleus is tonotopically organized. A prominent feature of NLL is neurochemical heterogeneity: approximately 85% of dorsal nucleus neurons are GABAergic; more than 80% of ventral nucleus neurons are glycinergic; only about 20% of intermediate nucleus neurons are inhibitory (either GABAergic or glycinergic; Saint Marie et al., 1997). The NLL are not an obligatory synaptic relay; some projections from cochlear nucleus and SOC terminate directly in IC. The specific role of the NLL in hearing is unknown. Similar to the superior olives, the output from the NLL terminates in the IC (Figure 2.7; Figure 2.9C).

The inferior colliculus (IC) is one of the largest auditory nuclei in the mammalian brain. It has three principle divisions as represented in Figure 2.9A: the central nucleus, which is exclusively auditory (Aitkin et al., 1994) and essential for normal hearing (Jenkins and Masterton 1982); the dorsal cortex, which receives substantial descending
projections (e.g., Winer 2005; Winer et al., 1998); and the lateral nucleus, which is multi-
sensory, and integrates auditory and somatic sensory information. To differentiate
between divisions, tuning curves and neural latencies differ. In central nucleus, tuning
curves are sharply tuned, while they are broader in other divisions of IC; also latencies
are shorter for central nucleus neurons vs. those of the other divisions (Syka et al., 2000).

In central nucleus, disc-shaped neurons (called flat cells in rat; Malmierca et al.,
1993) align to form frequency-band laminae (Figure 2.9B). Most laminae run from dorso-
lateral to ventro-medial. This laminar organization is the basis for tonotopic organization
in central nucleus of IC: low frequencies are represented dorso-laterally, high frequencies
ventro-medially (Merzenich & Reid, 1974; Schreiner and Langner, 1997). The axons and
dendrites of stellate cells interact with multiple laminae (Figure 2.9B).

Inferior colliculus is an important ‘hub’ of the auditory system. It is a major site
of convergence, receiving projections from almost all lower-level (binaural and
monaural) nuclei, as well as descending projections from all auditory cortical areas
(Winer et al., 1998; Figure 2.9C). It is an obligatory relay for ascending auditory
pathways to thalamus; neurons originating in IC are the principal source of innervation to
the medial geniculate body (MGB): they make both excitatory (presumed glutamatergic)
and inhibitory (GABAergic; Winer et al., 1996) projections bilaterally to MGB. Thus, IC
neurons are, indirectly, the principle source of innervation to cortex. In addition to these
extrinsic connections, IC also has substantial intracollicular connections (Malmierca et
al., 2003; 2005). Given its many connections, IC is an important link within the auditory
system, processing and integrating information from multiple sources and determining
the form auditory information takes as it is delivered to forebrain regions. It is plausible
that there can be significant neuroplasticity at this nucleus, and this, indeed, is the topic of the present thesis.

The medial geniculate body (MGB) receives most of its inputs from ipsilateral IC; projections from MGB are almost entirely ascending and ipsilateral (Figure 2.7). The degree of descending input to the MBG from cortex is greater than the ascending input received from IC, implying tight coupling between cortex and thalamus. MGB is divided into ventral, medial, and dorsal regions. Neurons in the ventral division of MGB have response properties similar to IC, its main source of input. The tonotopic arrangement of sound frequency is maintained in the ventral region (Imig and Morel 1984); neither of the other regions appears tonotopically arranged. Dorsal MGB receives most of its input from dorsal cortex of IC (Calford and Aitkin 1983). The dearth of intrathalamic connections to link the three divisions suggests the independence of these nuclei, compared with the rich intrinsic connections of the IC and cochlear nucleus. Auditory thalamus has monosynaptic projections to subcortical limbic regions such as the amygdala (LeDoux et al. 1985; Shinonaga et al. 1994), suggesting the role of MGB may be more than strictly auditory.
Figure 2.9: (A) Three divisions of the inferior colliculus from Golgi-impregnated material of the cat. CIC: central nucleus; DIC: dorsal nucleus; EIC: external nucleus of inferior colliculus. Scale bar indicates 0.5 mm. Adapted from Morest and Oliver 1984. (B) Schematic of the microanatomy of central nucleus of IC. D: disc-shaped neurons; S: stellate neurons. Adapted from Oliver et al., 1994. (C) The inferior colliculus (indicated by blue arrows) receives projections from almost all lower-level nuclei, and is the principle source of innervation to higher levels of the ascending auditory pathway. Adapted from Pollak et al., 2003. See text above or List of Abbreviations for full names.
Auditory cortex, located in superior temporal gyrus of the temporal lobe, is where auditory information interfaces with higher-order communication and cognitive networks. It has a number of subdivisions. A broad distinction can be made between core and belt auditory cortical regions. The core auditory cortex is considered the cortical entry point for the ascending auditory pathway. It is characterized by strong tonotopic organization and relatively short neural response latencies (Harel et al. 2000). Based on these features, primary auditory cortex (AI), among other areas, is considered part of core auditory cortex. Belt areas are less clearly delineated. They receive diffuse projections from belt regions of MGB, and therefore have a less precise tonotopic arrangement. Subcortical input to auditory cortex is primarily from ipsilateral thalamus (Figure 2.7). Primary auditory cortex receives point-to-point connections from the ventral region of MGB and is tonotopically organized. The grey matter of A1 has six layers that establish patterns of neural connections with other regions of the brain. For example, layers III and IV receive input from ventral MGB; layers I and VI receive input from medial MGB (Huang and Winer 2000). Layers V and VI project to MGB and IC, respectively. Layers are linked vertically by interneurons (Prieto and Winer 1999) or by specific groups of pyramidal cells (Ojima et al. 1992).

2.2 Neural plasticity

2.2.1 The importance of the early environment on development

Our sensory systems have evolved to provide central representations of the external (and internal) environment. During development, when there are normal patterns of sensory stimulation, sensory pathways develop normally. However, abnormal patterns
of neuronal activity during the maturation process result in the abnormal development of central sensory pathways. Perhaps one of the earliest examples of the way in which sensory experience during an early developmental period can “shape” brain activity is by Konrad Lorenz in the 1930s. He observed that in the absence of a mother goose, newly-hatched goslings would follow a human being (himself!) as if he were the parent, a phenomenon termed filial imprinting. This behaviour demonstrates the enormous impact that the early environment has on brain development.

### 2.2.2 Neural plasticity of sensory cortices

Numerous experiments over the past few decades have shown that sensory areas of cerebral cortex can be “re-programmed” as a result of peripheral lesions (reviewed in Lomber and Eggermont 2006). Neuronal connections can reorganize and synaptic strength can be modified such that changes in peripheral input result in changes in central representations of the periphery. These adaptations can be broadly termed: plasticity. For example, seminal work by Wiesel and Hubel (Wiesel and Hubel 1963; Wiesel and Hubel 1965; Wiesel 1982) demonstrated changes in the functional organization of monkey striate cortex. In conditions of monocular deprivation (for 18 months, from 2 weeks of age), the majority of cortical neurons were driven by the non-deprived eye (Figure 2.10). Subsequent work by Rasmusson (1982) provides evidence for reorganization in primary somatosensory cortex following digit removal. Sixteen weeks following amputation of the fifth digit in a raccoon model, the location of receptive fields suggested that the area of cortex that would normally represent the fifth digit was taken over by input from the fourth digit. These studies collectively show that areas of the cortex that no longer receive sensory input become re-wired to process sensory information from areas
adjacent to the lesion in the case of somatosensory studies, or from the non-deprived eye, in the vision experiments outlined above. Similar results have also been reported in auditory cortex following partial cochlear lesions. Thirty-five to 81 days after unilateral mechanical damage to the organ of Corti, the region of contralateral auditory cortex that would have normally represented frequencies in the range of the lesion, was occupied by an increased representation of sound frequencies adjacent to the lesioned frequency range (Robertson and Irvine 1989).

Figure 2.10: Developmental plasticity of monkey striate cortex. Ocular dominance columns from (A) a control subject, and (B) a subject monocularly-deprived for 18 months from 2 weeks of age. Cortical regions that would normally receive input from the deprived eye now receive projections predominantly from the non-deprived eye. Scale bar indicates 1 mm. Adapted from Wiesel 1982.
2.2.3 Cortical vs. subcortical plasticity in neonatal & adult models

Relevant to the present thesis is the distinction between adult and developmental plasticity, since the experiments described herein focus on experimental alterations to the auditory system during early post-natal development. Experiments cited in the previous section were carried out in adult (Robertson and Irvine 1989) and developing (Wiesel and Hubel 1963; Wiesel and Hubel 1965; Wiesel 1982) animals. These results indicate that cortical neurons in both mature and developing mammals have the capacity to reorganize when sensory input is restricted (e.g., partial cochlear lesions) or removed (e.g., monocular deprivation). In the adult auditory system, there is evidence for altered sound frequency representation in auditory thalamus. For example, following restricted unilateral cochlear lesions resulting in severe mid- to high-frequency hearing loss, there is an expanded representation of lesion-edge frequencies in the ventral region of medial geniculate body where mid- to high-frequencies would normally be represented (Kamke et al. 2003). Harrison (2001) reports less extensive sub-thalamic (midbrain) reorganization following cochlear lesions (Figure 2.11C) than that reported at the cortical level (Kakigi et al., 2000). There is also little evidence for reorganization of sound frequency representation in the cochlear nucleus of mature subjects following cochlear lesions (Kaltenbach et al., 1996; Rajan and Irvine 1998). The results of these studies suggest that in the mature animal, it is at the level of the cortex and/or thalamus at which reorganization mainly occurs in response to a peripheral acoustic deficit.

At the level of auditory midbrain, (i.e., inferior colliculus), reorganization of sound frequency representation following peripheral lesions has been shown to be more extensive in a developmental model compared with an adult model. By damaging the
basal cochlear sensory epithelium (hair cells), a high-frequency hearing loss was created in the neonate (shown in Figure 2.11B as the shaded area along cochlear length). Several months later, single-unit recordings made in inferior colliculus reveal abnormal development of sound frequency representation in the neonatal animal (Figure 2.11B) compared with controls (Figure 2.11A). Similar findings of over-representation of frequency regions corresponding to the edge of the cochlear lesion have also been shown at the level of the primary auditory cortex (cat: Harrison et al., 1995; chinchilla: Kakigi et al., 2000). Also included in Figure 2.11C are the results of the same experimental manipulation carried out in the adult chinchilla. Together, the results of the neonatal and adult lesion studies suggest that the auditory midbrain has a greater capacity for reorganization in an early developmental period rather than at a later, more mature stage of development.
Figure 2.11: Sound frequency representation in chinchilla inferior colliculus in (A) control subjects, and subjects in which partial cochlear lesions were induced by oto-toxic drugs (B) as neonates, and (C) as adults, yielding a hearing loss above 8 kHz. Cross-hatching in (B) indicates an iso-frequency region. Scale bar: 1mm. Adapted from Harrison 2006.

Experimental manipulations described thus far have created sensory deficits. Cochlear lesions resulting in partial deafferentation have reduced auditory input. While these animal models serve as useful models of sensorineural hearing loss, they are somewhat less informative about the development of auditory pathways in a normal-
hearing subject. Sensory deficits have been shown to alter the development of sound-frequency representation; do acoustically-enhanced environments have an influence? This idea was explored at the level of auditory cortex by rearing newborn kittens in a spectrally-enhanced acoustic environment (Stanton and Harrison 1996). The dominant sound source was a tonal stimulus centered at 8 kHz (Figure 2.12C). A moderate sound intensity, approximately 65 dB SPL, was selected so as to not compromise hair cell integrity (suggested by normal auditory brainstem response audiograms). Following the 3-month tone-rearing period, subjects were returned to a normal acoustic environment, and sound frequency representation was assessed at 1 year. A larger area of auditory cortex was found to be dedicated to the 8-16 kHz octave band (Figure 2.12B, cross-hatching) compared with controls (Figure 2.12A, cross-hatching).
Figure 2.12: (A) Normal representation of sound frequency. (B) Cortical overrepresentation of the 8-16 kHz octave band in a kitten reared in an enhanced acoustic environment. Cross-hatching in both (A) and (B) indicates the 8-16 kHz octave band region. (C) Spectrum of the sound-exposure (conditioning) stimulus. sf: sylvian fissure; aef: anterior ectosylvian fissure; pef: posterior ectosylvian fissure. Data after Stanton and Harrison 1996.

2.2.4 Mechanisms of neural plasticity

How do patterns of neuronal activity – whether spontaneous or driven by sensory experience – modify neural circuitry? In this section, we consider several mechanisms of neural plasticity occurring at the level of the synapse, such as long-term potentiation, as
well as those occurring at the level of the neuron, such as axon elaboration, and synapse elimination. We begin with a brief overview of Hebb’s postulate.

In 1949, the Canadian psychologist Donald Hebb put forth a number of ideas relating to the neural basis of learning (a form of neural plasticity). His main thesis, which has come to be known as Hebb’s postulate, is that, “When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased” (Hebb 1949, p. 50). This phrase is known colloquially as, “cells that fire together, wire together; cells that fire out-of-sync lose their link”. This casual summary does not, however, distinguish between important temporal correlations between action potentials of pre- and post-synaptic cells. Repeated arrival of presynaptic spikes milliseconds before postsynaptic spikes, in many synapse types, leads to long-term potentiation (spike-timing-dependent plasticity, Bi and Poo 1998; review: Caporale and Dan 2008).

Some of the first evidence for Hebb’s postulate is the discovery of long-term potentiation (LTP), first reported by Bliss and Lomo (1973). Synapses have the ability to undergo activity-dependent modifications in synaptic strength, called synaptic plasticity. LTP is one of the most well-studied examples of prolonged change in synaptic strength. It has been used as a model to study the synaptic basis of Hebbian plasticity. While the capacity for neural plasticity is especially prominent during development, LTP is not restricted to the developing central nervous system; it has also been reported in adult models. In inferior colliculus of auditory midbrain, there is some evidence that AMPA
and NMDA receptors mediate excitation (Ma et al., 2002) and that LTP can occur here (Hosomi et al., 1995, Zhang and Wu 2000).

In the mammalian brain, one of the best understood forms of synaptic plasticity is \textit{N}-methyl-D-aspartate-receptor- (NMDAR-) dependent LTP (Figure 2.13). This form of LTP requires the activation of NMDARs. NMDARs are fast, ligand-gated, ionotropic receptors. They are activated by coincident presynaptic release of glutamate, the main excitatory neurotransmitter in the brain, and significant depolarization of the post-synaptic membrane, which relieves the voltage-dependent Mg\textsuperscript{2+} block of the NMDAR. With the Mg\textsuperscript{2+} block removed, NMDARs are permeable to Ca\textsuperscript{2+} and monovalent cations such as K\textsuperscript{+} and Na\textsuperscript{+}. Ca\textsuperscript{2+} is an important second messenger critical to the induction of LTP. The increased concentration of Ca\textsuperscript{2+} in post-synaptic dendritic spines triggers intracellular signaling cascades that results in the insertion of AMPA receptors in the post-synaptic membrane (Figure 2.13). This is the mechanism primarily responsible for the increase in synaptic strength during NMDAR-dependent LTP. The application of NMDA antagonists prevents LTP. There is also some evidence to suggest that morphological changes accompany LTP, such as enlargement of dendritic spines and post-synaptic densities (review: Yuste and Bonhoeffer 2001).
In the auditory system, work by Knudsen and colleagues has revealed a mechanism of neuronal plasticity in the barn owl. Previous research from this lab investigated the tuning of neurons to binaural sound localization cues in subjects reared with altered input during an early post-natal period (Brainard and Knudsen 1993). Baby owls were reared from P14 to P18 (just as their eyes were opening) for at least 150 days, with prismatic glasses. This alteration shifts the visual field laterally such that there is a mismatch between auditory and visual maps in the optic tectum. The representation of interaural timing differences is shifted in the direction of the leading ear in both the optic tectum and the external nucleus of IC, but not in central nucleus of IC, establishing the

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3 The optic tectum is the homologue of the superior colliculus in avian species.
external nucleus of IC as a site of plasticity (Brainard and Knudsen 1993). A subsequent study provides evidence for axon elaboration (depicted schematically in Figure 2.14A) as a mechanism of plasticity that accompanies the functional changes reported in Brainard and Knudsen (1993). Axons were labeled from their source (central nucleus of IC) to their point of termination (external nucleus of IC) using an anterograde tracer. In prism-reared subjects, projection fields were denser and broader compared with controls (DeBello et al., 2001). Thus, in addition to changes that occur at the level of the synapse, described in the preceding paragraphs, neural plasticity can occur at the level of the neuron.

Another mechanism of neural plasticity – at the level of the neuron – is illustrated in a study of filial imprinting in domestic fowl (White Leghorn; Wallhausser and Scheich 1987). Chicks were imprinted to an acoustic stimulus (400 Hz, 3 bursts/s, presented continuously at 80 dB SPL) beginning on the 14th day of incubation until hatching. Subjects were considered imprinted when they moved towards the imprinting stimulus in an approach test, and when they preferred the imprinting stimulus to a new stimulus in a simultaneous discrimination task. The Golgi-Cox method was used to study neuron morphology. Analysis of dendritic segments in specific brain areas 7 days after hatching revealed a significant decrease in the frequency of dendritic spines in MNH neurons (mediorostral neostriatum/hyperstriatum). It was hypothesized that this spine loss (illustrated pictorially in Figure 2.14B) is a critical mechanism of filial imprinting.

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4 In the barn owl, interaural timing differences from frequency-specific channels ascend from auditory brainstem to central nucleus of IC. This information then converges on external nucleus of IC, and is subsequently conveyed to optic tectum.
### 2.3 Motivation for undertaking the present research

As outlined above in Section 2.2.2: Neural plasticity of sensory cortices, many studies describing sensory system plasticity have revealed alterations in cortical representation. However, one question that arises is whether cortical plasticity is intrinsically cortical, or whether it reflects, wholly or partially, reorganization at lower levels. One of the first steps to address this question is to study whether neurons of subcortical nuclei do indeed reorganize subsequent to altered peripheral input. Relatively normal thalamo-cortical projection patterns were observed following neonatal deafening (Stanton and Harrison 2000), suggesting that reorganization occurs at sub-thalamic levels. At the first sub-thalamic nucleus, inferior colliculus of auditory midbrain (Figure 2.7), there is some evidence for neural reorganization in response to sensory deficits (Harrison et al., 1998). In large part, the animal models in which midbrain plasticity has been demonstrated are in altricial species where neonatal manipulations are very early
in auditory system development. It remains unclear whether an enhanced acoustic environment has an effect at this level of the auditory brain in a precocious animal model (hearing onset in utero, compared with post-natal hearing onset of common laboratory species, such as rat and mouse). In the present thesis, we ask whether midbrain plasticity occurs in a precocious animal model in response to post-natal exposure to an enhanced sound environment.

### 2.3.1 Choice of the animal model

The chinchilla is a commonly used animal model, particularly for studies of auditory function. As a precocious species, the maturity of its auditory system, at birth, differs from that of altricious species. Newborn chinchilla’s cochleae are structurally and functionally mature. Most hair cells appear adult-like 24 hours after birth (Harrison et al., 1996). Tonotopic maps in primary auditory cortex and secondary auditory cortical fields are well-ordered and neurons are sharply tuned by P3 (Pienkowski and Harrison 2005a). In contrast, onset of cochlear function in altricious species occurs days after birth (P12-P14 in rat; Geal-Dor et al., 1993). For 2-3 weeks following hearing onset, tonotopic maps are poorly organized and neurons broadly tuned (Chang et al., 2005; Zhang et al., 2001). Furthermore, unlike other laboratory species (cat, rat, guinea pig), chinchilla audibility curves more closely resemble those of humans, also a precocious species (Querleu et al., 1988), across a broad range of frequencies (Heffner and Heffner 1991; Miller 1970). The state of the chinchilla’s auditory system, at birth, is similar to that of a human, at birth. For these reasons, we have selected the chinchilla as the animal model for our studies of auditory neuroplasticity.
2.3.2 Effects of an enhanced acoustic environment on IC neurons

To date, a few studies have examined the effects of an augmented sound environment on properties of developing collicular neurons and tonotopic maps, with differing results. One of the earliest studies (Moore and Aitkin 1975) reports no change in tuning curves or tonotopic organization after exposing newborn kittens to a continuous pure-tone, 8 hours/day for the first 50 – 75 days of life. Several papers report changes in rat tonotopic maps, specifically an increase in the proportion of neurons in central nucleus tuned to a moderately intense (60-70 dB SPL) sound-exposure frequency following 14-16-hr exposure to 25-ms tone pips from P9 to P28 (Oliver et al., 2011) and 12h/day exposure to continuous pure tones for 3 post-natal weeks (Poon and Chen 1992). Click-reared subjects (20/sec, 88.5 dB SPL, from P8 to P19-24) exhibit broader tuning curves and no change in spontaneous activity, response latency, or tonotopic maps (Sanes and Constantine-Paton 1985).

More recently, Miyakawa et al. (2013) reported a transient narrowing in tuning curve bandwidth following chronic tone-pip exposure (7.5 kHz, 100-ms pip duration, 6 pips in a train at 6 Hz, 1 train every 2 s, 60 dB SPL, from P9 to P25). Long-lasting changes in cortical (but not collicular) tonotopic maps using the same sound-stimulation pattern were observed. A two-tone rearing paradigm (16 + 40 kHz, 80 dB SPL, from P9 to P17, 22-23 hrs/day) revealed large-scale reorganization of tonotopic maps in IC as seen by MRI (Yu et al., 2007). These studies have all been done in altricious species (post-natal hearing onset). To the best of our knowledge, the effects of an enhanced acoustic environment on the development of tonotopic maps in inferior colliculus of a precocious animal model have not been reported, and form the topic of the present thesis.
2.3.3 C-fos immunohistochemistry for the precocious chinchilla

Immunolabeling of the c-fos protein has been used to study neuronal activity patterns in several systems of the vertebrate brain (e.g., vision: Beaver et al., 1993; somatosensation: Filipkowski et al., 2000; olfaction: Sallaz & Jourdan 1993; audition: Reimer 1993). C-fos is an immediate early gene (IEG), a family of genes characterized by rapid and transient induction following a variety of cellular stimuli, among them: the neuronal depolarization that follows sensory stimulation (reviewed in: Herrera & Robertson 1996; Hughes & Dragunow 1995; Loebrich & Nedivi 2009; Sheng & Greenberg 1990). IEG expression occurs in the presence of protein synthesis inhibitors (cyclohexamide/anisomycin), suggesting they do not require *de novo* protein synthesis or the activation of other genes prior to their activation (Cochran et al., 1984; Greenberg et al., 1986). For these reasons, it has been suggested that IEG expression offers the first genomic response to cell stimulation.

While experimental protocols exist for many altricial species (mouse (e.g., Brown and Liu 1995; Lu et al., 2009), rat (e.g., Friauf 1992; Nakamura et al., 2005), gerbil (e.g., Scheich and Zuschratter 1995), bat (e.g., Qian and Jen 1994)), our lab is the first (to our knowledge) to establish a protocol for the precocious chinchilla (D'Alessandro and Harrison 2014). The *c-fos* immediate early gene is activated following acoustic stimulation (e.g., Mello & Pinaud 2006). By chromatically labeling its protein product, c-fos, we obtain an indirect measure of global neural activation patterns elicited by acoustic stimulation. We developed a reliable, reproducible c-fos protocol in the chinchilla using commercially-available reagents. Advantages of using c-fos immunolabeling (vs., e.g., 2-deoxyglucose) are that it is much quicker (several days vs.
several weeks) and also allows the visualization of neural activity with cellular resolution.

Recall that the inferior colliculus is arranged tonotopically, with low frequencies represented dorso-laterally, and high frequencies represented ventro-medially. When a subject is stimulated with a pure-tone of sufficient duration, the resulting c-fos-labeled neurons in inferior colliculus lie in a band that corresponds to the frequency used to induce them (D’Alessandro and Harrison 2014). We use this characteristic of c-fos expression in inferior colliculus to study whether the neural representation of sound frequency is altered following rearing in the enhanced acoustic environment. We compare the width and number of c-fos-labeled neurons in the 2-kHz band in inferior colliculus of subjects who have heard the sound-exposure stimulus for 4 weeks to those of age-matched control subjects.

In addition to c-fos, a variety of immediate early genes have been used to map neuronal activation (NGFI-A: nerve growth factor induced-A, a.k.a. zif268, ERG-1, KROX-24, and ZENK; and arc: activity-related cytoskeleton). Among the most commonly studied IEGs, c-fos and NGFI-A exert an effect on the cell by encoding transcription factors that regulate the expression of downstream target genes termed late-response genes. Like c-fos, the expression of NGFI-A is highly sensitive to membrane depolarization. NGFI-A has been shown to regulate the expression of genes that encode pre-synaptic proteins involved in neurotransmitter release (synapsin I: Thiel et al., 1994; synapsin II: Petersohn et al., 1995; synaptobrevin II: Petersohn and Thiel 1996) as well as genes associated with structural stability (neurofilament: Pospelov et al., 1994). The NGFI-A transcription factor seems well-positioned to relate changes in cell surface
activation with genomic responses, perhaps as part of experience-dependent neuronal rewiring (review: Knapska and Kazcmarek, 2004).

Arc and immediate early genes like it (e.g., homer1a) are effector genes; they encode proteins that directly influence cellular function. The mRNA encoded by arc is transported rapidly to neuronal dendrites, where local translation occurs (Lyford et al., 1995; Steward et al., 1998; Steward & Worley 2001a). The selective targeting of arc mRNA to active synapses has been shown to require NMDA receptor activation (Steward and Worley 2001b). It has also been suggested that arc interacts with an actin-associated protein (Lyford et al., 1995). Together, these functions have led researchers to propose that arc is involved in activity-dependent dendritic reconfiguration (Steward and Worley 2001b). Arc has been widely studied in learning and memory related contexts (review: Bramham et al., 2008).

We selected c-fos immunohistochemistry from among other immediate early genes for several reasons. First, basal expression levels of this protein are relatively low, thus allowing us to observe possible increases in neural expression following neonatal rearing in an enhanced sound environment. The protein product of the NGFI-A immediate early gene, for example, is expressed at high basal levels (Kaczmarek and Chaudhuri 1997). Second, the c-fos protein is localized to the cell nucleus, thus each instance of c-fos-labeling indicates that that neuron has recently been activated (c.f. arc, which is localized to neuronal dendrites). It was our goal to quantify changes in the number of neurons activated by sound before and after our experimental treatment. We thus elected to use c-fos immunohistochemistry to study global neuronal activation
patterns in inferior colliculus of the precocious chinchilla following neonatal rearing in an augmented sound environment.

2.4 Hypothesis and Research Objectives

Our working hypothesis is that the development of neuronal network connections within the ascending auditory pathway is driven, in large part, by environmental sound stimulation during an early post-natal period, consistent with the idea that the brain is “programmed” to the prevalent acoustic environment. If environmental stimuli during an early post-natal period are abnormal, we predict alterations in functional organization. Specifically, we hypothesize that sound frequency maps in central nucleus of inferior colliculus are altered in neonatal subjects who hear a moderately-intense narrowband sound stimulus (centered at 2 kHz) for 4 weeks, compared with age-matched controls.

There are four objectives to this research:

1) To determine whether tonotopic maps recorded electrophysiologically in central nucleus of inferior colliculus differ in sound-exposed subjects compared with age-matched controls.

2) To establish a c-fos immunohistochemical protocol for the chinchilla.

3) Using this protocol, to compare whether there are differences in the band of c-fos labeled neurons present following 90-min of pure-tone sound stimulation between sound-exposed and control subjects.

4) To verify that the sound-exposure stimulus does not damage the cochlea. We will examine the structure of the hair cells of the cochlea (using SEM), and their function, by using auditory brainstem responses, ABRs, to estimate hearing thresholds.
Chapter 3

Experimental Methods & Analysis

3.1 Overview

We hypothesize that enhanced peripheral activation during an early post-natal period will alter the normal representation of sound frequency at the level of the inferior colliculus. To test this, neonatal chinchillas were chronically exposed to a moderately-intense (70 ± 5 dB SPL) narrowband (2 ± 0.25 kHz) acoustic stimulus for a period of at least 4 weeks. Subsequently, micro-electrode recordings of neural action potentials (spikes) were used to define tonotopic maps in central nucleus of inferior colliculus.

We also visualized global neural activation patterns in chinchilla IC using c-fos immunohistochemistry with the goal of detecting any abnormal patterns associated with neonatal sound exposure. This histological method has been used to reveal patterns of activated neurons in altricious species such as mouse and rat. However, to the best of our
knowledge, no c-fos protocol had been published for the precocious chinchilla. Thus, we first optimized a protocol for the chinchilla using commercially-available reagents (D'Alessandro and Harrison 2014).

The neonatal sound exposure paradigm was designed to provide significant excitation of cochlear neurons, while avoiding acoustic trauma. To study whether sound exposure altered cochlear function, frequency-specific auditory brainstem responses (ABRs) were recorded at frequencies around the sound-exposure stimulus. ABR thresholds of sound-exposed subjects were compared with (non-exposed) control subjects. To study whether sound exposure altered cochlear structure, the morphology of hair cell stereocilia and the pattern of hair cell distribution along the cochlear epithelium in the region of the sound exposure stimulus were assessed in control and sound-exposed subjects using scanning electron microscopy (SEM).

An overview of experimental timelines is shown in Figure 3.1, and sample sizes by experimental group for each experimental technique is shown in Table 3.1 below.

Table 3.1: Sample size by experimental group for each of the four experimental techniques employed. Con: Control; SE: Sound-exposed.

<table>
<thead>
<tr>
<th>ABRs</th>
<th>SEM</th>
<th>Micro-electrode recordings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>SE</td>
</tr>
<tr>
<td># of subjects</td>
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<tr>
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<td>25</td>
</tr>
<tr>
<td>tones</td>
<td>26</td>
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</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-fos immunohistochemistry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Sound</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2 kHz, 90 min</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6 kHz, 90 min</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>SE + 2 kHz, 90 min</td>
<td>199</td>
<td>158</td>
</tr>
<tr>
<td>SE + No Sound</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 1 coronal slice yields 2 samples
Figure 3.1: Timelines for (A) ABR, SEM, and extracellular electrophysiological experiments, and (B) c-fos immunohistochemistry experiments.

### 3.2 Animal Model

All procedures were approved under The Hospital for Sick Children Animal Care Committee protocols, following CCAC guidelines. We selected the chinchilla (*chinchilla laniger*) as our animal model for several reasons. Primarily, the chinchilla is a precocious animal: it experiences hearing onset *in utero*, similar to humans (c.f. altricious rats, who experience hearing onset between post-natal days 12 to 14 [Geal-Dor et al., 1993]; reviewed in Chapter 2, Section 2.3.1: Choice of the animal model). Thus, the state of the chinchilla’s peripheral auditory system is similar at birth to that of humans, allowing some confidence in cross-species extrapolation to humans. Moreover, several practical considerations contribute to our choice. It is usually free of middle ear infections ([Heffner and Heffner 1991](#)); [Miller 1970](#) and is fairly easily bred and housed. Essential for our developmental studies is that we can obtain newborn pups. Also, our laboratory has many
years of experience with this species. We have made extensive recordings in both inferior colliculus (e.g., Harrison et al., 1998) and auditory cortex (e.g., Brown and Harrison 2010; Brown and Harrison 2011; Pienkowski and Harrison 2005a; Pienkowski and Harrison 2005b).

3.3 Sound exposure stimulus and calibration

We created a narrow-band sound stimulus centered at 2 kHz (2 ± 0.25 kHz), 20 ms rise/fall time, 500 ms on, 1 s off (Adobe Audition 2.0, San Jose, CA, USA; sound frequency spectrum show in Figure 3.2). The stimulus was chronically presented (24 hrs/day), free-field (Sony Micro Hi-Fi Component System, CMT BX20i, coupled to Sony speakers, Model #SS-CBX20, Minato, Tokyo, Japan), for at least 4 weeks (28-33 days), beginning on post-natal day 0 (P0) or P1. The sound-exposure stimulus was calibrated to be 70 ± 5 dB SPL at the level of the animals’ ears. Calibration stimuli were 1000 ms in duration. Measurements were made at multiple locations in each cage. The ambient sound spectrum measured in the animal housing facility was relatively flat with no other significant peaks. Subjects did not exhibit abnormal behaviour; they appeared to feed normally. There was no difference in weight between control and sound-exposed subjects (reported as mean ± SD; controls: 168.3 ± 35.6 g; sound-exposed: 170.4 ± 33.9 g; p = 0.77, t-test).
Figure 3.2: Acoustic spectrum of the neonatal sound exposure stimulus (2 ± 0.25 kHz).

3.4 Cochlear Function and Structure

To verify that sound exposure did not damage hair cell function or structure, we measured auditory thresholds using auditory brainstem-evoked responses, and examined hair cell morphology, particularly around 2-kHz, using SEM. These experiments were carried out in anaesthetized subjects (ketamine: 15 mg/kg i.p.; xylazine: 2.5 mg/kg, i.p.)

3.4.1 Auditory Brainstem Responses

Auditory brainstem responses (ABRs) to both broadband noise (47-µs clicks; \( n = 50 \) & 25 for controls & sound-exposed, respectively) and pure tones around the sound-exposure stimulus (4 ms pips, Blackman enveloped, at 1, 1.5, 2, 2.5, 3, 4, and 8 kHz; \( n = 26 \) & 15 for controls & sound-exposed, respectively) were recorded (Smart EP, Intelligent Hearing Systems, Miami, FL, USA). Click data were averaged from 1024 sweeps; tone-pip data from 512 sweeps. Skin needle electrodes were placed in a standard mastoid – vertex configuration. Stimuli were presented monaurally (right ear) through an
insert earphone (ER-2, Etymotic Research, Elk Grove Village, IL, USA) at a range of intensities, in 10 dB steps. Threshold was taken as the level at which predominant ABR peaks were just discernible (asterisks in Figure 3.3). Subjects were included in the study when click thresholds were less than 30 dB SPL.

![Representative Auditory Brainstem Responses (ABRs) to 47-µs clicks](image)

Figure 3.3: Determination of ABR thresholds from an intensity series. Threshold was taken as the level at which predominant peak(s) were just discernible (indicated by asterisks, *); here, 20 dB SPL.

### 3.4.2 Scanning Electron Microscopy

Anaesthetized subjects (n = 4; 1 female, 3 males) were transcardially perfused with saline (0.9%) followed by cold fixative (2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.4, 4 °C). Cochleae were removed, slowly perfused with 2-3 mL of fixative, then incubated in fresh fixative for 2 hrs. Samples were post-fixed for 1.5 hours in 1%
osmium tetroxide, and dehydrated through graded ethanol incubations. After cochleae were dissected open, samples were critical-point dried, sputter-coated with gold and cochlear morphology was assessed.

In a normal-hearing subject, hair cells are regularly arrayed in 3 rows of outer hair cells and 1 row of inner hair cells (white arrowheads in Figure 3.4A). If a subject were to have elevated hearing thresholds, using SEM, we would detect absent hair cells (red arrows, Figure 3.4B), and hair cell morphology would be noticeably compromised. It is this level of qualitative comparison of hair cell distribution and morphology that was made between control and sound-exposed subjects, particularly near the frequency region of the sound-exposure stimulus (around 2 kHz; Figure 3.5). N.B.: The hair cells shown in Figure 3.4B are from a control subject who had ABR thresholds greater than 30 dB SPL, and hence was not studied further.

![Normal-hearing subject vs. High-threshold subject](image)

**Figure 3.4:** (A) Hair cell distribution is regularly-patterned in normal-hearing subjects (3 rows of outer hair cells, 1 row of inner hair cells, indicated by white arrowheads). (B) If a subject were to have elevated thresholds, using SEM, we would detect absent hair cells (red arrows), and that the morphology of hair cell stereocilia is noticeably compromised.
Figure 3.5: Chinchilla cochlear frequency-place map. Schematic illustrating the region of the cochlea corresponding to approximately 2 kHz (black arrow) from which hair cells were examined by SEM.

3.5 Electrophysiology methods and analysis

Following neonatal sound exposure, one of two methodologies used to examine possible changes in IC neural responses was micro-electrode recordings of spike activity in central nucleus of IC.

3.5.1 Subject preparation

Eleven chinchillas (chinchilla laniger; 8 females, 3 males) were obtained from Roseneath Chinchilla (Roseneath, Ontario, Canada). Subjects were aged between postnatal day 29 (P29) to P34 (140 – 250 g). Animals were anaesthetized with intraperitoneal injections of ketamine (15 mg/kg) and xylazine (2.5 mg/kg). Subjects were given a one-half dose of each approximately every hour to maintain sedation for the duration of data collection, after which the deeply anaesthetized subjects were decapitated. With this
anaesthetic regime, animals respire spontaneously. The avoidance of artificial respiration (pumping) was important to maintain brainstem stability required for microelectrode recordings. Body temperature was monitored with a rectal probe and maintained at 37°C with a thermostatically-controlled heating blanket.

### 3.5.2 Surgical technique to access auditory midbrain

The inferior colliculus (IC) is usually accessed surgically in one of two ways: either the overlying cortex is aspirated, or the cerebellum is removed. We developed a surgical technique to access IC that preserves the integrity of the overlying cortex and cerebellum. Following tracheotomy and intubation, the cranium was opened above the junction of the occipital lobe and the cerebellum, and the dura reflected. Surface vessels were cauterized. A flattened, surgical-grade compressed sponge (Otocell® Ear Wicks, Boston Medical Products, Westborough, MA, USA) was gently inserted between occipital cortex and cerebellum. When moistened, this material expands, creating a gap between them (indicated by the arrow in Figure 3.6), allowing direct visual access of IC for electrode placement. Silicone oil was applied to the preparation to prevent desiccation. This technique was a reliable method of accessing IC.
3.5.3 Extracellular recordings

Extracellular micro-electrode recordings were made on a vibration-isolation table in a sound-attenuating booth (IAC). A remotely-controlled microdrive (MCM Controller Module, FHC, Bowdoin, ME, USA), which was regularly calibrated, held two or four high-impedance (2 – 4 MΩ) tungsten microelectrodes. Electrodes were spaced ~0.5 mm apart and were advanced vertically in 50-µm steps to depths of about 3 mm, the approximate length of the IC, until auditory-responsive neurons were no longer encountered. We used 2 or 4 bundled electrodes so as to increase neural recording yield. Stimuli were generated and recordings were stored and analyzed using Tucker Davis Technology (Gainesville, FL, USA) hardware (System 3 components) and software (SigGenRP v. 4.4, BrainWare32 v. 9.19).

Once the IC was visible, electrodes were placed using microdrive coordinates. A search stimulus (50 ms broadband noise, 70 dB SPL) was used to detect auditory neurons. When responsive neurons were located, 50-ms pure-tones (¼-octave spaced...
from 0.1 - 0.4 kHz, ⅛-octave-spaced between 0.4 and 20 kHz) were used to obtain neural responses. Tones were presented at 4 levels in 10 dB steps starting from ~0 dB SPL, and at a rate of 3 – 4 stimuli/s. All stimuli were presented twice, pseudorandomly. Based on the diameter of an electrode tip (< 1 µm) and axon width (~50 µm), we estimate to have recorded from a few (1-5) units per electrode at each electrode depth.

Tones were delivered monaurally to the right ear using a high-frequency sound transducer (Intelligent Hearing Systems, Miami, FL, USA) via a short tube and foam ear tip. Recordings were made from the contralateral IC. Electrode signals were amplified, and band-pass filtered (0.3 – 5 kHz). Action potentials were discriminated online using voltage window thresholding. Voltage thresholds were selected to lie just above and below the noise floor (red horizontal lines in Figure 3.7). Voltages that crossed both thresholds, and whose voltages had maximum and minimum peaks not more than 700 µs apart, were considered to be neural action potentials (spikes).
Real-time (online) spike discrimination paradigm

Figure 3.7: Online spike discrimination from background noise by voltage thresholding. Action potential voltages must cross both thresholds (indicated by red horizontal lines), and have maximum and minimum peaks not more than 700 µs apart, to be considered a spike.

Electrode tracks were confirmed histologically to ensure we were recording from central nucleus of inferior colliculus. Since there are distinct divisions of IC, each with differing neural properties, it is important to know from which division we recorded. Therefore, electrode tracks were confirmed histologically. Upon completion of micro-electrode recordings, a blood vessel near the occipital cortex of the anaesthetized subject was ruptured. The blood pooled in the valley between cerebellum and cortex (created to visually place electrodes in IC, described in Section 3.5.2: Surgical technique to access auditory midbrain). Micro-electrodes were carefully retracted and advanced, using
Narishige micromanipulators, marking electrode tracks in IC through which the electrodes traversed during the experiment. Following decapitation, the brain was removed, and placed in fixative for 48 hrs at 4 °C. One-hundred-micron slices were cut with a Vibratome™. Slices were collected on gelatinized slides, dried overnight, coverslipped, then imaged with Mirax™. IC divisions, superimposed on the electrode tracks in Figure 3.8, were estimated from rodent stereotaxic atlases (rat: Watson and Paxinos 1982; mouse: Franklin and Paxinos 2008). All electrode tracks measured passed through central nucleus of IC.

Figure 3.8: Electrode tracks were verified upon experiment completion to ensure they passed through central nucleus. CIC: central nucleus of inferior colliculus; DIC: dorsal cortex of inferior colliculus; EIC: external nucleus of inferior colliculus. Scale bar: 500 µm.

3.5.4 Properties of collicular neurons

For each frequency-level combination, we obtained an average count of the number of action potentials (the height of the bars is proportional to the number of spikes; Figure 3.9A). From these frequency response areas, we obtained frequency
tuning curves (Figure 3.9B). A “trace” of the tuning curve allows us to measure the following neural properties: characteristic frequency (CF), threshold, and bandwidth 10 dB above threshold ($BW_{10}$; Figure 3.9C). These properties were determined for recordings made from each electrode, at each electrode depth.

Figure 3.9: (A) Sample frequency response area (the height of the bars is proportional to the number of spikes for that frequency/level combination), and (B) tuning curve for a single electrode depth. (C) A tuning curve “trace” from which we quantify several neural properties: characteristic frequency (CF); threshold; and $BW_{10}$: bandwidth 10 dB above threshold.
3.6 c-fos immunohistochemistry methods and analysis

A second, histological method was used to examine potential differences in global neural activation patterns in IC following chronic, neonatal exposure to a frequency-specific sound stimulus. This involved: first optimizing the c-fos methodology for the precocious chinchilla; then determining the appropriate experimental protocol to obtain tonotopic bands of c-fos-labeled neurons; and finally, quantifying changes in c-fos expression patterns following neonatal sound exposure.

3.6.1 Development of c-fos histological methods for chinchilla

To reveal nuclear sites where antigen (the c-fos protein) is present, a series of incubations in various solutions is required. The c-fos protein is localized to the cell nucleus (Curran et al., 1984; Roux et al., 1990), thus application of a detergent (Triton X-100) permeabilizes cell membranes, permitting subsequent reagents to access the cytosol and nuclear material. The general method involves incubations in primary antibody (binds to antigen) then secondary antibody (binds to primary antibody) solutions, shown schematically in Figure 3.10. To prevent non-specific binding of antibody to charged sites on the tissue surface, samples are incubated in a “blocking” solution. In addition to the detergent mentioned above, this solution contains charged protein molecules (goat serum, bovine serum albumin) that bind to available charged sites on the tissue surface, thus preventing non-specific binding. In the final step, DAB (diaminobenzidine) is oxidized by peroxidase. Since DAB is a peroxidase marker molecule, one of the first incubations (not shown in Figure 3.10) is in hydrogen peroxide, to block endogenous enzyme (peroxidase) activity.
Figure 3.10: Schematic illustrating some of the solutions in which tissue samples are incubated to visualize the c-fos protein, which is localized to the cell nucleus. To adapt this protocol for the chinchilla, we began by optimizing antibody dilutions.

Since no c-fos immunohistochemical protocol had previously been reported for the chinchilla, we began by ascertaining optimal dilutions for primary and secondary antibodies. Combinations of primary (1°), and secondary (2°) antibody dilutions tested are shown in Table 3.2. The secondary antibody dilution that produced the (qualitatively) clearest staining with the least background staining was found to be 1:100. We tested two primary antibody dilutions with the 1:100 secondary dilution and found that a primary antibody dilution of 1:500 produced the clearest staining (Figure 3.11). Cell staining with these dilutions, however, was not as clear as could be obtained in a mouse model (c.f., Figure 3.11A & B).
Table 3.2: Primary (1°) and secondary (2°) antibody dilution combinations tested. A combination of primary antibody dilution of 1:500 with a secondary antibody dilution of 1:100 was found to be optimal.

<table>
<thead>
<tr>
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<td>1:2000</td>
<td>1:500</td>
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</tr>
</tbody>
</table>
Figure 3.11: c-fos expression in a mouse model (A) is clearer than that in a chinchilla model (B) following optimal antibody dilutions, suggesting further refinement to the chinchilla c-fos protocol is needed. Scale bar in (A) is 2 mm, in (B): 3 mm. BC: barrel cortex; Cx: cerebral cortex; Hipp: hippocampus.
We next modified primary antibody incubation duration and temperature. We began with a temperature and duration that produced optimal results in a mouse model, then decreased temperature and increased incubation duration. Again, a qualitative assessment of the results suggests that incubation for 48 hours at 4° C produced the clearest cell staining with the lowest level of background activity (Figure 3.12).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cell Staining</th>
<th>Background Staining</th>
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</thead>
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<td>High</td>
</tr>
<tr>
<td>2</td>
<td>4° C</td>
<td>24 hrs</td>
<td>Good</td>
<td>Reduced but present</td>
</tr>
<tr>
<td>3</td>
<td>4° C</td>
<td>48 hrs</td>
<td>Best</td>
<td>Low</td>
</tr>
</tbody>
</table>

Figure 3.12: Primary antibody incubation duration and temperature experiments and results. The best cell staining with the lowest level of background staining was produced under the conditions reported in the 3rd experimental protocol, indicated by the red rectangle.
To summarize, a primary antibody dilution of 1:500 and a secondary antibody dilution of 1:100 were taken as optimal for the chinchilla animal model. Further, a primary antibody incubation duration of 48 hrs and at 4°C was found to be optimal. We now report on: the acoustic protocol used to obtain bands of c-fos labeled neurons (that correspond to the tone-frequency used to induce them); and analysis methods to quantify changes in the pattern of c-fos expression following post-natal rearing in an enhanced acoustic environment.

3.6.2 Subjects

Twenty-six chinchillas (*chinchilla laniger*; 12 female, 14 male) were obtained either on the day of birth, post-natal day 0 (P0), or on P1 from Roseneath Chinchilla (Ontario, Canada). Subjects were randomly assigned to one of five experiments: two control groups, where subjects received either no sound stimulation (*n* = 5), or heard a 2-kHz pure-tone for 90 minutes (to induce bands of fos-labeled cells; *n* = 5), or to one of two sound-exposed groups, where subjects were reared in an enhanced auditory environment (described above in Section 3.3 Sound exposure stimulus and calibration) for 4 weeks, then either heard a 90-min 2-kHz tone (*n* = 5), or received no further sound stimulation (*n* = 5). A fifth group heard a 6-kHz tone for 90 minutes (to induce bands of fos-labeled cells; *n* = 6). Subjects were 28 - 35 days old (94 – 225 g). Normal hearing was verified using auditory brainstem response (ABR) thresholds to broadband, 47-μs click stimuli (Intelligent Hearing Systems, Smart EP).
3.6.3 Acoustic stimulation to induce bands of labeled neurons

All subjects were kept overnight in a 40-dB sound-attenuating booth (IAC) with ambient noise levels < 20 dB SPL. To induce bands of fos-labeled cells, some subjects were then stimulated free-field with gated pure tones (2- or 6-kHz; 100 ms on time, 200 ms off, 5-msec cos²-gated) for 90 minutes. All signals were calibrated to be 60 dB SPL at the level of the subject’s head (Larson Davis, Model 831, with B&K #4230 reference signal). Silence controls and sound-exposed subjects who received no further sound stimulation were treated similarly; however, instead of hearing the 90-min pure tone, they remained in silence for 90 min. Ten minutes before the end of the 90-min period, subjects were anaesthetized with ketamine (15 mg/kg i.p.) and xylazine (2.5 mg/kg, i.p.) and returned to the sound field (or silence).

3.6.4 C-fos immunolabeling protocol

At the end of the 90-min stimulation period (or 90-min period of silence), anaesthetized subjects were transcardially perfused with physiological saline followed by cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were removed and kept in fixative overnight at 4º C. Coronal slices (40 µm) were cut (VibratomeTM) and treated to visualize c-fos as follows. To block endogenous peroxidase activity, sections were incubated in hydrogen peroxide (0.3%), and rinsed with phosphate buffer (0.1 M; pH 7.4). To block non-specific antibody binding, sections were incubated in a blocking solution, (0.1% bovine serum albumin; 0.2% Triton X-100; 2% goat serum in 0.1 M phosphate buffer). Sections were immersed in the following solutions, each followed by buffer rinses: primary antibody (rabbit anti-Fos polyclonal IgG, Santa Cruz Biotechnology Inc.; diluted 1: 500) for 48 hours at 4º C, secondary antibody (goat anti-
rabbit IgG, Jackson Laboratories Inc.; diluted 1:100) for 1.5 – 2 hours at room temperature, and avidin-biotin complex (Vector Laboratories Inc., diluted 1:50) for 1.5 hours at room temperature. The diluent for antibody solutions and for the avidin-biotin complex solution was blocking solution. Sections were incubated in 3, 3’-diaminobenzidine (DAB, 0.05%) for 8 minutes, and staining revealed by application of 0.001% H2O2. Samples were then rinsed with phosphate buffer, mounted on gelatinized slides, dried, dehydrated, cleared in xylene, and cover-slipped.

Figure 3.13A shows the results of an experiment in which the primary antibody was omitted (sections were incubated in fresh “blocking” solution for 2 hours). As expected, no cell staining was observed; the tissue is visibly void of colour, in marked contrast to sections that undergo the primary antibody incubation (Figure 3.13B). These results indicate specificity of reagent binding; when an incubation step is omitted, immunolabeling is abolished.
Figure 3.13: Specificity controls. (A) When primary antibody is omitted, there is no substrate for the chromogenic reaction; hence neurons expressing the fos protein are not labeled. (B) Representative section from the same subject in which primary antibody was included. The 10x magnifications are raw images, obtained prior to ImageJ™ thresholding. Scale bars indicate 3 mm. Adapted from D’Alessandro and Harrison 2014.

### 3.6.5 Image analysis

Slides were digitally imaged using Mirax Scan™, which allows visualization of samples at a range of magnifications, and saved for further analysis. Using ImageJ™, thresholds were selected which best captured the pattern of fos-labeled neurons in IC. Cells were included in counts based on size and circularity. Images presented here (unless otherwise specified) are originals overlaid with “masks” of labeled neurons detected by ImageJ™ thresholding. To quantify the density of labeled neurons, we defined a region of interest (ROI) that consists of a grid of discrete, equal-sized columns overlaid on the centre of rotated collicular slices such that the edges of the ROI lay approximately 200-
400 µm away from the edges of the colliculus (see bottom panel, Figure 3.14). The columns of the grid are parallel to iso-frequency regions. Using a custom-made macro created in ImageJ™, we obtained counts of labeled neurons within each column of the ROI (top panel, Figure 3.14). To construct the scale of the abscissa in the resulting histograms (Chapter 5: C-fos Immunohistochemistry Results; Figures 5.3, 5.5, 5.6), we related position along the tonotopic axis to frequency as follows. Since we presented tonal stimulation at 2 different frequencies, we noted the average column in which the peak of the 2-kHz band lay in representative slices, and that in which the peak of the 6-kHz tonotopic band lay. We then built the scale such that the distance between the peaks (2- and 6-kHz) was maintained. The frequency range of the resulting scale relates well to the tonotopic map in chinchilla IC measured previously in our laboratory via electrophysiology (Harrison et al., 1998).
We next present results from our functional (auditory brainstem-evoked responses) and structural (SEM) analyses of the cochlea, as well as results from electrophysiological (micro-electrode) studies.
Chapter 4

Results of studies of the cochlea, and of electrophysiological recordings in IC

The main experimental manipulation in this thesis was prolonged (4-week) exposure of neonatal animals to a moderately-intense, narrowband sound stimulus. To verify that sound-exposure did not induce cochlear threshold elevations or hair cell damage, we assessed cochlear response thresholds using auditory-evoked brainstem responses, and imaged hair cell stereocilia using scanning electron microscopy (SEM) post-sound exposure. These results are described in Section 4.1.

We recorded tone-pip-evoked responses from neurons in inferior colliculus of sound-exposed subjects. We report on several properties of those neurons, relative to control subjects, in Section 4.2, as well as on the tonotopic representation of sound frequency in central nucleus of IC.
4.1 Cochlear thresholds and hair cell morphology in control and sound-exposed subjects

4.1.1 ABR thresholds

In Figure 4.1, thresholds of frequency-specific auditory brainstem responses (ABRs) are plotted for control (blue symbols) and sound exposed (red symbols) subjects. At all tone frequencies measured (1 – 8 kHz), average ABR thresholds of sound-exposed subjects are not different from those of controls \( (p = 0.98, \text{ANOVA}) \). These data suggest that neonatal sound-exposure did not cause temporary or permanent threshold changes.

![Figure 4.1: Average ABR thresholds for sound-exposed subjects (red symbols) are not statistically different from those of controls (blue symbols). Error bars are ± SD.](image)

4.1.2 Cochlear hair cell structure

Hair cells were imaged along the length of the cochlea using SEM. In Figure 4.2, we show representative images of hair cells in the region of the cochlea corresponding approximately to the sound-exposure stimulus, near 2 kHz (indicated schematically in
Figure 3.5). The SEM analysis is a qualitative study, in which we examined the cochlear sensory epithelium for loss of hair cells, and for any disruption of the stereociliar bundle. Hair cells of all subjects appear in the characteristic pattern of 3 rows of outer hair cells, 1 row of inner hair cells (indicated by white arrowheads in Figure 4.2E). The pattern of hair cells along the sensory epithelium is similar between control and sound-exposed subjects (Figure 4.2E). Hair cells of sound-exposed subjects have similar morphology as those of controls (inner hair cells: cf. Figure 4.2A & B; outer hair cells: cf. Figure 4.2C & D). That is, we were unable to detect any signs of unusual morphology in hair cells of sound-exposed subjects. Taken together with ABR results, these data suggest that the enhanced acoustic environment does not affect cochlear hair cell function or structure.
Figure 4.2: Hair cells in the 2-kHz region of the cochlea. Morphology of inner hair cells (c.f. (A) and (B)) and outer hair cells (c.f. (C) and (D)) are similar between groups. (E): The pattern of hair cells along the sensory epithelium (3 rows of outer hair cells, 1 row of inner hair cells, indicated by white arrowheads) is also similar between groups.
4.2 Electrophysiological responses in IC neurons

We were interested in whether long-term neonatal sound exposure had an influence on the development of sound representation in auditory midbrain. In this regard, we examined response properties as well as the tonotopic distribution of auditory neurons in central nucleus of IC. We recorded from multiple animals, from multiple litters.

4.2.1 Response properties of neurons in central nucleus of IC

We characterized several response properties of IC neurons from which we recorded, namely minimum threshold, and bandwidth 10 dB above threshold (BW_{10}), as described in Chapter 3: Methods, Section 3.5.4: Properties of collicular neurons, and illustrated in Figure 3.9. We also qualitatively compared the shape of neural response areas between control and sound-exposed subjects.

Minimum thresholds of neurons in central nucleus of IC are shown in Figure 4.3. Data reported are from 426 multi-units sampled from control subjects (Figure 4.3A) and from 983 multi-units recorded from neonatally sound-exposed subjects (Figure 4.3B). Near the region of the 2-kHz-centered sound-exposure stimulus (specifically, from 1-3 kHz), there was no difference in neural threshold (reported as average ± standard deviation; controls: 6.7 ± 9.8 dB; sound-exposed: 6.2 ± 7.3 dB, t(163) = 0.46, p = 0.65). This finding of similar neural thresholds in sound-exposed and control subjects is consistent with functional studies of the cochlea (Section 4.1.1: ABR thresholds) suggesting no ABR threshold differences between groups (Figure 4.1).
Figure 4.3: Minimum thresholds of IC neurons. There is no difference in neural thresholds near the region of the sound exposure stimulus (specifically, from 1 – 3 kHz) in neonatally sound-exposed subjects (B) compared with controls (A). Results shown are from 426 multi-units in (A), 983 multi-units in (B).

Neural bandwidths 10 dB above threshold (BW$_{10}$, measured in octaves) are plotted as a function of characteristic frequency in Figure 4.4. Data reported are from 422 multi-units sampled from control subjects (Figure 4.4A) and from 968 multi-units recorded from neonatally sound-exposed subjects (Figure 4.4B). In the region of the sound-exposure stimulus (from 1 – 3 kHz, as indicated in Figure 4.4A and B), there was no significant difference in BW$_{10}$ between groups (reported as average ± standard deviation; controls: 1.7 ± 1.1 octaves; sound-exposed: 1.7 ± 1.0 octaves, $t(175) = 0.02$, $p = 0.98$).
Figure 4.4: Neural bandwidth 10 dB above threshold (BW\textsubscript{10}). There is no difference in BW\textsubscript{10} near the region of the sound exposure stimulus (specifically, from 1 – 3 kHz) in neonatally sound-exposed subjects (B) compared with controls (A). Results shown are from 422 multi-units in (A), 968 multi-units in (B).

Representative tuning curves from low-, mid- and high-frequency regions are shown in Figure 4.5, for control (left panels) and sound-exposed (right panels) subjects. Qualitatively, tuning curve shape and off-frequency levels of activity were not different between groups over the frequency range from which we recorded.
4.2.2 Neural representation of sound frequency in IC

The increase in characteristic frequency (CF) with increasing electrode depth observed in control subjects (Figure 4.6A) is consistent with the literature (Harrison et al., 1998). We calculated the proportion of the tonotopic map that lay within octave-wide bands with center frequencies as shown in Figure 4.6 (B, D, and F). CF is fairly
evenly distributed over a range of frequencies in control subjects (from 0.25 to 8 kHz; Figure 4.6B). By contrast, in sound-exposed subjects, neurons with low CFs respond over a greater electrode excursion (indicated by arrows in Figure 4.6C & E; all electrode tracks for representative subjects are shown), and thus a greater proportion of the tonotopic map is tuned to neurons with low characteristic frequencies (Figure 4.6D, F).
Figure 4.6: CF vs. depth for controls (A) and representative sound-exposed subjects (C, E; all electrode tracks recorded from the subject are shown). (B), (D), and (F): The fraction of the tonotopic map which lies in octave-wide bands (centre frequencies as indicated) for corresponding CF vs. depth graphs; (A), (C), and (E), respectively.
Results from all electrode tracks from all subjects are shown in Figure 4.7. For control subjects, we recorded from a total of 426 multi-units from 16 electrode tracks; for sound-exposed subjects: 983 multi-units from 20 electrode tracks. As a group, sound-exposed subjects show increased representation of frequencies beginning an octave below the sound-exposure stimulus, between 1 and 0.1 kHz, relative to controls (cf. Figure 4.7A & C). In general, there is a shift in neural representation of sound frequency towards lower frequencies (Figure 4.7E), except at the octave band centered at 4 kHz, where there is an increase in neural representation (although not significant) that will be discussed further in Ch. 6: Discussion. The increase in the proportion of the tonotopic map devoted to the octave band centered at 125 Hz is significant \( t(7) = 2.39, p < 0.05 \); Figure 4.7E). There is a significant decrease in the fraction of the tonotopic map devoted to the octave band centered at 2-kHz, near the region of the sound-exposure stimulus \( t(23) = 3.19, p < 0.05 \). The decrease in the octave band centered at 8 kHz is also significant \( t(14) = 2.72, p < 0.05 \); Figure 4.7E).
Figure 4.7: Grouped CF vs. depth plots show increased representation of low frequencies beginning an octave below the sound-exposure stimulus for sound-exposed subjects (B) compared with controls (A). Corresponding histograms: shown separately for controls (C) and sound-exposed (D); plotted together in (E). All electrode tracks from all subjects are shown. * p < 0.05
4.2.3 Properties of neural tuning curves in the low-frequency region

Here we report on characteristics of neural tuning curves in the low-frequency region, where, in the previous section, we reported an expanded neural representation in sound-exposed subjects. Specifically, in the frequency region between 0.1 and 1 kHz, neural thresholds were lower for sound-exposed subjects (reported as average ± standard deviation; -4.3 ± 10.3 dB cf. 2.8 ± 11.2 dB for controls; \( t(311) = 8.0, p < 0.001 \); Figure 4.3). There was no significant difference in \( BW_{10} \) in this region (reported as average ± standard deviation; controls: 1.4 ± 0.5 octaves; sound-exposed: 1.4 ± 0.6 octaves; \( t(416) = 1.54, p = 0.12 \); Figure 4.4).

4.3 Summary

In this chapter, we have provided evidence that cochlear function and structure are not compromised following chronic presentation of the moderately-intense sound exposure stimulus during the first 4 post-natal weeks of life. Hearing thresholds (estimated from auditory brainstem evoked responses), hair cell morphology, and the pattern of hair cells along the sensory epithelium (imaged with SEM) are not different from age-matched controls. Near the region of the sound exposure stimulus, specifically between 1 and 3 kHz, electrophysiological data suggest there is no difference in neural thresholds or bandwidth 10 dB above threshold (\( BW_{10} \)). Qualitative comparisons of tuning curves in low- mid-, and high-frequency regions suggest that tuning curve shape and off-frequency levels of neural activity are not different between groups. Tonotopic maps in inferior colliculus are altered following neonatal sound-exposure: there is a
marked increase in neural representation of low frequencies (< 1 kHz), and a decreased proportion of the tonotopic map devoted to frequencies with octave bands centered at 2- and 8 kHz. In the low frequency region where we observed increased neural representation (between 100 Hz and 1 kHz), neural bandwidths 10 dB above threshold (BW_{10}) were not different; neural thresholds were lower for sound-exposed subjects in this region.

Next, we present c-fos expression patterns in inferior colliculus: at basal levels; subsequent to 90-min pure-tone stimulation; and following neonatal rearing in the narrowband-sound-enhanced acoustic environment.
Chapter 5

c-fos Immunohistochemistry Results

In this chapter, we first present data that suggest that the c-fos immunohistochemical protocol has been successfully optimized for the precocious chinchilla. Using this method, we then establish a technique to view bands of c-fos labeled neurons following pure-tone sound stimulation. The location of these bands is compared with characteristic frequency/electrode depth results from electrophysiological recordings made in a separate set of subjects. Finally, we assessed neural activation patterns in inferior colliculus in subjects that were neonatally sound exposed compared with those of controls.

5.1 Tonotopic bands in chinchilla IC

Bands of fos-labeled neurons are visible following 90-minutes of pure-tone sound stimulation (indicated by arrows in Figure 5.1A and B). Bands produced with a 6-kHz sound stimulus lie ventro-medial to bands present following a 2-kHz stimulus, in
accordance with the known tonotopic organization of IC (e.g., Merzenich and Reid 1974). Interestingly, we observed decreased cell labeling adjacent to the tonotopic band, which, to our knowledge, has not been reported using this technique, and is further discussed in Chapter 6, Section 6.2: Global neuronal expression patterns: c-fos immunolabeling. Basal levels of c-fos active neurons with no tonotopic banding are observed in silence controls as illustrated in Figure 5.1C.

Figure 5.1: Representative sections for a subject who received (A) 90 minutes of 2-kHz sound stimulation, (B) 90 minutes of 6-kHz sound stimulation, and (C) a silence control. Arrows indicate bands within the inferior colliculus. Scale bars: 500 µm. Adapted from D’Alessandro and Harrison 2014.
5.2 Electrophysiological data corroborate location of c-fos bands

Next, we verified the position of c-fos banding with functional electrophysiological recordings of tonotopic maps. In a separate set of subjects, we determined neuron characteristic frequency vs. electrode depth. Bands of c-fos labeled neurons observed following 90-min, pure-tone stimulation (black arrows, Figure 5.2A and C) fall within average minimum and maximum depths (represented by vertical lines, Figure 5.2A and C) at which we recorded from neurons tuned to 2- and 6 kHz. Representative tuning curves from neurons with characteristic frequencies of 2- and 6 kHz are shown in Figure 5.2B and D, respectively. These results serve to confirm that a band of fos-labeled neurons induced by specific tonal sound stimulation corresponds to activity of auditory neurons tuned to that sound frequency.
Figure 5.2: (A) and (C): Bands of c-fos labeled neurons (indicated by black arrows) following 90 min of pure tone sound stimulation lie within average minimum and maximum depths (represented as black lines) at which we recorded from neurons tuned to 2 kHz and 6 kHz. (B) and (D): Representative tuning curves from neurons tuned to 2- and 6-kHz, respectively. Immunohistochemical and electrophysiological experiments were conducted on distinct subjects. Scale bars indicate 500 µm. From D’Alessandro and Harrison 2014.

5.3 Quantification of c-fos data

To quantify c-fos labeled cell density, a grid aligned along the tonotopic frequency axis in IC and perpendicular to the labeled band was used as shown in Figure
5.3A (described in Chapter 3, Section 3.6.5 Image Analysis). Cell counts so determined are shown for two experimental groups and one silence control group in the histograms of Figure 5.3B, C, and D, respectively. For the 2 kHz frequency band in Figure 5.3B and the 6 kHz band in Figure 5.3C, there is a clear increase in the number of labeled cells compared with off-frequency areas and silence controls (Figure 5.3D). Pooled data from the 11 experimental and 5 control animals are presented in Table 5.1. Compared with controls, there is a significant (40%) increase in labeled cells in the 2-kHz region and a 72% increase in the 6-kHz region.

Table 5.1: Average counts of c-fos labelled neurons. Values reported as average ± SEM. ROI: region of interest.

<table>
<thead>
<tr>
<th>Region</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (no sound stim.)</td>
</tr>
<tr>
<td></td>
<td>(5 subjects; 199 samples)</td>
</tr>
<tr>
<td>2-kHz band</td>
<td>76.2 ± 2.8</td>
</tr>
<tr>
<td>6-kHz band</td>
<td>64.9 ± 1.2</td>
</tr>
<tr>
<td>Entire ROI</td>
<td>306.1 ± 7.7</td>
</tr>
</tbody>
</table>

*aOne coronal slice yields 2 samples

*Significant (p < 0.001) increase, compared with controls in the same region
Figure 5.3: (A) Representative sample, rotated, with grid overlaid to obtain cell counts (see Chapter 3, Methods, Section 3.5.4 Image Analysis). Average cell counts (± SEM) for subjects who received (B) 90-min 2-kHz sound stimulation, (C) 90 min 6-kHz sound stimulation, and (D) silence control subjects. Arrows denote regions of decreased cell labeling. Scale bar in (A) indicates 500 µm. Adapted from D’Alessandro and Harrison 2014.
5.4 Sound-exposed subjects

Subjects reared in the enhanced sound environment who then heard a 2-kHz pure tone for 90 min show an increase in c-fos labeled cells over a broad range of the inferior colliculus compared with control subjects who heard only the 90-min 2-kHz pure tone (Figure 5.5A; qualitative comparison: Figure 5.4A & B). The increase in both the number of c-fos-labeled cells in the 2-kHz band (Figure 5.5B), and over the entire region of interest (Figure 5.5C) is significant, compared with controls subjects who heard only the 2-kHz tone for 90 min (values listed in Table 5.2). There was no difference in the width of the 2-kHz band (taken as the distance between the two “troughs”, or regions of decreased neural labeling; Figure 5.6) between groups.

Table 5.2: Average counts of c-fos labelled neurons (± SEM) by experimental group. Statistical tests were performed between pairs of groups, as indicated; corresponding p-values are reported.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-kHz band</td>
</tr>
<tr>
<td>2 kHz, 90 min</td>
<td></td>
</tr>
<tr>
<td>Sound-Exposed + 2 kHz, 90 min</td>
<td>106.9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>No Sound</td>
<td>184.9 ± 3.8</td>
</tr>
<tr>
<td>Sound-Exposed + No Sound</td>
<td>76.2 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>p = 0.4</td>
</tr>
<tr>
<td>No Sound</td>
<td>79.2 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>p = 0.8</td>
</tr>
</tbody>
</table>
Figure 5.4: Representative patterning of fos-labeled cells, by experimental group.
Figure 5.5: (A) Cell counts for subjects who received 2-kHz, 90 min sound stimulation (red bars; 158 samples from 5 subjects), and from those who were reared in an augmented acoustic environment, then received 90 min of 2-kHz sound stimulation (blue bars; 194 samples from 5 subjects). One coronal slice produces 2 samples. Error bars show SEM. Differences in cell counts both in the 2-kHz band (B) and over the entire region of interest (ROI; C) are significant, * $p < 0.001$. 
Figure 5.6: The width of the 2-kHz band – taken as the distance between “troughs”, or regions of decreased c-fos labeling – is not different between control (A) and sound-exposed (B) subjects.

Next, we wanted to discern whether this increase in c-fos-labeled neurons is due to sound-exposure alone. The sound-exposed subjects described in the previous paragraph have, in effect, received 2 “treatments”: sound-exposure, and the 2-kHz tone for 90 min. Thus, we conducted a separate set of experiments where subjects were exposed to the enhanced sound environment, then received no additional sound stimulation. Quantitatively, there is no difference in the number of labeled neurons in
either the region where the 2-kHz band would be (Figure 5.7B) or over the entire region of interest (Figure 5.7C). These results suggest that there is no effect of sound-exposure alone on basal levels of c-fos-active cells (cf. Figure 5.4C & D).

Figure 5.7: (A) Cell counts for subjects who received no sound stimulation (neither the 4-week sound-exposure stimulus, nor any subsequent pure-tone stimulation; red bars; 199 samples from 5 subjects), and from those who heard the sound-exposure stimulus, then received no additional sound stimulation (blue bars; 249 samples from 5 subjects). One coronal slice yields 2 samples. Error bars show SEM. There is no significant difference in cell counts where the 2-kHz band would be (B), or over the entire region of interest (ROI; C).
5.5 Summary

In this chapter, we have demonstrated the viability of the c-fos immunohistochemical protocol for the precocious chinchilla. We reported on resting levels of c-fos activity in inferior colliculus, and the bands of c-fos-labeled neurons present following 90 minutes of pure-tone stimulation of differing frequencies. Bands present following higher-frequency stimulation (6 kHz) lay ventro-medial to those present following lower-frequency stimulation (2 kHz). These findings are consistent with the tonotopic organization of central nucleus. Electrophysiological recordings made in a distinct set of subjects corroborated the location of these bands. Subsequent to the 4-week neonatal sound exposure period, we observed a significant increase in the number of c-fos-labeled neurons, both in the 2-kHz band, and over the entire region of interest. There was no difference in the width of the 2-kHz band. The 4-week sound exposure period does not affect basal levels of c-fos expression: subjects who heard the sound exposure stimulus then received no further sound stimulation had c-fos expression patterns similar to controls. There was no difference in the number of c-fos-labeled cells either where the 2-kHz band would be, or within the larger region of interest. These data suggest that sound-exposure has changed the way in which neurons in IC subsequently respond to prolonged (90-min) periods of pure-tone sound stimulation.

We now discuss the implications of all reported results, and suggest mechanisms by which they may have occurred.
Chapter 6

Discussion

In this thesis, we sought to examine whether neural activation patterns initiated at the periphery could influence the normal representation of sound frequency in central auditory structures. We hypothesized that increased activity during an early post-natal period would alter representation of sound frequency in inferior colliculus, the first sub-thalamic auditory nucleus. We discuss evidence from both electrophysiological and immunohistochemical experiments, both of which support the hypothesis. We present conclusions, and discuss future work.
6.1 Electrophysiological recordings from neurons in IC

6.1.1 Changes near the region of the sound-exposure stimulus

During development, when there are natural patterns of sensory stimulation, ascending pathways develop normally. However, unusual patterns of stimulus-driven neuronal activity can result in the abnormal development of central sensory maps (e.g., Miyakawa et al., 2013; Schreiner and Polley 2014). Both sensory deficits and enhanced sensory environments can be viewed as different-from-normal environments, and hence result in abnormal central maps. We found a significant decrease in neural representation around the sound exposure stimulus (2 ± 0.25 kHz) in the inferior colliculus of the precocious chinchilla. We describe this change in neural representation around 2 kHz as a form of plasticity and not due to a noise-induced hearing loss: neural thresholds, neural bandwidth 10 dB above threshold (BW₁₀), ABR thresholds, and hair cell morphology for sound-exposed subjects in this region were not different from those of controls. We cannot report, with the experimental measurements used in the present thesis, on the state of the cochlear nerve.

In a study by Kujawa and Liberman (2009), the authors exposed 16-week-old mice to an octave band of noise (8-16 kHz) for 2 hours at 100 dB SPL. Level and duration were adjusted such that measures of cochlear thresholds (among them, ABRs) were elevated for several days before returning to normal. They report that in regions where temporary threshold shift was maximal (around 32 kHz), despite evidence of normal hair cell function, Wave I ABR amplitudes were less than half their pre-exposure values. In frequency regions where threshold shifts were less pronounced (around 12 kHz), Wave I amplitudes recovered more fully. They also report degeneration of pre- and
post-synaptic elements of the inner hair cell in the basal half of the cochlea (high frequency regions). These results suggest that although ABR thresholds recover, there may be downstream effects on auditory nerve anatomy and function. The sound exposure stimulus used in the present study was presented at a considerably lower level (70 dB SPL, vs. 100 dB SPL). Nonetheless, the intriguing results reported in Kujawa and Liberman (2009) suggest that future work involving protracted presentation of a sound stimulus be accompanied by measurements of Wave I ABR amplitudes to study the function of cochlear neurons, in addition to techniques that examine hair cell integrity (ABR thresholds, SEM). With the data presented herein, we can make no assertions about the integrity of the cochlear nerve.

One plausible mechanism for the decrease in neural representation we observed at 2 kHz may relate to homeostatic decreases in synaptic strength following potentiation of neurons in the 2-kHz region. Turrigiano and colleagues (Turrigiano et al. 1998) measured the magnitude of AMPA-mediated synaptic currents as a function of activity in cultured pyramidal neurons in rat visual cortex. When synaptic activity was reduced by growing cells for 48 hours in culture containing tetrodotoxin (TTX), a Na+ channel blocker, the amplitude of miniature excitatory post-synaptic currents (mEPSCs) was significantly increased. Conversely, enhancing activity by growing cells in culture containing the GABA_A antagonist bicuculline resulted in a significant decrease in the amplitude of mEPSCs. These results suggest that the amplitude of the mEPSCs depends on prior activity levels of the neuron. In the present study, the increased neural firing associated with prolonged exposure to the sound stimulus may have induced a homeostatic decrease in synaptic strength at those frequencies such that when tones in the region of 2 kHz are
subsequently presented to determine frequency response areas, neural responses recorded at the level of IC are decreased.

There were several octave bands in the low-frequency region where neural representation was increased in sound-exposed subjects (125 Hz, 500 Hz). The increase at 125 Hz was significant. At higher frequencies, sound-exposed subjects had greater neural representation at the 4-kHz octave band. This difference, however, was not significant. The putative decrease in synaptic strength around the 2-kHz region may lead to a release from lateral inhibition (or disinhibition) of neurons neighbouring the sound-exposure stimulus, thus leading to an increase in representation of frequencies at the “edges” of the sound exposure stimulus.

In both developing and adult animal models, neural representation of sound frequency can be altered by exposing animals to an enriched sound environment. However, methods to induce these changes differ. To induce changes in sound frequency representation in developing animals, passive exposure to the sound stimulus suffices (e.g., de Villers-Sidani et al., 2007; Stanton and Harrison 1996). As a result of the pulsed pure-tone and frequency-modulated stimulation, respectively, cortical representation of the stimulus frequency increases. Adult animals must be trained to attend to the acoustic stimulus (e.g., Recanzone et al., 1993) or the stimulus must be paired with electrical microstimulation of cholinergic or dopaminergic systems (nucleus basalis: Kilgard & Merzenich 1998; ventral tegmental area: Bao et al. 2001) in order to observe changes in sound frequency maps. These results suggest that in adult models, acoustic stimuli must be behaviourally relevant to induce plastic change, at least at the level of the cortex.
Recent studies by Eggermont and colleagues challenge the notion that the mature auditory system is immune to changes resulting from passive exposure to a sound stimulus. Passively exposing 75-day old cats to multitone complexes (Norena et al., 2006) and adult cats to noise bands (Pienkowski & Eggermont 2009, 2010, review: Pienkowski & Eggermont 2011) result in decreased responses of cortical neurons in A1 to the majority of frequencies comprising the exposure stimulus. The results of these experiments together with those in the previous paragraph suggest that the structure of the auditory stimulus may be an important determinant of possible sound-exposure-induced neural reorganization.

Following this line of thought, another potential mechanism for the decrease in neural representation at 2 kHz reported in the present thesis may relate to how different types of auditory stimuli have differing effects on the auditory system. In previous studies that report increased neural representation of the sound-exposure frequency in IC following rearing in an enhanced acoustic environment (e.g., Oliver et al., 2011; Poon and Chen 1992), the authors presented either pulsed or continuous pure tones. In the present study, we used a narrowband noise stimulus centered at 2 kHz. We report significant decreases in neural representation of the octave band centered at 2 kHz. In a study by Merzenich and colleagues (de Villers-Sidani et al., 2008), neonatal rats were exposed to a 70 dB SPL bandlimited-noise (extending from 5-20 kHz) or notched noise (extending from 0-5 kHz and 20-30 kHz, with a noiseless notch from 5-20 kHz) from P7 (before hearing onset) to P20. In both cases, the authors report, among other findings, a significant decrease in frequencies that lay within the noise bands, and marked over-representation of frequencies outside the noise-exposure regions. Taken together with the
previous IC studies, including the present one, these results suggest that pure tones and noise are perceived differently by the auditory system. Pure tones strictly stimulate the auditory system. Noise serves to mask relevant environmental sound inputs, raising the threshold of the subject in that frequency range, and preventing them from hearing the normal patterning of sound that would promote normal development of sound frequency maps there. In clinical audiology for example, noise is used to mask hearing in a “non-test ear”, effectively raising thresholds in that ear, to test the hearing in the other “test ear”, much as an optometrist covers one eye while testing vision in the other eye (Katz and Lezynski 2002). Masking also prevents acoustic cross-over between ears (measured in a mouse model: Harrison, Negandhi, Allemang, D’Alessandro, & Harrison 2013). In the study discussed above by Merzenich and colleagues (de Villers-Sidani et al., 2008), neural responses in cortex were investigated. Our study is the first, of which we know, to report changes in sound frequency maps in inferior colliculus, a sub-cortical region, following rearing in an environment enhanced with a moderately-intense narrowband noise.

We did not observe an increase in neural representation at the sound exposure frequency. This may relate to the developmental state of the animal model used. Altricious animal models, such as the rat used in the aforementioned studies, have auditory systems that are less mature at birth than a precocious species such as the chinchilla (see Chapter 2: Background, Section 2.3.1: Choice of the animal model). Results reported here for the chinchilla reflect changes that occur in a more developmentally-mature auditory system at the time of the experimental treatments, and thus may differ from those reported in altricious species.
6.1.2 Tonotopic map changes in a low-frequency region

To gain a more accurate impression of the pattern of neural excitation caused by the neonatal exposure stimulus, it is instructive to consider the frequency response areas of auditory neurons. Tuning curves elucidate how neurons respond to tones of varying frequency/level combinations. Rather than having a fixed shape, tuning curve shape varies with frequency (Figure 6.1). Neurons with low characteristic frequencies (CFs) tend to have V-shaped tuning curves, while those with high CFs tend to have a steep, high-frequency cut-off region and a longer low-frequency “tail”. At threshold, the pure-tone frequency that activates the neuron is called the characteristic frequency. Supra-threshold sound stimulation activates neurons with tuning curves that lie over a broader frequency range. Thus, it is not implausible that we see changes in tonotopic maps at a broader range of frequencies following the 70 dB SPL, 2-kHz-centered narrowband sound stimulation.
Figure 6.1: Schematic of chinchilla frequency tuning curves spanning a range of frequencies. Note how tuning curve shape varies with frequency. The red oval indicates the 70 dB SPL, 2-kHz-centered narrowband sound exposure stimulus. Cross-hatching indicates neural tuning curves that would be activated by the sound-exposure stimulus, for the given neural tuning curve thresholds shown.

Results from electrophysiological experiments suggest that there is an increase in the neural representation of frequencies beginning approximately 1 kHz below the 2 ± 0.25 kHz sound-exposure stimulus, spanning 0.1 – 1 kHz. While it has been previously reported that early post-natal exposure to an enhanced acoustic environment increases cortical representation of the octave band containing the exposure frequencies (Stanton and Harrison 1996), these experiments were carried out in an altricial species, compared with the precocious chinchilla used in the present experiments. A critical examination of the literature suggests that increased cortical and sub-cortical (inferior colliculus) areas dedicated to frequencies below the exposure frequency is not uncommon following post-natal rearing in an enhanced acoustic environment.
In an early report by Clopton and Winfield (1976), rats were exposed for the first 4 months of life to a 1-s frequency sweep ("up": 6 – 9 kHz, "down": 9 – 6 kHz; 65 dB SPL) followed by a 1-s noise burst at 40 dB SPL. This pattern was repeated for an average of 5 hours/day. Units in inferior colliculus were sampled. Data from Figures 3 and 5 of their paper (Clopton and Winfield 1976) are shown re-plotted in Figure 6.2. The greatest number of responses occurred between 4 and 5 kHz; that is, about 1 kHz below the lowest frequency of the exposure sweep.

Figure 6.2: Data from Figures 3 & 5 of Clopton & Winfield (1976) re-plotted. Subjects were exposed for the first 4 months of life to patterned sound: a 1-s upward (or downward) tone sweep from 6 – 9 kHz (or 9 - 6 kHz) at 65 dB SPL, followed by a 1-s 40-dB-SPL noise burst, repeated for an average of 5 hours/day. The majority of responses occurred at characteristic frequencies between 4- and 5-kHz, 1-kHz below the lower limit of the exposure tones. The black horizontal line indicates frequencies in the exposure sweep.
More recently, close inspection of Fig. 2 in Oliver et al. (2011), shows increased representation of 13 kHz in adult rat IC (recordings made at P212) following 60-70 dB exposure to a 14-kHz pure tone (25 ms, every 250 ms) from P9 – P28 (Figure 6.3).

In a study of primary auditory cortex by Merzenich and colleagues (Nakahara et al., 2004), rats were exposed to a sequence of 30-ms 65-dB-SPL tone pips from P9 to P30. The first sequence consisted of 2.8-, 5.6-, 4.4-kHz pips that began at 0, 150, and 300 ms, respectively; the second sequence: 15-, 21-, and 30-kHz pips at 500, 650, and 800 ms, respectively. The greatest percent change in cortical representation of adult rats was at 1.7 kHz, about an octave below the lowest tone stimulus presented, with a 140% increase in cortical area dedicated to these frequencies (bin width, 0.6 octaves) compared
with controls. These studies provide evidence that developmental changes in tonotopic maps following neonatal exposure to tonal stimulation are not limited to the exposure frequency. Changes at low-frequency “edges” of the sound stimulus have been reported.

6.2 Global neuronal expression patterns: c-fos immunolabeling

6.2.1 Technique development

We have developed a reliable method to label c-fos-active cells in auditory midbrain of chinchilla. Our protocol is based on methods similar to those developed for other animal species (Brown and Liu 1995; Harrison and Negandhi 2012; Lu et al., 2009). However, these studies focus on altricial laboratory subjects (e.g., mouse, rat), who undergo hearing onset after birth (P12-P14 in rat; Geal-Dor et al., 1993), whereas the chinchilla is precocious: cochlear function begins in utero, similar to humans (Querleu et al., 1988). Moreover, our protocol uses only commercially-available reagents, thus it is accessible to the wider research community.

To use c-fos labeling to reveal functional organization in the auditory midbrain: the subject must be maintained in a (near) silent environment for many hours prior to hearing the acoustic stimulus of interest; the subject has to hear the stimulus for an adequate period of time; and the brain must be fixed at within a time window corresponding to the dynamics of c-fos expression. Our protocol has achieved this. We can histologically image tonotopic bands present following pure-tone sound stimulation. The location of these bands is consistent with the known sound-frequency organization of auditory midbrain. The presence of bands agrees with reports in the literature from other
animal species measured both with c-fos immunolabeling (e.g., Brown and Liu 1995; Friauf 1992) and 2-deoxyglucose measurements (e.g., Ehret and Romand 1994). To our knowledge, the correlation of these bands with electrophysiological recordings, and the potentially inhibitory sidebands observed adjacent to these bands are new findings. Taken together, these results suggest that the c-fos labeling technique can be used to study tonotopic organization in chinchilla.

Although the relationship between neuronal activation and immediate early gene induction is not fully understood, part of the signal transduction pathway involves the influx of Ca^{2+} either via activation of NMDA-type glutamate receptors or via L-type voltage-gated Ca^{2+} channels (Platenik et al., 2000; Sheng and Greenberg 1990). The c-fos protein is translated from c-fos mRNA in the cytoplasm, then moves into the cell nucleus. It is at the level of the cell nucleus that the c-fos protein is labeled in the present thesis (Curran et al., 1984; Roux et al., 1990). In the cell nucleus, the c-fos protein dimerizes with other transcription factors (members of the Jun family) to form part of the activator protein-1 (AP-1) transcription factor (Curran and Franza 1988). AP-1 upregulates transcription of a wide variety of genes involved in cell proliferation and differentiation (Angel & Karin 1991), tumorigenesis, and apoptosis (Preston et al. 1996).

In addition and of some interest, we have observed regions of decreased cell labeling adjacent to the bands of labeled neurons, which has not been previously reported using this technique. It is plausible that this decrease represents an inhibitory region. Lateral (or sideband) inhibition is a rather ubiquitous property of auditory neurons and has been studied at the level of IC by a number of authors (Alkhatib et al., 2006; Ehret and Merzenich 1988; Palombi and Caspary 1996a). Furthermore, iontophoretic
application of bicuculline and strychnine -- GABA_A and glycine receptor antagonists, respectively -- increases firing rate and tuning curve width of neurons in IC (LeBeau et al., 2001; Mayko et al., 2012; Xie et al., 2005). Anatomically, neurons in the central nucleus of inferior colliculus are arranged in iso-frequency laminae that are orthogonal to the tonotopic axis (Morest and Oliver 1984; Oliver and Morest 1984; Schreiner and Langner 1997). Malmierca et al. (1993) describe two types of neurons observed in the IC of adult rat: flat neurons (called disc-shaped neurons in other species) have dendritic arbors that are thinner and denser, and form laminae; less flat neurons (stellate cells, in other species) have thicker arbors, are less dense, and are found to populate the inter-laminar compartments. Regions of inhibitory activity may occur in these interlaminar compartments: inhibitory connections between laminae have been reported in the bat (Pollak and Park 1993) and cat (Palombi and Caspary 1996b). While it is reasonable to posit that the regions of decreased cell labeling adjacent to the bands of fos-labeled cells correspond to inhibitory regions, further studies are needed to test this hypothesis.

Various studies have examined how brief periods of sensory stimulation alter c-fos expression patterns. In somatosensory cortex, vibrissae stimulation has been shown to increase c-fos expression in rat barrel cortex. Following 15-20 minutes of manual whisker stimulation in anaesthetized (Mack & Mack 1992) and awake subjects (trained to sit atop a copper cylinder; Filipkowski et al. 2000), c-fos expression levels in barrel cortex were increased relative to unstimulated controls. For more “natural” whisker stimulation, in a second experimental paradigm, subjects were placed in a new wire cage (where they exhibited increased sniffing and whisking behaviour). Again, significant c-fos expression was reported in barrel cortex in these subjects compared with controls.
(Filipkowski et al. 2000). In visual cortex, experiments to study the effects of sensory stimulation on IEG expression often involve delivering visual input following a period of dark-rearing (review: Kaczmarek & Chaudhuri 1997). C-fos expression levels in visual cortex were increased in dark-reared kittens (birth to 5 weeks, then 1 hr of light stimulation: Rosen et al. 1992; birth to 30 days, then 0.5, 1, 2, 4, or 6 hrs of light stimulation: Beaver et al. 1993). Maximum c-fos induction occurred following the 2-hr period of light stimulation (Beaver et al. 1993). In the auditory system, brief (1-2 hr) pure-tone sound stimulation induces tonotopic bands in a number of animal models (mouse: Brown and Liu 1995; rat: Lu et al., 2009), and is the stimulation duration used in the present thesis to elicit bands of c-fos-labeled neurons.

We note that the spatial extent of the region of c-fos labeled cells is more than would be expected if a near threshold tonal stimulus was used for c-fos activation. Our stimulation tone to induce bands of fos-labeled cells was presented suprathreshold (at 60 dB SPL), and thus activated a wider neuron array (Figure 6.1). It is also possible that the c-fos labeled band width is determined by the laminar neuro-architecture of IC central nucleus as described by Malmierca et al. (1993) and Winer and Schreiner (2005). This laminar neuronal organization in IC translates functionally into "frequency band laminae", and may relate to observations of a discontinuous, or stepwise increment in tonotopic organization (Malmierca et al., 2008; Schreiner and Langner 1997), which has been reported as ~0.3 octaves per frequency step, independent of frequency (rat IC: 0.29 octaves per frequency step, Malmierca et al. 2008; cat IC: 0.28 octaves per frequency step, Schreiner & Langner 1997).
Immunohistochemical techniques complement electrophysiological studies. Much progress has been made in understanding neural function throughout the brain with multi-electrode, multi-site recordings in both anaesthetized and awake subjects. While electrophysiological studies have high temporal resolution, they are invasive, and have low spatial coverage. Previous histological activity markers that have been employed (e.g., \( ^{14}\text{C}\)-2-deoxyglucose, and the mitochondrial enzyme cytochrome oxidase) have supplemented results from electrophysiological methodologies by permitting visualization of global neuronal activation patterns. These methods have high spatial resolution, but low temporal resolution. C-fos immunohistochemistry also allows mapping of global neural activation patterns, with the additional benefit of single-cell-level resolution. Additionally, during stimulation and subsequent induction of immediate early genes, subjects are awake, with minimal interruption to their “normal” behavior. In the present study, we have employed these complementary techniques; results observed from each technique are not incompatible.

6.2.2 c-fos expression patterns following sound-exposure

Subjects who heard the sound exposure stimulus for 4 weeks, then received no additional sound stimulation did not exhibit c-fos expression patterns that differed from age-matched controls (Figure 5.4C & D; Figure 5.6). These results imply that sound-exposure does not change basal levels of neural activity in inferior colliculus. This includes neurons that are spontaneously active, as well as those that are auto-evoked (e.g., from the subject’s movement within the cage, breathing rhythms). These results are in accordance with qualitative electrophysiological results, seen in the tuning curves of
Figure 4.6, which also show that off-frequency levels of neural activity were not different between groups over a broad frequency range.

Sound-exposed subjects who subsequently heard the 90-min, 2-kHz pulsed tone exhibited increased c-fos expression levels, both near the 2-kHz region of interest and over a broad range of inferior colliculus (Figure 5.4A & B; Figure 5.5). It is important to note here the difference in timing between the two main experimental techniques used in this thesis (Figure 6.4). Electrophysiological recordings were made in IC neurons of sound-exposed subjects in response to 50-ms tone pips (Figure 6.4A), while sound-exposed subjects in this set of c-fos expression experiments essentially underwent 2 experimental “treatments”: the 4-week sound exposure, then the 90-min, 2-kHz pulsed tone exposure (to induce bands of fos-labeled neurons, Figure 6.4B). Taken together with the results of the previous paragraph, these data suggest that the sound-exposure stimulus alters the way in which neurons respond to a subsequent, prolonged presentation of a sound stimulus in the frequency range of the sound exposure stimulus (90 min pulsed 2-kHz tone; shown schematically in Figure 6.4B).
Figure 6.4: Schematic emphasizing the difference in timing between (A) micro-electrode electrophysiological recordings, and (B) c-fos immunohistochemical experiments for sound-exposed subjects who subsequently heard the 90-min, 2-kHz pulsed pure-tone stimulus.

While the mechanism by which extracellular stimuli result in induction of the c-fos immediate early gene is not clear, part of the signal transduction pathway is known to include activation of NMDA receptors (Platenik et al., 2000; Sheng and Greenberg 1990). During development, there are changes in glutamatergic transmission. “Silent synapses” (those that contain only NMDA receptors) have been proposed to predominate in early development (Durand et al., 1996; Wu et al., 1996). Sensory experience and NMDAR activation converts these synapses to functional ones via NMDA-receptor-mediated insertion of AMPA receptors (Isaac et al., 1997; Quinlan et al., 1999). Additionally, NMDAR sub-unit composition switches: NMDARs composed of NR1 and NR2B sub-units dominate in sensory cortices; during development, NR1- and NR2A-containing sub-units emerge (Quinlan et al., 1999; Sheng et al., 1994). It has been
suggested that NMDAR sub-unit composition influences the degree of synaptic plasticity during both developmental and adult periods, since \( \text{Ca}^{2+} \) permeability is determined by subunit composition (review: Lau et al., 2009). Moreover, synaptic transmission can be maintained in an immature state by delaying sensory experience (e.g., dark-rearing in the visual system: Quinlan et al., 1999).

In a study by Hogsden and Dringenberg (2009), the authors reliably induced long-term potentiation (LTP) in rat primary auditory cortex by theta-burst stimulation of the medial geniculate nucleus (of thalamus). During early post-natal life, a second set of subjects were reared in continuous white noise (from P5 to P50-P60; 70-80 dB SPL), which effectively masked patterned auditory input. LTP was enhanced in these white-noise-reared subjects compared to age-matched controls reared in an unaltered sound environment. The enhanced LTP was abolished following application of NR2B-subunit antagonists (Ro 25-6981 or ifenprodil). The authors suggest that post-natal white-noise exposure maintains higher, juvenile-like levels of synaptic plasticity. It is plausible that the sound-exposure stimulus used in the present thesis preserved regions of the IC in an immature state (greater proportion of NR2B sub-unit composition), and that subsequent stimulation – of sufficient duration – induced an enhanced neural response, similar to that reported by Hogsden and Dringenberg (2009), seen here as an increase in the number of c-fos labeled neurons (Figure 5.4A & B; Figure 5.5).

### 6.3 Methodological considerations

The results reported herein should be viewed in light of various methodological considerations. First, electrophysiological results were obtained from anaesthetized subjects. There are several advantages to this experimental preparation: factors such as
attention and comfort level that would be present in awake subjects are eliminated; stable recordings can be made for longer periods of time in anaesthetized subjects; and with the chosen anaesthetic regime (ketamine/xylazine), subjects respire spontaneously, eliminating the need for artificial respiration, which facilitates the brainstem stability needed for micro-electrode recordings. Considerations include that the consistent administration of intraperitoneal anaesthetic may be subject to error. Care must be taken when comparing results from awake subjects to those from anaesthetized subjects, and also when comparing results from studies employing differing anaesthetic regimes. For example, in a study of response properties of IC neurons in the guinea pig, Syka and colleagues (Astl et al., 1996) report that spontaneous activity was increased in ketamine-anaesthetized subjects compared with urethane- or pentobarbital-anaesthetized subjects. Thresholds of units with a sustained response were lower in ketamine- and urethane-anaesthetized subjects than units with an onset response. Frequency tuning was not different between the anaesthetic types used (Astl et al., 1996).

Second, electrophysiological data were recorded, for the most part, from multi-units. We estimate to have recorded from approximately 1-5 units per electrode at each electrode depth. Recall that characteristic frequency increases with increasing electrode depth in the inferior colliculus. Given this tonotopic arrangement, and that electrode tracks were oriented orthogonal to the tonotopic axis, it is probable that at a given electrode depth, the few neurons from which we recorded would have similar characteristic frequencies. Also, neural responses from collicular neurons are quite robust, extending well beyond the noise floor (Figure 3.7), making spike discrimination relatively straight-forward with a simple voltage-thresholding technique. This differs
from neural discrimination at the level of the cortex, for example, where neural responses are not as easily discernible from the noise floor, and more complex analysis techniques are needed to discern neural activity from electrode noise (such as statistical comparisons between noise floor and electrode voltages, e.g., Brown and Harrison 2009).

Regarding the c-fos immunohistochemical technique, we re-iterate its low temporal resolution: c-fos protein synthesis peaks 30-90 minutes after stimulation, and has a half-life of a few hours (Morgan and Curran 1991; Herdegen and Leah 1998). Thus, the technique is not ideally suited for short-lasting behavioural states, nor when stimulation lasts longer than several hours. In the present set of experiments, we studied changes in neural activation patterns to a 90-min tone stimulus before and after the 4-week sound-exposure period, thus working within the time frame of highest c-fos protein expression. C-fos studies were complemented with micro-electrode recordings to measure changes in neural response patterns to short-duration (50-ms) tones, which would not have been possible with the low temporal resolution of the c-fos technique.

It is important to note that c-fos protein expression is not initiated by changes in cell energy metabolism unlike, for example, 2-deoxyglucose uptake. We mention this because it is sometimes debated as to whether inhibitory processes use less or equivalent energy to neural excitation. If c-fos labeling was dependent on ATP use, we would need to consider this issue. However, it is accepted that the initiation of c-fos expression is triggered by calcium channel modulation directly associated with cell excitation (depolarization; reviewed in Finkbeiner & Greenberg 1998) and not linked to energy metabolism. C-fos immunohistochemistry does not label neurons whose activity is inhibited.
6.4 Conclusions, Future Work

The data presented in this thesis support the hypothesis that changes in sound stimulation patterns at the periphery (the ear) affect change in central sensory representations. We provided evidence that the sound exposure stimulus does not alter cochlear function or structure: in the frequency range surrounding the sound-exposure frequencies, we observed similar ABR thresholds, hair cell morphology, and neural thresholds between control and sound-exposed subjects. Prolonged (4-week), passive exposure to the sound exposure stimulus was shown to decrease neural representation of 2- & 8-kHz octave bands, and increase neural representation of low-frequency regions (< 1 kHz) in auditory midbrain.

Compared with age-matched controls, we observed a significant increase in the number of c-fos-labeled neurons both in the 2-kHz region (corresponding to the sound-exposure frequency) and over a broad frequency range in inferior colliculus of sound-exposed subjects who subsequently heard the 90 min, 2-kHz pulsed pure-tone. There were no differences in c-fos expression in sound-exposed subjects who heard no additional sound, suggesting that sound-exposure does not alter basal levels of neural activity. These data support the hypothesis that persistent exposure to an abnormal sound environment -- during an early post-natal period -- can alter tonotopic organization in auditory midbrain. More broadly, this study contributes to a growing body of literature that suggests that sub-cortical levels of the ascending auditory pathway reorganize following changes in peripheral input during development.

Several additional research questions arise from this work. Are the changes in sound frequency representation following sound exposure reported in this thesis long-
lasting? That is, would they be present several weeks or months following the end of the
sound-exposure period? Answers to these questions would give us insight as to whether
sound exposure maintains the auditory system in an immature state, effectively extending
the critical period, as reported in Chang & Merzenich (2003). Can returning the subjects
to a “normal” sound environment reverse the changes reported herein, thus restoring
normal sound processing, as in Zhou et al. (2011)? Also, our experiments were carried
out in developing subjects. It remains to be seen whether passive exposure to an
enhanced sound environment in a precocious adult subject suffices to alter neural
responsiveness. We have seen some evidence for this in mature altricial subjects
(Pienkowski and Eggermont 2010). Further experiments are needed to explore these
research questions.
References


Kaltenbach, J.A., Meleca, R.J., Falzarano, P.R., 1996. Alterations in the tonotopic map of the cochlear nucleus following cochlear damage. In Auditory System Plasticity and


LeBeau, F.E., Malmierca, M.S., Rees, A., 2001. Iontophoresis in vivo demonstrates a key role for GABA(A) and glycnergic inhibition in shaping frequency response areas in the inferior colliculus of guinea pig. J Neurosci. 21, 7303-7312.


Mayko, Z.M., Roberts, P.D., Portfors, C.V., 2012. Inhibition shapes selectivity to vocalizations in the inferior colliculus of awake mice. Front Neural Circuits. 6, 73.


Moore, D.R., Aitkin, L.M., 1975. Rearing in an acoustically unusual environment -
effects on neural auditory responses. Neurosci Lett. 1, 29-34.

Morest, D.K., Oliver, D.L., 1984. The neuronal architecture of the inferior colliculus in
the cat: Defining the functional anatomy of the auditory midbrain. J Comp Neurol. 222,
209-236.

Morgan, J.I., Curran, T., 1991. Stimulus-transcription coupling in the nervous system:
Involvement of the inducible proto-oncogenes fos and jun. Annu Rev Neurosci. 14, 421-
451.

cortex processing by sound exposure in the "critical period". Proc Natl Acad Sci U S A.
101, 7170-7174.

stimulation of the cochlear nerve in rats: Analysis of c-fos expression in auditory
brainstem nuclei. Brain Res. 1031, 39-55.

acoustic environment disrupts frequency representation in cat auditory cortex. Nat
Neurosci. 9, 932-939.

circuitry in the cochlear nuclei studied with intracellular recordings from brain slices. In

infragranular pyramidal neurons in cat primary auditory cortex. Cereb Cortex. 2, 197-
216.


J Comp Neurol. 222, 237-264.

Palombi, P.S., Caspary, D.M., 1996a. GABA inputs control discharge rate primarily
within frequency receptive fields of inferior colliculus neurons. J Neurophysiol. 75, 2211-
2219.


