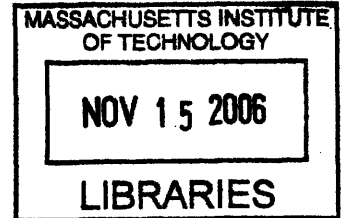


# Tone-evoked Fos labeling in the Central Auditory Pathway: Effects of Stimulus Intensity and Auditory Fear Conditioning

by

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Licentiate in Biology  
Faculty of Sciences, University of Lisbon, 1999



**ARCHIVES**

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## **ABSTRACT**

Understanding intensity coding and auditory learning are basic concerns of research on the auditory central pathway. There is no unifying model of intensity coding but several mechanisms have been proposed to play a role. The first aim of this thesis was to determine the mechanisms of intensity coding in the central auditory pathway from the cochlear nucleus to the auditory cortex. The Fos labeling method was used to assess neuronal activation in the central auditory system. This technique allows one to study large regions of the brain in awake animals. Increasing sound pressure level led to: (1) spreading of labeling towards neurons with higher best frequencies; (2) spread of labeling orthogonal to the tonotopic axis; (3) and increased density of labeling within the tonotopic band.

In addition to encoding the physical features of a stimulus, it is fundamental for survival that we learn about the meaning of sounds and put them in a behavioral context. The second aim of this thesis was to study how learning, in particular auditory fear conditioning, changes the pattern of neuronal activation of neurons, as measured with Fos labeling, in the central nervous system. Conditioning led to an increase in Fos labeling in central auditory nuclei. This increase in labeling was similar to the effects of increasing sound intensity. The present results support the idea that auditory fear memories are stored in the auditory pathway.

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*What is this thing that builds our dreams yet slips away from us*  
*Brian May (Queen)*

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# Chapter 1: Introduction and Background

## INTRODUCTION

Understanding how the brain encodes intensity is a fundamental research question in the field of auditory neuroscience. The objective of the first aim of this thesis was to investigate how the spatial pattern of activation of a large population of neurons, at different levels of the auditory pathway, changes as a function of sound pressure level. This spatial information could potentially be used for encoding sound intensity.

Our perception of a sound goes far beyond listening to spectral and magnitude variations across time. It is fundamental for survival that we learn about the ‘meaning’ of sounds and put them in a behavioral context. Understanding learning and memory processes is a particularly fascinating research area because of the huge contribution of these processes to the evolutionary success of the human species. One of the basic questions in the field of learning and memory is the identification of the loci in the brain that are involved in the acquisition and storage of information. The second aim of this thesis was to help clarify the role of nuclei that are critical for one type of learning, auditory fear conditioning. We investigated the pattern of neural activation of auditory and association nuclei while the animal became conditioned. The pattern of neural activation in these critical nuclei was compared with the behavioral effects of learning.

The Fos labeling technique was used in both aims 1 and 2 to assess neuronal activation in the central nervous system. This technique allows one to study neuronal responses of large regions of the brain, with single-cell resolution and in awake animals (Sagar et al, 1988).



In the following section of this chapter the motivation and rationale of this thesis will be briefly discussed. The results for the two aims are presented as self-contained research papers in chapters 2 and 3. Appendix figures presented in these two chapters consist of figures that will not be submitted for publication. Chapter 4 contains a brief summary of results and a general discussion aiming to integrate the present results with current ideas of how the brain processes intensity and auditory fear conditioning. Moreover, the putative function of the Fos protein will be discussed.

## BACKGROUND

### **Fos labeling**

#### What is Fos labeling?

In the late 80s a new type of neuronal signal, a wave of gene expression, was observed immediately following *in vivo* stimulation of the central nervous system (Sagar et al, 1988). These genes are rapidly induced following stimulation and their expression does not depend on new protein synthesis (Morgan et al, 1989; Sheng et al, 1990). Consequently, this family of genes has been called immediate early genes. *c-Fos* is part of this family of genes and encodes the transcription factor Fos. After the Fos protein has been produced in the cytoplasm it migrates to the nucleus where it forms heterodimeric transcription complexes and modulates the expression of other genes. *In vitro*, *c-fos* expression can be induced by a variety of extracellular signals including neurotransmitters, growth factors and depolarization (Morgan et al, 1989; Sheng et al, 1990; Herdegen et al, 1998).

Acoustic stimulation *in vivo* leads to the expression of the gene *c-fos* in auditory neurons throughout the auditory pathway (Ehret et al, 1991; Friauf, 1992; Sato et al, 1993; Adams, 1995; Brown and Liu, 1995; Scheich et al, 1995; Iversen, 2001; Santos et al, 2004). Sound-evoked *c-fos* expression occurs in different auditory neuronal types, but in only a small subset of the total number of cells (Adams, 1995; Yang et al, 2005). Stimulus-evoked Fos production is dependent on the temporal pattern of stimulation

(Fields et al, 1997; Yang et al, 2005). In contrast to stimulus-induced *c-fos* expression, the basal level of expression of this gene in most cells of the nervous system, including the auditory pathway is very low (Herdegen et al, 1995). Several lines of evidence suggest that the Fos protein plays an important role in the stabilization of long-term memories (Clayton, 2000; Guzowski, 2001).

### Advantages of using Fos labeling

In this study we chose to use the Fos labeling technique because we were interested in studying the responses of a large population of neurons, in behaving animals. Recording simultaneously from large numbers of neurons in one animal is technically very difficult. Furthermore, most techniques that allow one to measure neuronal responses of large areas of the brain such as 2-deoxyglucose or functional MRI do not have single-cell resolution. In contrast, the Fos labeling technique allows one to study neuronal activation in large areas of the brain, with single-cell resolution, in awake animals. In addition, recording from very small cells such as granule cells is extremely difficult. But, *in vivo* stimulation evokes Fos labeling in very small cells and therefore this technique can give us some insight into these cells' responses and function.

It is important to note that we do not know the specific cascade (or cascades) of events that leads to Fos production in the auditory pathway. Furthermore, Fos labeling is not equivalent to electrophysiology (see chapter 4). For example, fusiform cells respond to sound but only rarely show stimulus-induced Fos labeling (Adams, 1995; Yang et al, 2005). Nevertheless, the pattern of sound-evoked Fos labeling follows important functional principles of auditory physiology such as tonotopy. Consequently, Fos labeling seems to be an appropriate neuronal marker to study the overall pattern of neural activation in the central auditory pathway.

## **Chapter 2: Tone-evoked Fos labeling in the central auditory pathway: Effects of stimulus level**

Intensity is an important sound parameter, and intensity coding is one of the basic concerns of auditory neuroscience. Human ears can operate over a remarkably large

dynamic range. In contrast, the dynamic range of auditory nerve fibers is limited to about 20-50 dB SPL and in most cases rates of discharge reach saturation at relatively moderate sound levels (Ruggero, 1992). Therefore, a model of intensity coding where the magnitude of sound is simply proportional to the magnitude of discharge of auditory neurons does not seem viable. Alternative models have been proposed where additional types of information are used to encode stimulus intensity (Plack et al, 1995). For example sound pressure changes could be coded by: (1) differing thresholds; (2) the spatial pattern of excitation; and (3) neural synchrony. In this paper, we were particularly interested in studying how the spatial pattern of neuronal activation changes as a function of sound level. With this study we aim to better understand the contribution of spatial information for coding of sound intensity. In addition, this study will be useful to better understand sound-evoked Fos labeling in the auditory pathway and will serve as a reference for the study of conditioning effects on the auditory pathway (chapter 3).

### **Chapter 3: Effects of auditory fear conditioning on Fos labeling in the auditory pathway, posterior intralaminar nucleus and amygdala**

#### Auditory fear conditioning

Fear conditioning is a simple form of associative learning that has been described in many animal models and that is particularly important for survival (LeDoux, 2000; Maren, 2001). Following fear conditioning, an animal learns that a neutral stimulus, the conditioned stimulus (CS, e.g. a tone), predicts an aversive event, the unconditioned stimulus (US, e.g. a shock). The animal will quickly develop a conditioned response (CR, e.g. freezing), to the CS that was previously elicited by the US only (Blanchard et al, 1969). In addition to freezing, the CR, consists of other behavioral and physiological responses such as increased heart rate and blood pressure, and release of hormones (Fendt et al, 1999; Maren, 2001). A few CS-US pairings are enough to produce fear conditioning. For all the above it is clear that using fear conditioning to study learning and memory has several advantages including: ease of implementation, good understanding of necessary conditions to produce it, and well known behavioral and autonomic responses elicited by it. Freezing is a widely used and reliable metric of

conditioning (Fendt et al, 1999; LeDoux, 2000). An observer can easily score freezing. Several groups or commercial companies have also developed automatic methods to measure freezing (e.g. Agnostaras et al, 2000; Coulbourn Instruments). Thus, we chose to use freezing as our behavioral metric of conditioning. We developed a semi-automatic method of measuring freezing by comparing successive frames acquired from a digital recording of the animal's behavior.

Fear conditioning is critically dependent on the following structures: (1) the CS pathway, which for auditory fear conditioning is the auditory pathway, (2) the amygdala, and (3) the brainstem nuclei that produce the CR (Blanchard et al, 1972; LeDoux et al, 1983; LeDoux et al, 1988; Romanski et al, 1992; Campeau et al, 1995; Muller et al, 1997) (Fig.1.1). The exact role of the different auditory and amygdaloid nuclei is still not clearly understood and further research is required to determine the exact loci of plasticity associated with auditory fear conditioning. Thus, the second aim of this work was to help clarify the role of critical nuclei for acquisition and storage of auditory fear conditioning. We decided to look at nuclei that have been referred as strong candidates for auditory (tone)-somatosensory (shock) convergence and plasticity (posterior intralaminar nucleus or PIN; basolateral amygdaloid complex or BLA; and central amygdala or CE). In addition, we are interested in studying the auditory pathway because it could be the site of storage of auditory fear memories (Weinberger, 1998). Furthermore, this work aims to determine if auditory fear conditioning can induce changes at lower levels of the auditory pathway.

#### Methods: Choice of conditioning paradigm and behavioral controls

1. *The Conditioning Paradigm:* We used a trace-conditioning paradigm (the CS and US do not overlap) over a delay-conditioning paradigm (the US is given before the CS terminates) because this type of paradigm is known to be very efficient and does not activate the CS and US channels simultaneously, decreasing the chances of possible interaction effects due to non-associative factors (Lieberman, 1993). We used a variable interstimulus time interval because subjects can become conditioned to temporal intervals and develop behavioral responses at the times a CS is scheduled and not to the CS itself (Weinberger, 2004). The number of trials used (=36), the CS-US onset interval (=20 sec),

and the average interval between trials (=3 mins) was decided according to a literature review that indicates that the ratio of intertrial interval to CS-US onset interval is fundamental in determining the success of conditioning (Rescorla, 1988).

The behavioral paradigm was designed so as to produce a strong conditioning response to the CS tone and to significantly evoke Fos labeling in the auditory pathway. Several preliminary experiments with tones of different durations were done and it was observed that a short tone burst (20-30 secs) repeated many times clearly evokes Fos labeling in the auditory pathway while simultaneously conditioning the animals. Initially the animals were conditioned with 60 dB SPLs tone bursts. Later on, during our study of the effects of SPL on Fos labeling it was observed that in animals stimulated with tones above 45-50 dB SPL new bands of labeling appear in higher CF regions. To avoid spreading of labeling to other regions other than the CS frequency region, the level of the CS tone was lowered to 45 dB SPL, and the memory experiments were done at this level. Behavioral and Fos data from animals trained at 45 dB SPL was not qualitatively different from cases trained at 60 dB SPL.

2. *The Unstimulated, Shock Only and Tone Only Controls:* The Unstimulated control is used to determine the baseline level of freezing and Fos in the absence of any stimulation. The Tone Only and Shock Only controls are designed to evaluate the effects of the CS and the US alone on the experimental animals (Rescorla, 1967).

3: *The Unpaired Control:* It is not enough to control for the effects of tone and shock alone, because the presence of tone and shock together can lead to synergistic effects that cannot be predicted by looking at tone and shock separately (Lieberman, 1993; Rescorla, 1967). An explicitly unpaired control (the shock never occurs when the tone is on) was chosen over the truly random control (the shock can occur when the tone is on or off) as a control for possible 'non-associative' effects. The truly random control leads to the development of some level of persistent excitatory conditioning to the CS early in training (Rescorla, 2000). Current thinking explains the equivalence in behavioral responses to CS and US following a truly random control by the existence of competition of conditioning to the CS cue and to the background cues that occurred during individual pairings with the US. Thus, such a control in which excitatory conditioning to the CS is occurring could lead to similar physiological effects, in particular in the CS (auditory)

pathway in the control and in the conditioning paradigm. In contrast, in an explicitly unpaired control this excitatory conditioning to the CS is not observed, but some degree of inhibitory conditioning can develop. Inhibitory conditioning only tends to be developed when many (>40) CS and US trials are given (Weinberger, 2004) (No. of trials in our experiments=36). The development of some level of inhibitory conditioning was regarded as preferable because it would still allow us to compare very clearly distinct learning situations (excitatory conditioning vs inhibitory conditioning). In contrast, the truly random control would not allow for a clearly distinctive comparison with the conditioned group (lesser degree of excitatory conditioning vs higher degree of excitatory conditioning).

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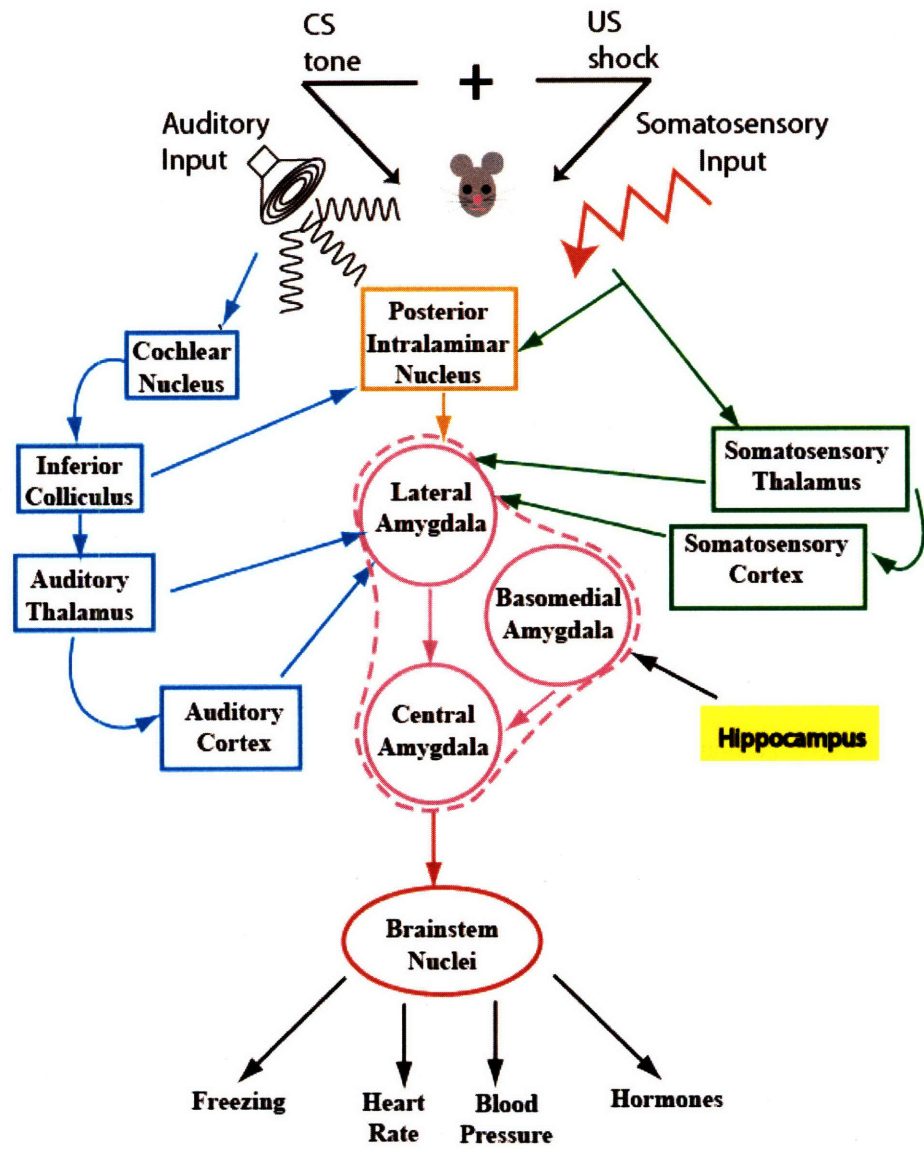


Figure 1.1. Simplified diagram of the neural pathway of auditory fear conditioning.

## **Chapter 2: Tone-evoked Fos labeling in the central auditory pathway: Effects of stimulus level**

### **INTRODUCTION**

Understanding intensity perception and coding is a basic concern of auditory research. Human ears can operate over a remarkably large dynamic range, as much as 120 dB in normal listeners (Viemeister et al, 1988). In contrast, the dynamic range of auditory nerve fibers is much narrower (40-50 dB SPL), and in most cases their rates of discharge reach saturation at moderate stimulus levels (e.g. Taberner et al, 2005). This is what has been called the dynamic range problem. A minority of auditory nerve fibers has wider dynamic ranges, higher thresholds, and low spontaneous rates of discharge (Liberman, 1978; Winter et al, 1990). A plausible hypothesis is that these low spontaneous rate, high threshold, fibers are particularly important for intensity coding at high sound levels (Shofner et al, 1986; Viemeister, 1988). But the fact that there are relatively low numbers of low spontaneous rate fibers is problematic for models where intensity coding at high levels is solely accounted by their rates of discharge.

Several alternatives to rate models of intensity coding have been proposed (Plack et al, 1995). For example, spreading of excitation to unsaturated higher CF fibers could provide information about the magnitude of a tonal stimulus (Evans, 1981). Spatial response profiles of auditory neurons in the periphery show spreading of excitation at moderate and high sound levels (Kim et al, 1979; Evans, 1981; Shofner et al, 1986; Kim et al, 1989; Kim et al, 1990). At low sound pressure level (20 dB SPL) there is a narrow peak of neural excitation centered at the stimulus frequency. In contrast, at medium-to-high sound pressure levels (45 and 70 dB SPL) the spatial pattern of neural excitation spreads along the tonotopic axis and becomes broad and more complex (e.g. appearance of secondary peaks). No similar studies have been done above cochlear nucleus level due to the technical difficulty of recording large populations of cells, in particular in the CNS.

The dorsal cochlear nucleus (DCN), inferior colliculus (IC) and auditory cortex (AC) contain units with complex tuning curves and non-monotonic rate-level functions (Evans et al, 1973; Phillips et al, 1985; Rhode et al, 1986; Ehret et al, 1988). Such nonlinearities could shape the pattern of neural excitation in central auditory nuclei and make it different from the spatial response profile of peripheral auditory neurons.

Furthermore, possible differences in the response properties of units along directions other than the tonotopic axis have been observed in different auditory stations particularly at higher levels of the pathway (Semple et al, 1979; Stiebler et al, 1985; Schreiner et al, 1988; Ghoshal et al, 1996). For example, units in IC are topographically organized in a threshold map and spatial information relating activation of units as a function of stimulus level could contribute to intensity coding (Stiebler, 1986). In the cochlear nucleus some regions also seem to contain more units with higher thresholds (Ghoshal et al, 1996). Fibers with differing spontaneous rate have differences in their projection pattern into the cochlear nucleus (Liberman, 1991; Liberman, 1993). Therefore, in addition to the putative role of low spontaneous rate fibers in rate-intensity models, it is possible that activation of the specific areas highly innervated by this type of fiber is a source of information about the magnitude of a stimulus. Low and medium spontaneous rate fibers send a relative higher number of inputs to rostro-dorsal and rostral areas of anteroventral cochlear nucleus (AVCN) and to the fusiform cell layer in DCN. Consequently, we could expect to see increased neural activation in these areas at high sound pressure levels.

The first aim of this work was to study the spread of neuronal activation along the tonotopic axis with increasing sound pressure level in the central auditory pathway. The second aim of this work was to investigate if spreading of neuronal activation occurs along other directions of the auditory pathway, in particular perpendicular to the tonotopic axis and in areas highly innervated by low and medium spontaneous rate fibers.

The Fos labeling method was used to investigate the pattern of neuronal activation as a function of sound pressure level because it allows one to study large areas of the brain, with single-cell resolution in awake animals (Sagar et al, 1988). Acoustic stimulation leads to production of the Fos protein in auditory neurons in cochlear nucleus, superior olivary complex, nuclei of the lateral lemniscus, inferior colliculus, and auditory cortex

(Ehret et al, 1991; Friauf, 1992; Friauf, 1994; Sato et al, 1993; Adams, 1995; Brown et al, 1995; Scheich et al, 1995; Iversen, 2001; Santos et al, 2004; Yang et al, 2005). In contrast to stimulus-induced *c-fos* expression, the basal level of expression of this gene in most of the nervous system is very low (Herdegen et al, 1995; Adams, 1995). The gene *c-fos* encodes a transcription factor, and is rapidly induced following stimulation (Morgan et al, 1989; Sheng et al, 1990). The Fos protein acts in the nucleus, where it regulates expression of other genes (Herdegen et al, 1998). *In vitro*, *c-fos* expression can be induced by a variety of signals including neurotransmitters, growth factors, and depolarization. The exact cellular cascade of Fos induction in auditory neurons following *in vivo* stimulation is not known. But, Fos induction following excitation of neurons seems to involve calcium influx (Kovacs, 1998) and is dependent on the temporal pattern of stimulation (Yang et al, 2005). The exact function of this gene is still unknown but it might be important for the stabilization of long-term memories (Clayton, 2000; Guzowski, 2002). Despite our incomplete understanding of this gene's function, Fos labeling is an excellent tool and has been widely used as a marker of neuronal activation.

## MATERIAL & METHODS

### Animals

CBA/J adult male mice (n=19), 2-6 months old, from Jackson Labs (Bar Harbor, ME) were used in these experiments. This particular strain has excellent hearing and has been used as a standard in auditory research (Willot, 2001). 16 animals were stimulated with tone bursts (two at each sound level used). In addition, to determine the basal level of expression in the auditory pathway, 3 animals were subjected to the same procedure except that they were not stimulated. The animals were housed in the animal facility of Massachusetts Eye and Ear Infirmary, 3-5 per cage, on a 12h-light/12h-dark cycle, with food and water *ad libitum*. On the afternoon before the experiment day, each mouse was put into an individual cage, with food and water, inside a sound isolation box. In most cases there was only one animal per isolation box but in some cases there were two animals (each in their individual cage) per isolation box. No differences were found in

Fos labeling between animals that were kept in isolation one per box or two per box. 3-4 animals were used and sacrificed per experiment.

### **Sound stimulation**

On the day of the experiment, each animal was stimulated alone inside an isolation box. Animals were binaurally stimulated while awake and freely moving. The acoustic stimuli consisted of free-field, 12 kHz tone bursts, 14-84 dB SPL, 50 msec duration and 2.5 rise-fall time, at a repetition rate of 10/sec. The tone bursts were generated with a Krohn-Hite oscillator (4031R) connected to an amplifier (Crown D-75) and an attenuator. The sound source was a Radio Shack tweeter (#40-1310B) placed just above the animal's cage (~ 5 inches from cage floor). Free-field sound levels varied  $\pm 10$  dB in different cage locations. Sound calibrations were obtained using a spectrum analyzer (HP 35660A) at the approximate position of the head of the animal with a  $\frac{1}{4}$  or 1 inch microphone. The group of animals used on a particular experiment day and processed as a group included animals stimulated with the same and different sound level.

### **Anesthesia/Perfusion/Fixation**

One hour and 45 minutes after the onset of acoustic stimulation mice were anesthetized with an intramuscular injection of ketamine (~600 mg/kg) and left within the isolation box. 15 minutes later they were perfused with saline (with 0.1% of NaNO<sub>2</sub>) followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer; pH 7.3). The brains of mice were left in fixative for about 2hs, after which they were immersed in 30% sucrose in phosphate buffered saline (PBS) overnight.

### **Histology**

On the morning of the day following the experiment the brains of mice were cut with a freezing microtome into 80  $\mu$ m sections and placed into PBS. The sections were then processed free-floating, with the following solutions (plus interspersed PBS washes): 10% methanol in distilled water with 3% H<sub>2</sub>O<sub>2</sub> (1 hour); blocking solution of 2% RIA grade bovine serum albumin (BSA) in PBS with 0.03% Triton-X 100 (1 hour); primary antibody solution (polyclonal anti-Fos antibody; Santa Cruz Biotechnology, sc-52) of

1:3000 dilution in 1% BSA in PBS (overnight); 0.1% glutaraldehyde in PBS (5 mins); secondary antibody solution (biotinylated donkey anti-rabbit antibody; Jackson ImmunoResearch) of 1:200 dilution, 1% BSA in PBS (1 hour); avidin-biotin-horseradish peroxidases (ABC kit; Vector Labs) (1 hour); 0.15 g diaminobenzidine in 300 ml 0.1 phosphate buffer (pH 7.3), with 0.01% H<sub>2</sub>O<sub>2</sub> (until Fos labeling became visible; usually ~3 minutes). Sections were then washed in PB, mounted on subbed slides, dehydrated, and coverslipped with permount. The primary antibody used was a polyclonal antibody (it recognizes more than one epitope) and this reactivity should be termed Fos-like immunoreactivity (FLI). For simplicity we will call FLI the “Fos labeling”.

### **Cell counts and data analysis**

Tissue photomicrographs were acquired with a Hamamatsu CCD camera on a Nikon microscope. These photomicrographs were processed (background subtraction and normalization of gray scale) and thresholded using the program Metamorph. The threshold was set to be above background darkness and to detect medium-to-dark labeled cells, but not lightly labeled cells (set at ~60% of darkness scale).

A region of interest was manually drawn and the number of labeled neurons inside the region of interest was automatically counted. Additionally, the size (area, width and height) of labeled nuclei and the darkness of labeling were measured. The region of interest for the different nuclei included: the whole DCN except the strial corner; the whole AVCN except the superficial layer and subpeduncular corner; layers III and IV of dorsal cortex and central IC; and primary auditory cortex. First we looked at Nissl stained material to define the boundaries of the regions of interest in these nuclei. In Fos labeled sections, DCN and AVCN could be clearly distinguished from neighboring regions by differences in contrast. The medio-ventral border of IC was surrounded by the periaqueductal gray and was clearly distinguishable from this structure by differences in contrast. The ventro-lateral border of the region of interest in IC was positioned at ~ 100 μm of the free edge of the nucleus in order to exclude the lateral nucleus and layers I-II of dorsal cortex. Auditory cortex regions were as defined in Franklin et al, 1997 and Iversen, 2001. The exact borders of AI could not be clearly distinguished but a region of interest was defined using several anatomical landmarks. Counts of AI labeling were

restricted to sections where the medial geniculate was seen and the hippocampus spread over the entire dorso-caudal axis. The region of interest was 300  $\mu\text{m}$  wide along the rostro-caudal axis and 600  $\mu\text{m}$  high along the dorso-ventral axis in order to exclude the surrounding ventral and dorsal auditory cortices. The region of interest was placed at a distance of  $\sim 100$   $\mu\text{m}$  from the rhinal fissure. Counts included all cortical layers.

In all cases stimulated below 80 dB SPL, a tonotopic band of labeling could be distinguished and a contour could be drawn around it. The shape and position of the band and labeled cell density were slightly different in the most caudal and rostral sections from intermediate sections. Therefore, the most caudal (1-2 sections) and rostral (1-2 sections) sections were excluded from analysis of the position and width of tonotopic band and labeled cell density in DCN. The remaining intermediate sections used amounted to 3-5 in cochlear nuclei in each side, and 4-6 in IC. The position of the tonotopic band of labeling was determined by measuring the distance at midpoint of the lower frequency edge of the band (ventrally located in DCN and AVCN, and dorsally located in IC) to a fixed point (DCN: granule cell lamina; AVCN: ventral free edge of nucleus; IC: lateral free edge of nucleus). The spread of the band along the tonotopic axis was determined by measuring the distance between the most dorsal and most ventral labeled cell in the band. Similarly, the spread of labeling along the medio-lateral axis was determined by measuring the distance between the most lateral and most medial labeled cell in the band. The extent of labeling in the rostro-caudal direction was quantified as the number of sections with labeling (sections were cut along this axis).

Labeled cell density measurements were done by using a rectangular area as an approximation of the area occupied by labeling. Labeled cell density was defined as the number of labeled neurons measured in the rectangle of interest divided by its area. For example, the area of the band of labeling at 14 dB SPL in figure 2.5 was approximated as a rectangle with an area of  $1800 \mu\text{m}^2$  (160  $\mu\text{m}$  wide and 60  $\mu\text{m}$  high).

Deep DCN, fusiform cell layer in DCN, rind of AVCN and granule cell areas were identified in Nissl stained material first. Then, counts of Fos labeled neurons in the equivalent positions in sections without Nissl staining were performed. Measurements of deep DCN were centered at  $\sim 35\mu\text{m}$  from the medial edge of DCN and measurements of fusiform cell layer at  $\sim 25\mu\text{m}$  from the lateral edge. Rind AVCN was restricted to the area



within 20  $\mu\text{m}$  of the free edges of AVCN along the medio-lateral axis. Measurements of core AVCN were centered at the midpoint of the medio-lateral axis of AVCN. To compare rostral and caudal sections of AVCN two clearly distinct positions along the caudal-rostral extent of the nucleus were picked. Caudal sections were positioned at ~90% of the caudal-rostral extent and rostral sections at ~35% of the caudal-rostral extent (similar to Liberman, 1991). Only two of the seven granule cell areas could be clearly distinguished with light microscopy: the superficial layer and the lamina. The medial sheet and islands of granule cells in the cochlear nerve root do not form a continuous layer and were difficult to identify. The exact borders of the subpeduncular and strial corners were also not clear. Therefore we only counted the number of Fos labeled neurons in the lamina and superficial layer. We decided to do manual counts of cells in these two areas because the overall background in the superficial layer surrounding ventral cochlear nucleus can be quite dark and trash particles can accumulate here. Consequently, the signal-to-noise ratio wasn't good enough to do reliable automatic counts with Metamorph. Neurons were counted only if they were medium-to-darkly labeled (clearly darker than DCN processing background), had a round or elliptical shape and were inside the borders of the lamina or the superficial layer. In a few cases, we also made automatic counts to get an idea of the characteristics of the labeled nuclei in these regions (e.g. nucleus area, darkness, etc). Putative granule cells in DCN were counted as part of the tonotopic band.

The data were analyzed and plotted using Matlab, Excel and Adobe Illustrator. Diagrams of labeled sections were done using Adobe Illustrator. For statistical analysis we used the Kruskal-Wallis test (a non-parametric test adequate for analysis of small populations).

## RESULTS

### 1. Tone-evoked Fos labeling in central auditory nuclei

Fig.1.1A shows tone-evoked Fos labeling in dorsal cochlear nucleus (DCN). Fos labeled neurons (thin arrows) are clearly darker than unlabeled neurons (thick arrow) and

single labeled neurons are easily distinguished. Some of the labeled neurons are extremely small (thin arrow, upper left corner).

Stimulation with tones at low sound pressure levels results in narrow bands of labeling as seen for example in IC (Fig.1.1B). Some labeling can be seen outside the tonotopic band but these few scattered labeled cells are generally lighter. Similar levels of scattered, lighter labeling in IC were seen in unstimulated cases. At low stimulation levels narrow bands of labeling are also seen in DCN (Fig.2.2A), anteroventral cochlear nucleus (AVCN) (Fig.2.2B) and auditory cortex (AC) (Fig.2.2D). This band of labeling appears in the lower frequency region of these auditory nuclei consistent with the known tonotopic map of the mouse (Stiebler et al, 1985; Stiebler et al, 1997; Ehret et al, 1991; Friauf, 1994; Iversen, 2001; Müller et al, 2004). Stimulus-evoked Fos labeling can be seen in DCN, IC and AC with tone bursts as low as 14 dB SPL. In contrast, Fos labeling in AVCN is only evoked with tone bursts starting at 24 dB SPL. Overall, we observed that 12 kHz tone bursts evoke more Fos labeling in DCN than AVCN. Unstimulated animals have very low numbers of Fos labeled neurons in these auditory nuclei in particular in cochlear nuclei (Fig.2.3). Some labeling was seen in the superior olivary complex and in the nucleus of the lateral lemniscus. Only rarely was labeling seen in the medial geniculate nucleus.

## **2. Spreading of labeling along the tonotopic axis and appearance of secondary bands**

Increasing sound pressure level leads to an increase in the total number of Fos labeled neurons in DCN, AVCN, IC and AC (Fig.2.3 and 2.4). Counts of With increasing sound level, the band of labeling becomes wider and spreads along the tonotopic axis at all levels of the pathway (Fig.2.2). The spread was dorsally in DCN and AVCN or dorso-laterally in IC towards higher CF regions. Little spread was seen ventrally or ventro-medially respectively (Fig.2.5).

The spread of labeling in AC was not quantified because we could not define an adequate landmark to determine the precise position of the lower frequency edge of labeling. Nevertheless, spreading of labeling towards higher CF regions with increasing sound level can be clearly seen in this nucleus (Fig.2.2D).

At moderate stimulus levels (44-54 dB SPL) a new, secondary narrow band of labeling appears around the 24 kHz region in DCN and IC (thin arrows in Fig. 2.2B, C). This secondary band is not observed in all animals stimulated at these sound levels. At higher stimulus levels other secondary bands are observed (Fig.2.2B, D). At the highest stimulus levels used the pattern of Fos labeling becomes widespread and clear secondary bands cannot be distinguished. In AC, even at moderate sound levels, clear secondary bands of labeling are not seen (Fig.2.2D). The pattern of secondary bands in DCN and IC is similar to each other. In contrast, in AVCN spread of labeling towards higher CF regions is only observed at relatively high sound levels.

No acoustic distortions are seen in the stimulus that could account for the secondary band of labeling observed at 54 dB SPL (Fig.2.6A). The acoustic distortions seen at and above 48 kHz, at 74 dB SPL (Fig.2.6B), are below the neural auditory threshold of CBA/J mice (Taberner et al, 2005) and it seems unlikely that they are responsible for the secondary bands seen at this sound level.

### **3. Cell density within the area of the tonotopic band**

At 14 dB SPL the area of the tonotopic band was approximated to a rectangle and the number of labeled neurons inside this rectangle was counted. Labeled cell density was calculated by dividing the total number of labeled cells by the area of measurement. At higher sound levels labeling was measured within the same area and at an equivalent position. Up to 44 dB SPL increasing the level of our stimulus led to an increase of the labeled cell density in the area of the tonotopic band in DCN (Fig.2.7). Labeled cell density in this area seems to reach a limit at 44 dB SPL. A similar effect was observed in AVCN (data not shown). In IC and AC the band of labeling is not rectangle-like. In these regions labeled cell density was measured within a curved contour drawn around the tonotopic area. Similarly results were obtained.

### **4. Spreading of labeling perpendicular to the tonotopic axis**

Increasing sound pressure level also leads to spread of Fos labeling along the two axes perpendicular to the tonotopic axis. The width of the band of labeling in DCN was defined as the distance between the most medial and most lateral labeled cells in the

band. In IC the width of the band (oriented dorsomedial-to-centrolateral) was similarly calculated. Increasing the sound level led to an increase of the width of the band of labeling in DCN and IC (Fig.2.8), with labeling spreading mostly towards lateral or ventrolateral regions. The width of the band of labeling in AVCN was not measured, because below 44 dB SPL most labeling is actually seen in rind areas. The rind was defined as the area within 20  $\mu\text{m}$  of the lateral and medial edge of AVCN. At higher sound levels more labeling was seen in core areas.

The spread of labeling along the rostro-caudal axis was measured by counting the number of sections with labeling. It was observed that below 34 dB SPL the most caudal and most rostral sections of DCN, AVCN and IC did not have labeling (Fig.2.9A). In contrast, at higher stimulus levels Fos labeling is seen practically in the totality of the rostral-caudal extent of these nuclei (Fig.2.9A, B).

In AC, the overall pattern of labeling becomes more widespread with increasing sound level and more labeling is seen in directions perpendicular to the tonotopic axis (Fig. 2.2D).

### **5. Fos labeling in areas highly innervated by low spontaneous rate fibers**

The fusiform cell layer in DCN, rostral AVCN and rind areas of caudal AVCN are highly innervated by low and medium spontaneous rate fibers (Liberman, 1991; Liberman, 1993) and seem to contain more high threshold units (Ghoshal et al, 1996). Therefore, we could expect to see more Fos labeling in these areas with increasing sound level.

We determined the density of Fos labeling in equivalent areas in deep and fusiform cell layer in DCN (Fig.2.10A). At low stimulus levels (14 dB SPL) significantly more labeling is seen in deep DCN than in the fusiform cell layer, with virtually no labeling in the second (Kruskal-Wallis test;  $*p < 0.05$ ) (Fig. 2.10A). At and above 34 dB SPL, the density of labeling in these two areas is very similar.

In AVCN, we determined the density of Fos labeling in caudal and rostral sections in a restricted area of AVCN. The measured area spanned ~15% of the total height of AVCN (~maximal spread of labeling) and its ventral limit was the lower frequency edge of the band. Caudal sections are at ~90% of the caudal-rostral extent and rostral sections

at ~35% of the caudal-rostral extent. At low stimulus levels (24-34 dB SPL), we see there is less Fos labeling in rostral AVCN when compared to caudal AVCN (Fig.2.10B), while at higher sound pressure levels the two areas have similar values of labeled cell density.

Finally, we compared Fos labeling in rind and core areas of AVCN. Measurements of labeled cell density in the rind of AVCN include the medial and lateral rind, within 20  $\mu\text{m}$  of the edge. Measurements of the core of AVCN correspond to a central area spanning 60  $\mu\text{m}$  in width and with the same height as rind density measurements. The rind of AVCN clearly shows Fos labeling at low sound levels (24 and 34 dB SPL) (Fig.2.10C and D). In fact, at these low stimulus levels the majority of labeling is seen in rind areas, in particular in caudal sections.

## **6. Fos labeling in granule cell areas in cochlear nucleus**

There is virtually no Fos labeling in granule cell areas of unstimulated animals and of animals stimulated with 14 dB SPL tone bursts (except possibly as part of the tonotopic band in DCN). At 24 dB SPL, a few Fos labeled neurons are seen in the lamina, superficial layer, and medial sheet of cochlear nucleus. At moderate sound pressure levels abundant labeling is seen in these regions (Fig.2.11A). The superficial layer and the lamina can be easily distinguished in the mouse with light microscopy and Fos labeling counts were obtained in these areas (Fig.2.11B). The number of Fos labeled neurons in these two granule cell domains increases as a function of sound level. In the subpeduncular and strial corners labeling was only observed starting at 44-54 dB SPL. Fos labeling in granule cell domains does not form a tonotopic band, with the exception of possible labeled granule cells part of the 12 kHz band of DCN and labeling in the superficial layer close to the 12 kHz band in core AVCN (Fig.2.11A). Some of the labeled neurons seen in granule cell domains were very small, were similar to granule cells identified in Nissl stained material and were thus very likely granule cells (Fig. 2.1A). We can thus infer that neurons in granule cell areas including granule cells respond to sound.

## 7. Population of Fos labeled neurons

The population of Fos labeled neurons in DCN, AVCN, IC and AC is heterogeneous in nucleus size and darkness of labeling (Appendix Figs.2.1 and 2.2). The vast majority of labeled cells have small-to-medium sized nuclei with an area smaller than  $45\mu\text{m}^2$  (Appendix Figs.2.1 and 2.2). At least 80% of labeled neurons have nuclei smaller than  $25\mu\text{m}^2$ . An example of a labeled small cell can be seen in Fig.2.1A (thick arrow). We could not distinguish clear populations of cells as a function of nucleus area or labeling darkness but instead we see a continuum over the range of these features. Most labeled nuclei have a circular shape, as the nuclear width-height ratio of the majority of labeled neurons is relatively close to 1 (Appendix Fig.2.1A and 2.2). In all nuclei, we observed a small tendency of more neurons larger nuclei being recruited with increasing sound pressure level (Fig.2.12).

## DISCUSSION

Overall, we observed that increasing sound pressure level leads to increased neuronal activation in auditory nuclei as measured with Fos labeling. The measured increase in the number of Fos labeled neurons is in part due to spreading of labeling along the tonotopic axis and perpendicular to it. In addition, increasing sound pressure level leads to the recruitment of neurons with higher thresholds within a particular frequency region.

These results suggest that the spatial pattern of neuronal activation in combination with information about the magnitude of population and individual responses (as the total number of active neurons and the individual rate of discharge) could contribute to the encoding of stimulus level.

### Neuronal activation spreads towards higher CF regions with increasing sound level

In the present study we have shown that increasing sound pressure level leads to spreading of labeling along the tonotopic axis, towards higher CF regions in cochlear nucleus, inferior colliculus, and auditory cortex. In agreement with our results, previous studies show that at moderate and high sound levels the spatial response profile of

auditory nerve fibers and cochlear nucleus neurons consists of a broad pattern of excitation biased towards higher CF regions (Kim et al, 1979; Evans, 1981; Shofner et al, 1986; Kim et al, 1989; Kim et al, 1990). The present study suggests that spreading of neuronal activation along the tonotopic axis as a function of sound level is a common phenomenon throughout the auditory pathway from the periphery to the auditory cortex. This spreading of labeling to higher CF regions with increasing sound pressure level could contribute to intensity coding at high sound levels. The idea that spreading of neural excitation contributes to intensity coding is further supported by perceptual studies showing that the presence of a high frequency noise masker impairs intensity discrimination to a tone at high sound levels (Moore et al, 1974; Carlyon et al, 1984).

In Fos studies using narrow noise bursts, spread of labeling to high CF regions is only observed at very high stimulus levels (~100 dB SPL; maybe lower, see figure 7 in Saint-Marie's paper) (Saint-Marie et al, 1999). Noise bands at a particular SPL have an overall lower peak of acoustic energy than pure tones at the same level (SPL is a function of the frequency bandwidth times the overall level). This difference in the overall peak of energy could at least partially account for the differences observed in the spreading of labeling with noise versus tone bursts. In addition, sound-induced basilar membrane displacement is a nonlinear phenomenon and its response differs to tone and noise bursts. Furthermore, it is reasonable to think that less information is contained in the spread of excitation produced by broadband stimuli in comparison to tonal stimuli. Consequently, spread of excitation could have a smaller contribution to intensity coding of broadband stimuli and a bigger contribution for intensity coding of tonal stimuli. Further support of the idea that spread of labeling contributes differently to intensity coding of noise and tones comes from perceptual studies. Viesmeister (1983) showed that intensity discrimination of a band of noise in the presence of a high frequency noise masker is not impaired at high sound levels.

#### Secondary bands in higher CF regions were observed at moderate and high sound levels

As level was increased new bands of labeling appeared in higher CF regions. These secondary bands of labeling cannot be explained by acoustic distortion in the stimulus. The appearance of higher CF secondary bands following stimulation with tone bursts has

been observed in previous Fos studies (Reimer, 1993; Brown et al, 1995; Kandiel et al, 1999). In Reimer's study, the acoustic distortion in the stimulus was measured and it is unlikely that the distortion accounts for the secondary peaks seen at very high frequency regions. Similarly, it has been repeatedly observed that moderate-to-high tone levels evoke multiple secondary higher CF peaks in the spatial profile of auditory nerve fibers' responses (Kim et al, 1979; Evans, 1981; Shofner et al, 1986; Kim et al, 1989; Kim et al, 1990).

Kim et al (1990), have suggested that the secondary peaks seen in the spatial response profile of auditory neurons might correspond to higher-order resonances in cochlear mechanics. It is possible that the secondary bands that we observed in the present study are also due to resonances in cochlear responses. Harmonic distortion has been observed in basilar membrane responses to tones (Cooper, 1998). The largest component of the distortion products was seen at twice the frequency of the stimulus. Interestingly, at moderate stimulus levels the secondary band of labeling that we observed was around 24 kHz, which is twice the frequency of the stimulus. At high sound pressure levels the responses of the basilar membrane to tones are more broadly tuned and the magnitude of the distortions decreases. At this high sound pressure levels we observed that the secondary bands of labeling could not be as clearly distinguish as at moderate sound levels. Inhibition mechanisms might sharpen these secondary bands. The spatial response profile of PVCN units exhibited inhibition in flanking regions at a certain distance away from the frequency of the stimulus (Kim et al, 1990). Moreover, the observed secondary peaks in PVCN were clearer and sharper than in AN. Many DCN and IC units show response inhibition at frequencies near the best frequency (Evans et al, 1973; Rhode et al, 1986; Ehret et al, 1988; Ramachandran et al, 1999).

Interestingly, spreading of labeling with increasing sound pressure level was unidirectional and the lower frequency edge of the band of labeling was the same for all stimulus levels. Central auditory nuclei could use information relating the position of the lower frequency edge of excitation produced by a pure tone to encode its frequency.



### Fos labeled neurons include cells with different thresholds

The density of Fos labeling increases within a particular frequency area as a function of sound pressure level. This finding suggests that labeled neurons within a frequency region include cells with different thresholds. Similarly, the electrophysiological threshold of neurons to sound with a particular best frequency can widely vary (e.g. Taberner et al, 2005). It is possible that some of these neurons activated at higher sound pressure levels are non-monotonic neurons that respond selectively to a small range of amplitudes, as such neurons are seen in DCN, IC and AC (Phillips et al, 1985; Rhode et al, 1986; Ehret et al, 1988).

### Spreading of labeling perpendicular to the tonotopic axis

In the present study, it was observed that most core, central regions show labeling at low sound levels while more peripheral regions tend to only have tone-evoked Fos labeling at higher levels. Fos labeling was only seen in the most caudal and rostral sections of the auditory brainstem starting at relatively moderate sound pressure levels. Furthermore, Fos labeling was only seen in the most lateral, medial and ventrolateral regions of DCN, AVCN, and IC respectively at the same moderate sound levels. These results suggest that above mentioned regions contain more units with higher thresholds. This pattern of labeling is consistent with a previously described tone-threshold map in IC (Stiebler et al, 198; Stiebler, 1986). Stiebler et al found that the lowest tone thresholds are located in the center of a given isofrequency band and that threshold values rise in every direction to the periphery (i.e. towards more caudal, rostral, lateral and medial parts of the IC). Most importantly, the present study strongly suggests the existence of similar tone-threshold maps in DCN and AVCN. In DCN this putative threshold map seems to be very similar to the one found in IC. In AVCN, the threshold map seems to be slightly different. Here, the most caudal and rostral sections also seem to contain more high-threshold fibers, as no labeling is seen in these regions at low sound pressure levels. But in contrast to DCN and IC, more Fos labeling is seen medially with increasing sound pressure level, thus suggesting that more higher threshold neurons can be found here.

It is more difficult to define clear axes in AC. But we observed that the overall pattern of labeling in AC became more widespread with increasing sound pressure level

indicating that spreading of labeling in directions other than the tonotopic axis also occurs here. These results strongly suggest the existence of topographic maps of intensity at all levels of the auditor pathway. Such maps could play an important role in the coding of stimulus intensity.

#### Fos labeling in areas highly innervated by low and medium spontaneous rate fibers

The fusiform cell layer in DCN and rostral sections of AVCN are highly innervated by low and medium spontaneous rate fibers (Liberman, 1991; Liberman, 1993). Fusiform cells receive additional input from all types of fibers in their basal dendrites. These two areas only show Fos labeling starting at relatively moderate sound pressure levels. The rind of AVCN is another area highly innervated by low spontaneous rate fibers. Interestingly, at low stimulus levels most labeling in AVCN was actually seen in the rind of AVCN, in particular in caudal sections of this nucleus. These results suggest that the fusiform cell layer in DCN, and rostral AVCN might contain units with particularly high thresholds. These areas could play a special role in the processing of acoustic stimuli at higher sound pressure levels.

The overall pattern of labeling in AVCN is very similar to the pattern of innervation of low and medium spontaneous rate fibers in AVCN (Liberman, 1991). A possible explanation for this similarity is that Fos is essentially produced in neurons that receive inputs from units with low and medium spontaneous rates. *In vitro* studies, using cells that had no spontaneous activity, have shown that maximal Fos expression is produced by short bursts of activity repeated at short intervals (Fields et al, 1997). These results, suggest that Fos induction depends on the occurrence of a cellular event that is distinctly above background. Moreover, areas with high levels of neuronal activity such as the visual cortex do not show significant Fos expression (Kovacs, 1998)

#### Fos labeling studies show that neurons in granule cell areas respond to sound

Sound stimulation led to the production of Fos in neurons within granule cell areas. Sound-evoked Fos labeling in these areas has been previously observed (Rouiller et al, 1992; Sato et al, 1992; Adams, 1995; Brown et al, 1995; Carreta et al, 1999; Yang et al, 2005). In addition, in the present study it was observed that the number of Fos labeled

neurons in granule cell domains is a monotonic function of sound level. Fos labeling in the subpeduncular and strial corners is only seen starting at moderate stimulus levels suggesting that these areas might be particularly important for processing sounds at higher levels. Some of the labeled neurons seen in these areas were very likely granule cells suggesting that these cells respond to sound. Sound-evoked Fos labeling in granule cells has been previously reported (Adams, 1995; Brown et al, 1995; Yang et al, 2005).

#### Population of Fos labeled neurons

The population of Fos labeled neurons is heterogenic relative to cell size as measured by nucleus area. The size of nuclei is likely to be positively correlated with the total size of cells suggesting that the majority of labeled neurons are of small-to-medium size. Similar results have been observed in previous studies (Adams, 1995; Brown et al, 1995; Yang et al, 2005).

At high sound pressure levels, there was a tendency to see an increase in the relative number of Fos labeled neurons with bigger nuclei. A similar phenomenon has been observed in Yang et al's paper (2005). These results could suggest that a particular group of larger neurons might only be active at high stimulus levels. An alternative explanation is that this phenomenon is due to a dilution effect. At low sound levels the amount of Fos protein inside a big nucleus might be too diluted to be detected. At higher sound levels more Fos might be produced and the concentration of the protein inside bigger cells would reach the threshold of detection.

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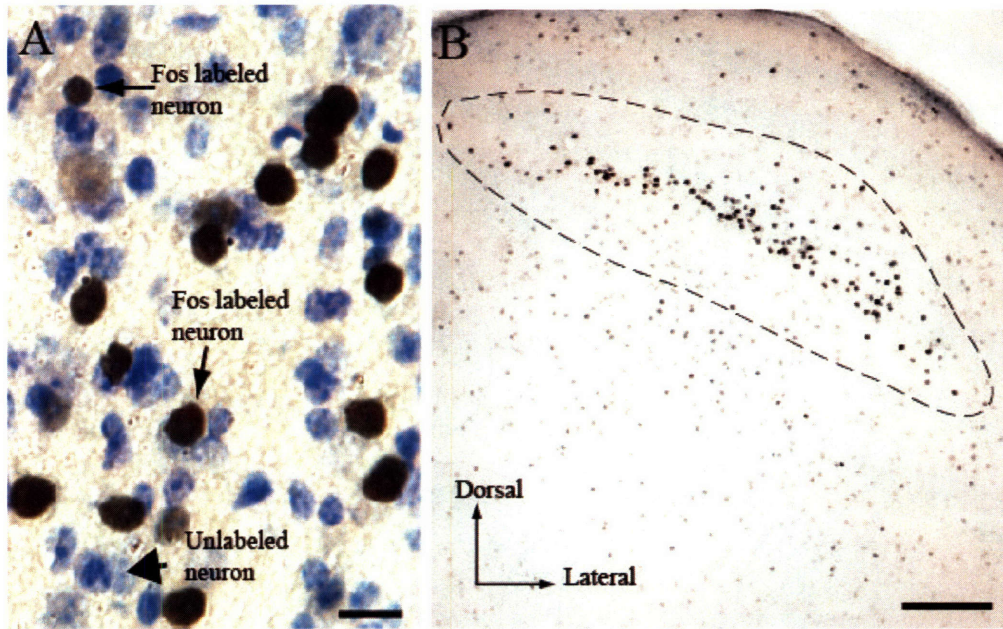


Figure 2.1. Photomicrographs of Fos labeled neurons. A: Tone-evoked Fos labeling in dorsal cochlear nucleus. This section was counterstained with Nissl. The stimulus was a 25 kHz tone burst at 85 dB SPL. Thin arrows indicate reaction product in the nucleus of labeled neurons. Labeled neuron in upper left corner is very small, and could be a granule cell. Thick arrow indicates one unlabeled neuron. Scale bar, 10  $\mu$ m. B: Band of Fos labeling in inferior colliculus evoked by a 12 kHz tone burst at 14dB SPL. The location of the band of labeling is consistent with tonotopic map of inferior colliculus. This section, like most of the material of the study, was not counterstained. The threshold for counting cells was set to detect only medium-to-darkly labeled neurons like the ones in the circled area. Scale bar, 100  $\mu$ m.

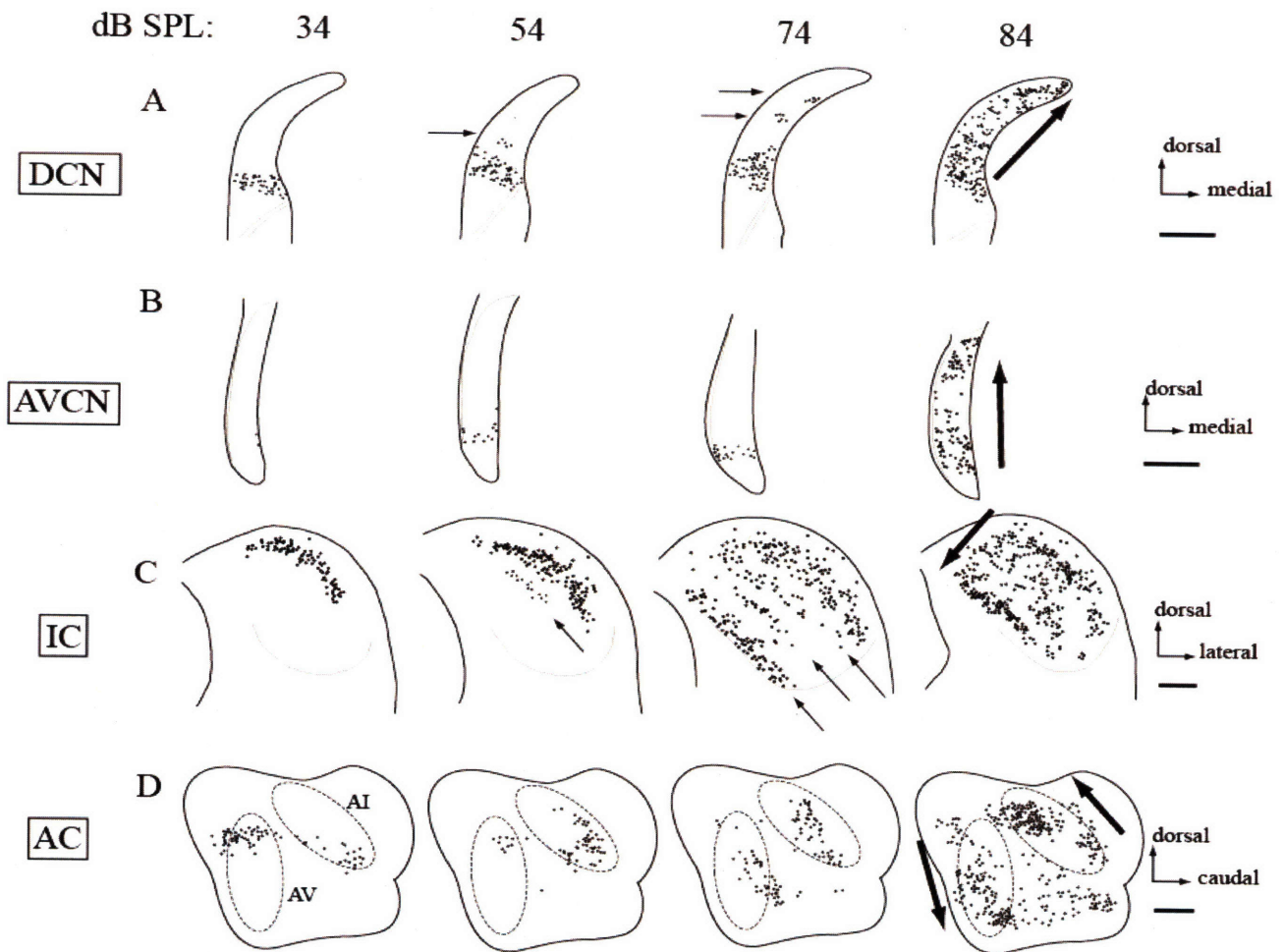


Figure 2.2. Drawings of representative sections from mice stimulated with 12 kHz tone bursts at 34, 54, 74, and 84 dB SPL. Each dot represents one Fos labeled neuron. Auditory cortex regions as defined in Iversen, 2001 (AI: primary auditory cortex; AV: anteroventral cortex). Additional labeling in the granule cell domains in cochlear nucleus, in periaqueductal gray and perirhinal cortex was not drawn. Thin arrows indicate appearance of bands in higher CF regions (secondary bands). Thick arrows indicate the low-to-high frequency map of the nucleus. Scale bar, 250  $\mu$ m.

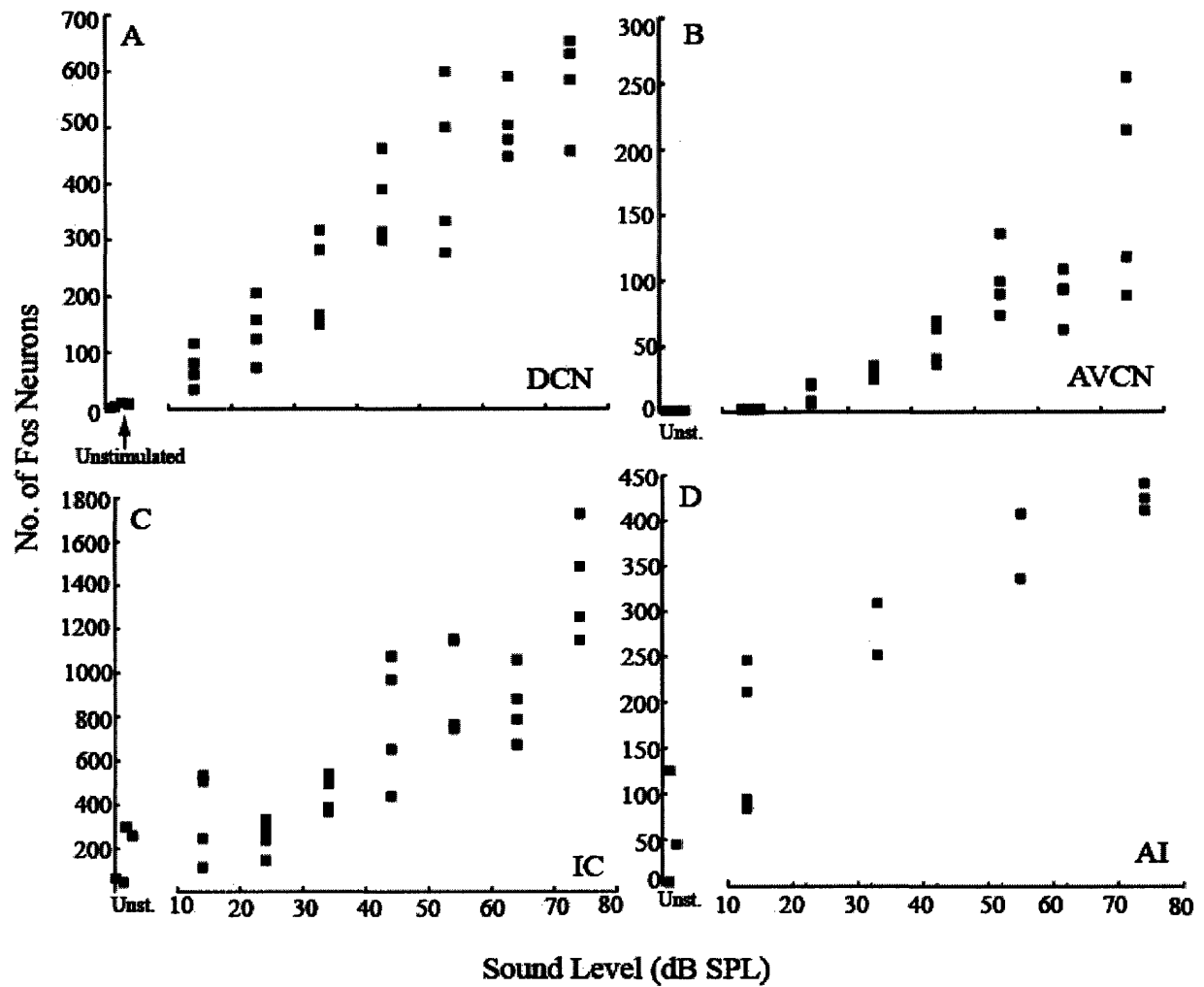


Figure 2.3. Total Number of Fos labeled neurons in DCN (A), AVCN (B), IC (C) and AI (D) as a function of sound level. Each data point represents one side (entire nucleus; left or right) from one mouse.

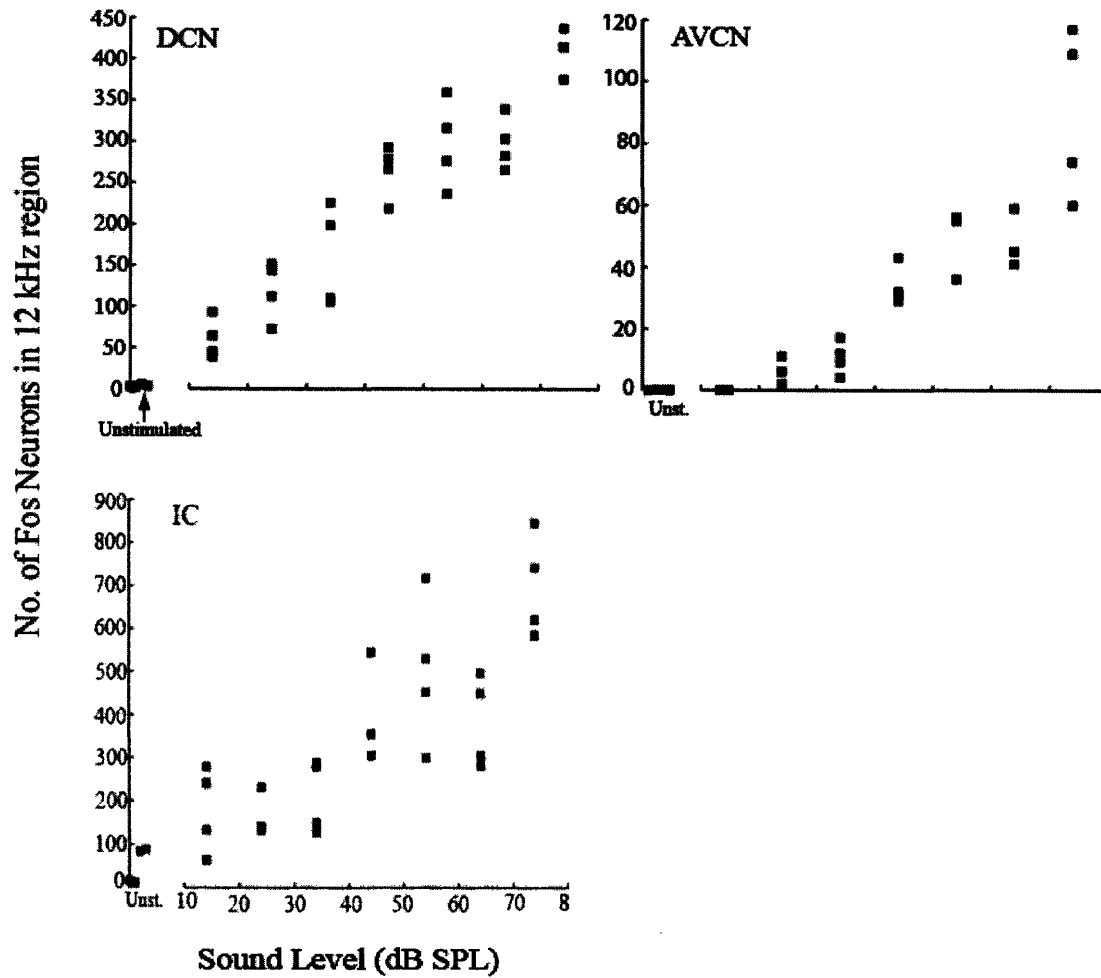


Figure 2.4. Counts of Fos labeled neurons in the 12 kHz region (tonotopic band) in DCN, AVCN and IC as a function of sound level. Each data point represents counts from one side (left or right from one mouse).

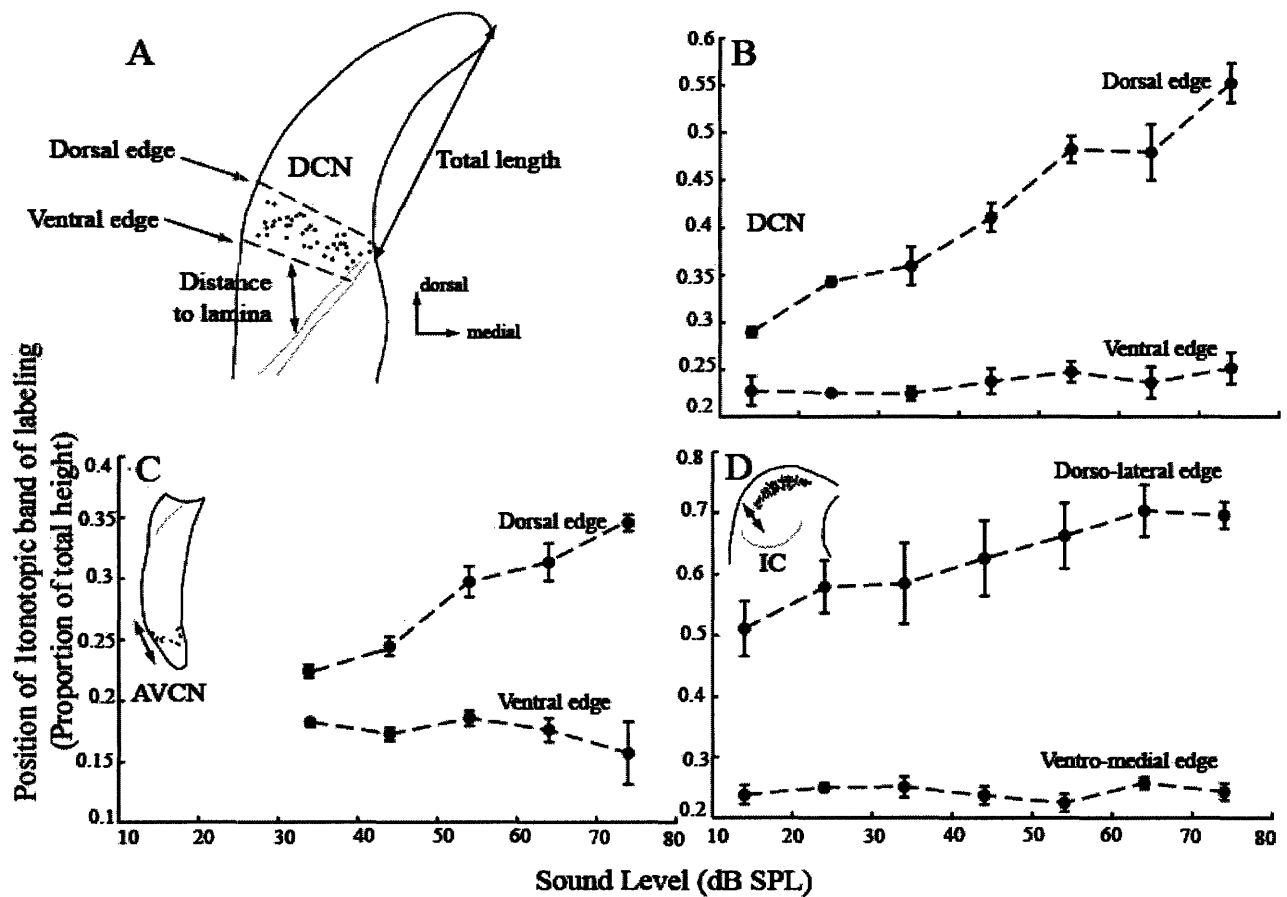


Figure 2.5. A: Diagram illustrating the method used to measure the spread of the tonotopic band of labeling along the tonotopic axis in DCN. A similar method was used for AVCN and IC, except that the position of the lower frequency edge was measured relative to the ventral edge of AVCN, and centrolateral edge of IC. B, C and D: Position (mean  $\pm$  s.e.) of tonotopic band of labeling in DCN, AVCN and IC. Each data point consists of the average of 4 measurements (left side, animal 1; right side, animal 1; left side, animal 2; right side, animal 2). Due to shifts of the position of the band along the rostro-caudal axis the most caudal and rostral sections were excluded from this analysis. 3-5 sections were used for each measurement (1 side). All measurements were normalized relative to the total length of the axis of measurement (in this case the tonotopic axis).

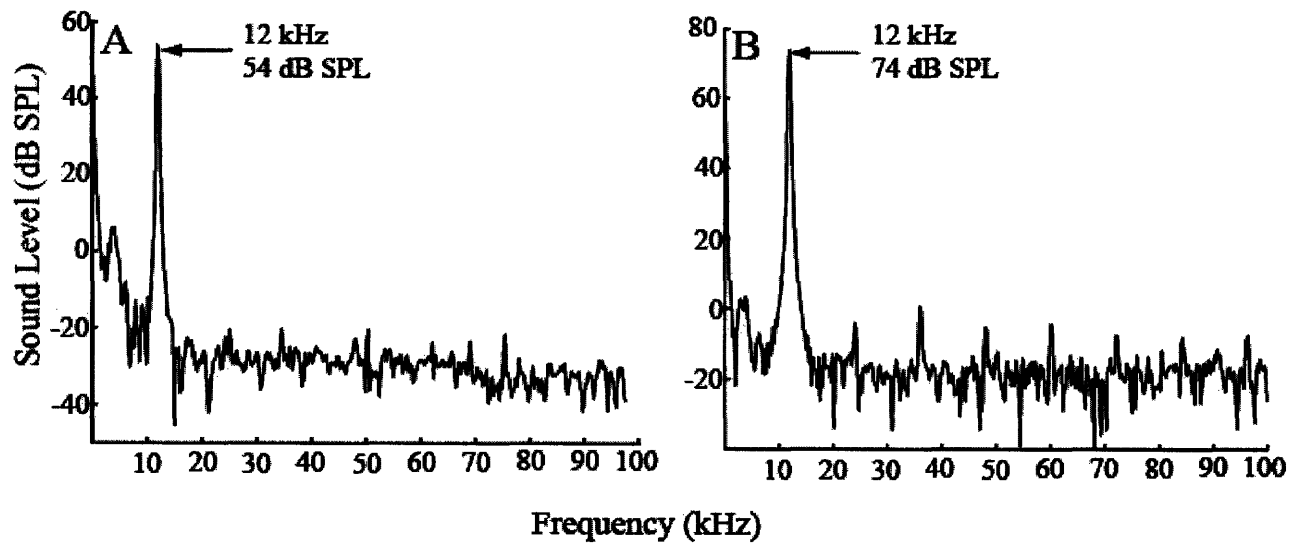


Figure 2.6. Spectrum of sound output for the acoustic stimulus used in the experiments (12 kHz tone).

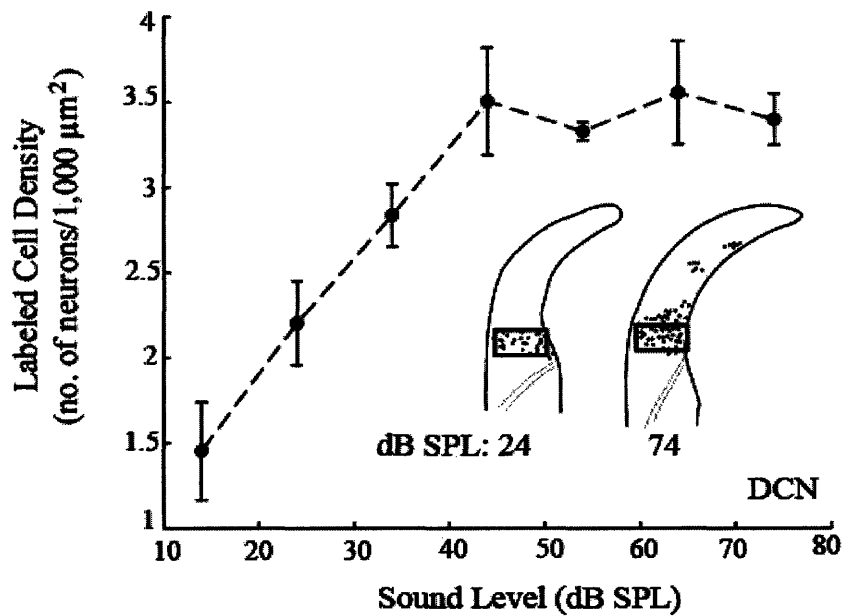


Figure 2.7. Labeled cell density (mean  $\pm$  s.e.) in the area of the tonotopic band as a function of sound level. At 14 dB SPL, the area of the tonotopic band was defined as a rectangle encircling the observed labeling. At higher levels, the number of labeled neurons in a rectangle with the same area and equivalent position was measured. Each data point consists of the average of 4 measurements (as figure 4). Because the shape of the band was slightly different in the most caudal and rostral sections, these sections were excluded from the analysis. Section thickness, 80  $\mu$ m.



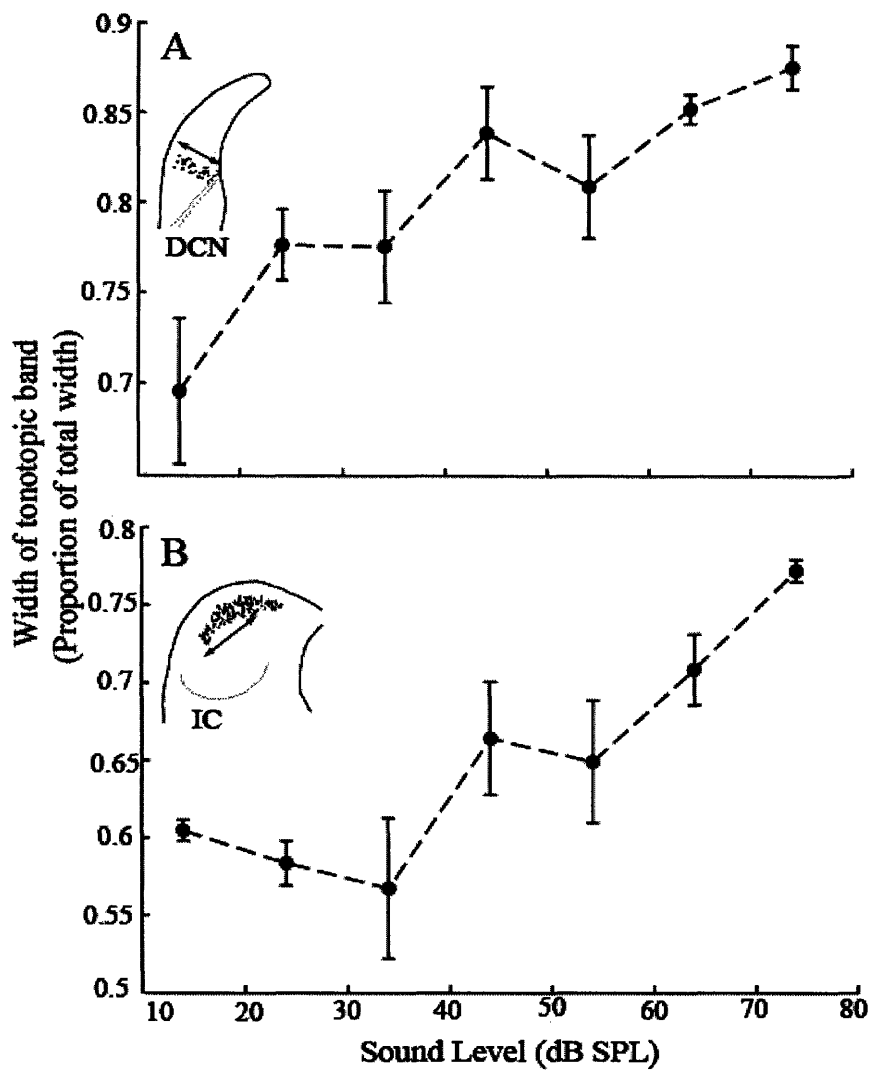


Figure 2.8. Width (mean  $\pm$  s.e.) of the tonotopic band of labeling in DCN (A) and IC (B) as a function of sound level. See figure 4 for details of measurements.

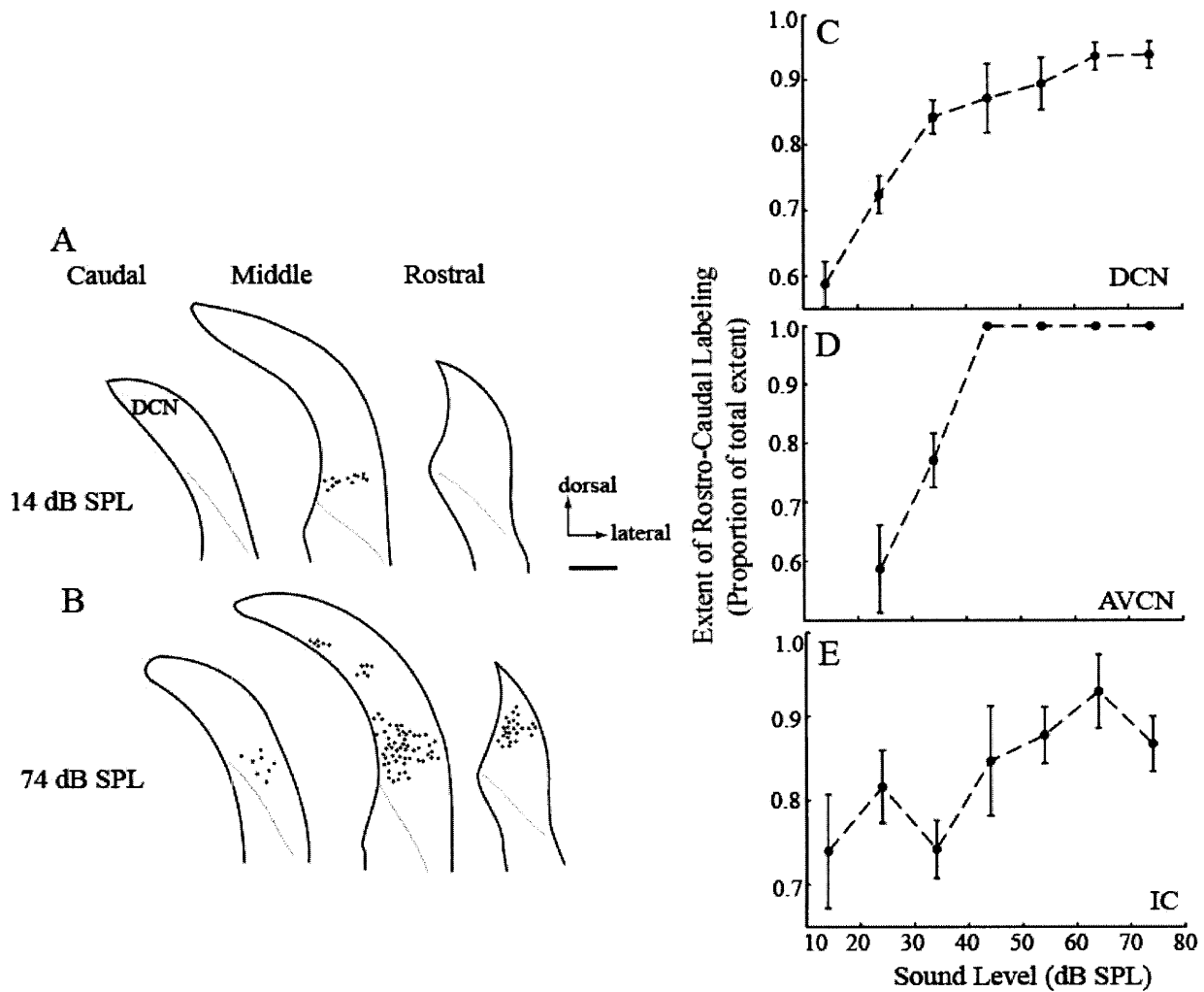


Figure 2.9. A and B: Drawings of representative caudal, middle and rostral DCN sections from mice stimulated with 12 kHz tone bursts at low (A) and high (B) sound level. Scale bar, 100  $\mu\text{m}$ . C, D and E: Extent (mean  $\pm$  s.e.) of spread of labeling in rostro-caudal axis for DCN (C), AVCN (D) and IC (E). Sections were cut along the rostro-caudal axis. Consequently the extent of rostro-caudal labeling was defined as the number of sections with labeling. Each data point corresponds to the average of 4 measurements (as figure 4). Measurements were normalized relative to the total extent of the nucleus.

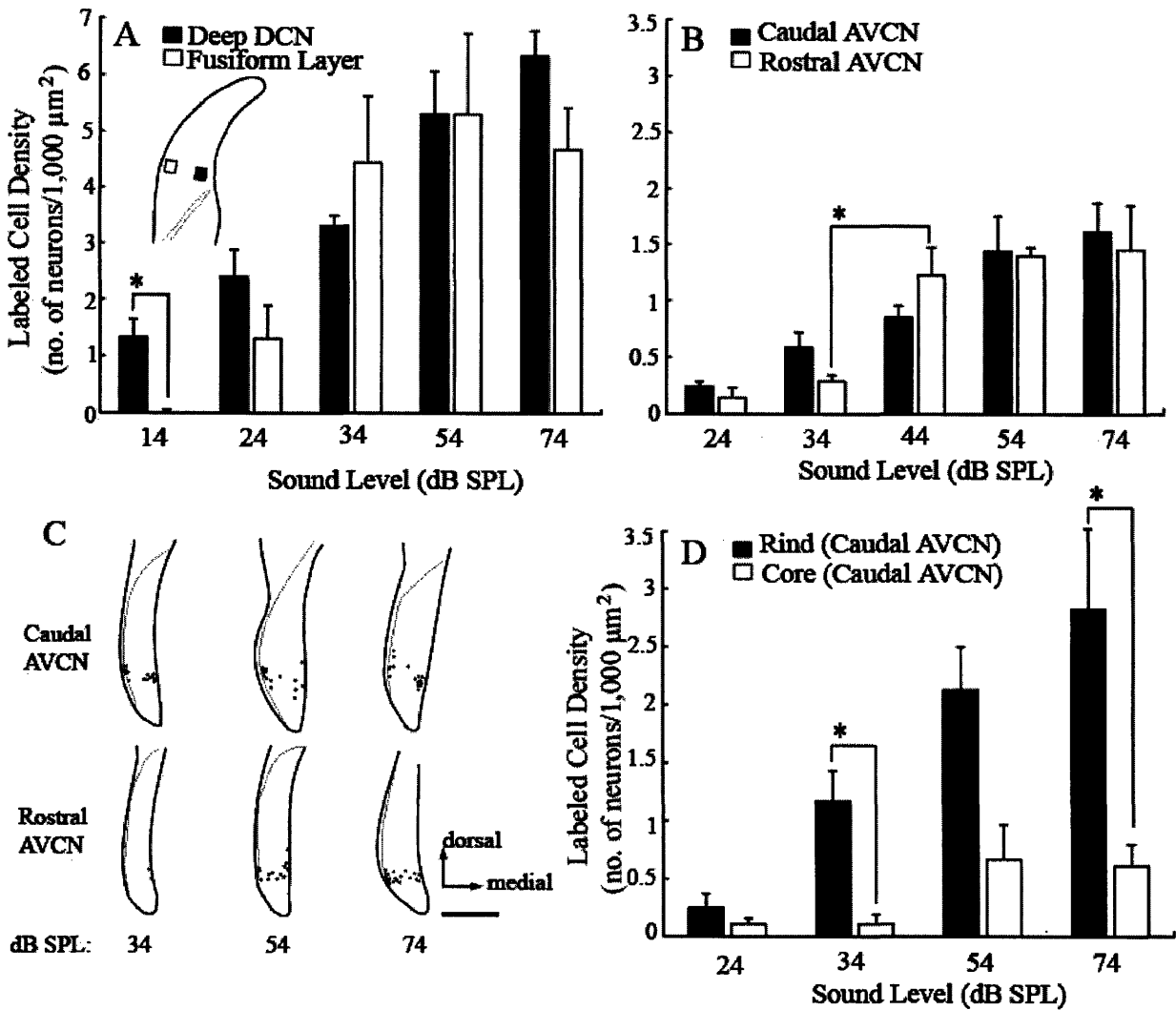


Figure 2.10. Fos labeling in cochlear nucleus areas highly innervated by low spontaneous rate fibers. A: Comparison between density of labeling (mean  $\pm$  s.e.) in deep and fusiform cell layer of DCN. B: Comparison between density of labeling (mean  $\pm$  s.e.) in caudal and rostral AVCN (Caudal AVCN  $\approx$  90% of total caudal-rostral extent. Rostral AVCN  $\approx$  35% of total caudal-rostral extent). Fos labeling was measured in a rectangular area equivalent to the area of the band of labeling at high levels ( $\approx$  15% of total AVCN area). C: Representative drawings of the pattern of labeling in caudal and rostral AVCN at low (34), medium (54) and high (74) sound level. Labeling in granule cell areas (superficial layer and lamina) was not drawn. Scale bar, 250  $\mu\text{m}$ . D: Comparison between density of labeling (mean  $\pm$  s.e.) in rind and core in caudal AVCN. In A, B and D, each data point consists of the average of 4 measurements (as figure 4). Kruskal-Wallis test, \* $p < 0.05$ .

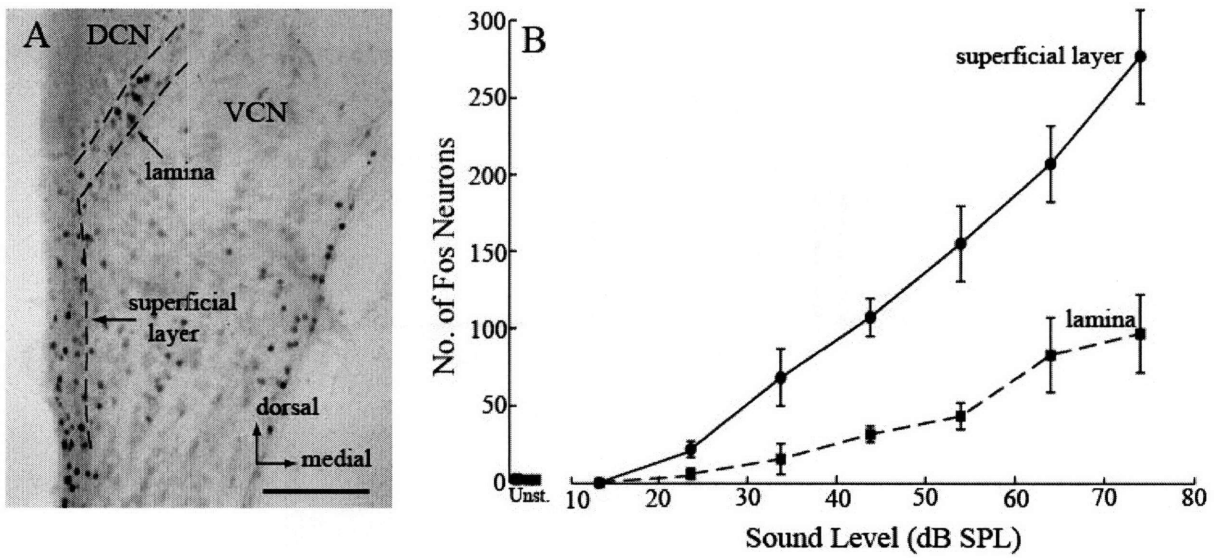


Figure 2.11. A: Representative photomicrograph of Fos labeling in two granule cell domains (lamina and superficial layer) in cochlear nucleus. Animal was stimulated with a 12 kHz tone burst at 64 dB SPL. Scale bar, 100  $\mu$ m. B: Number (mean  $\pm$  s.e.) of Fos labeled neurons in lamina and superficial layer. Counts of the total number of labeled neurons in each region were obtained for each side (left or right) separately of one animal. Data consists of the average of 4 counts (2 animals, 4 sides).

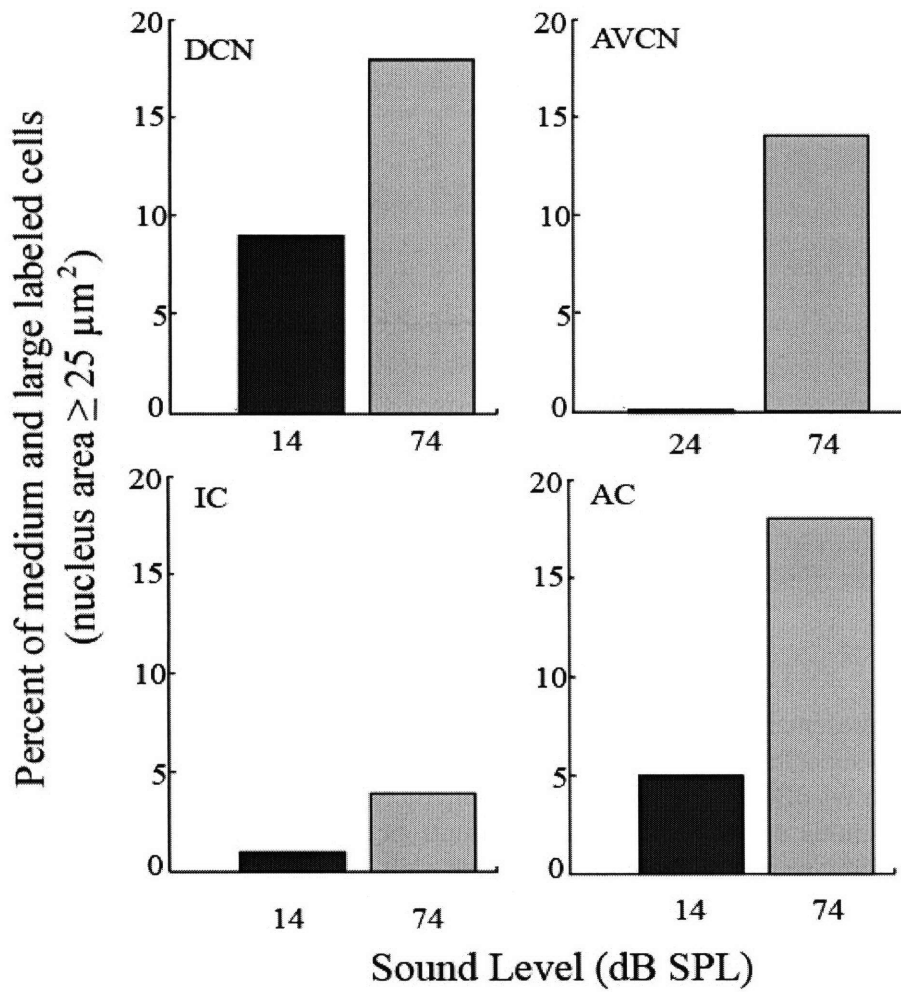
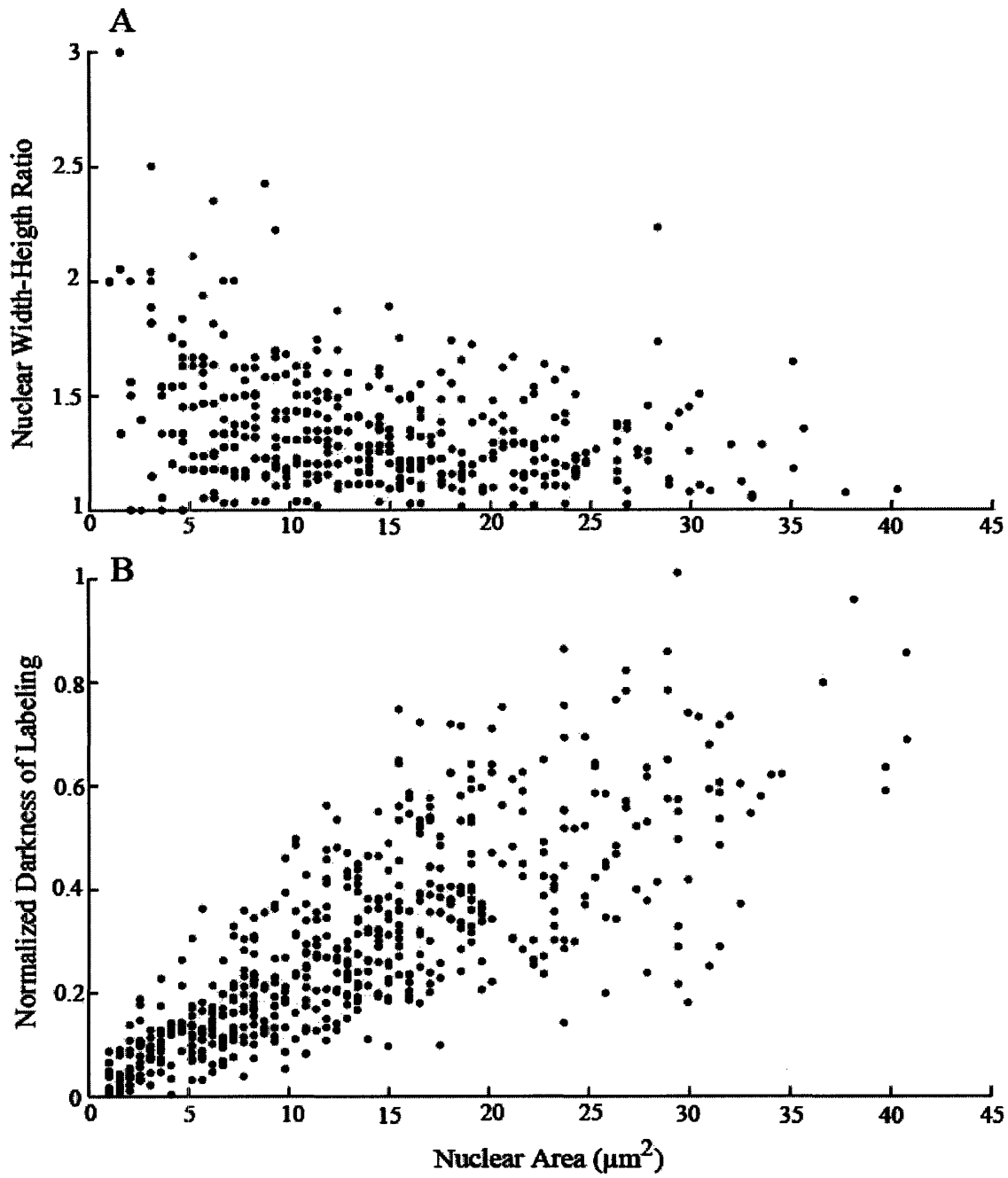
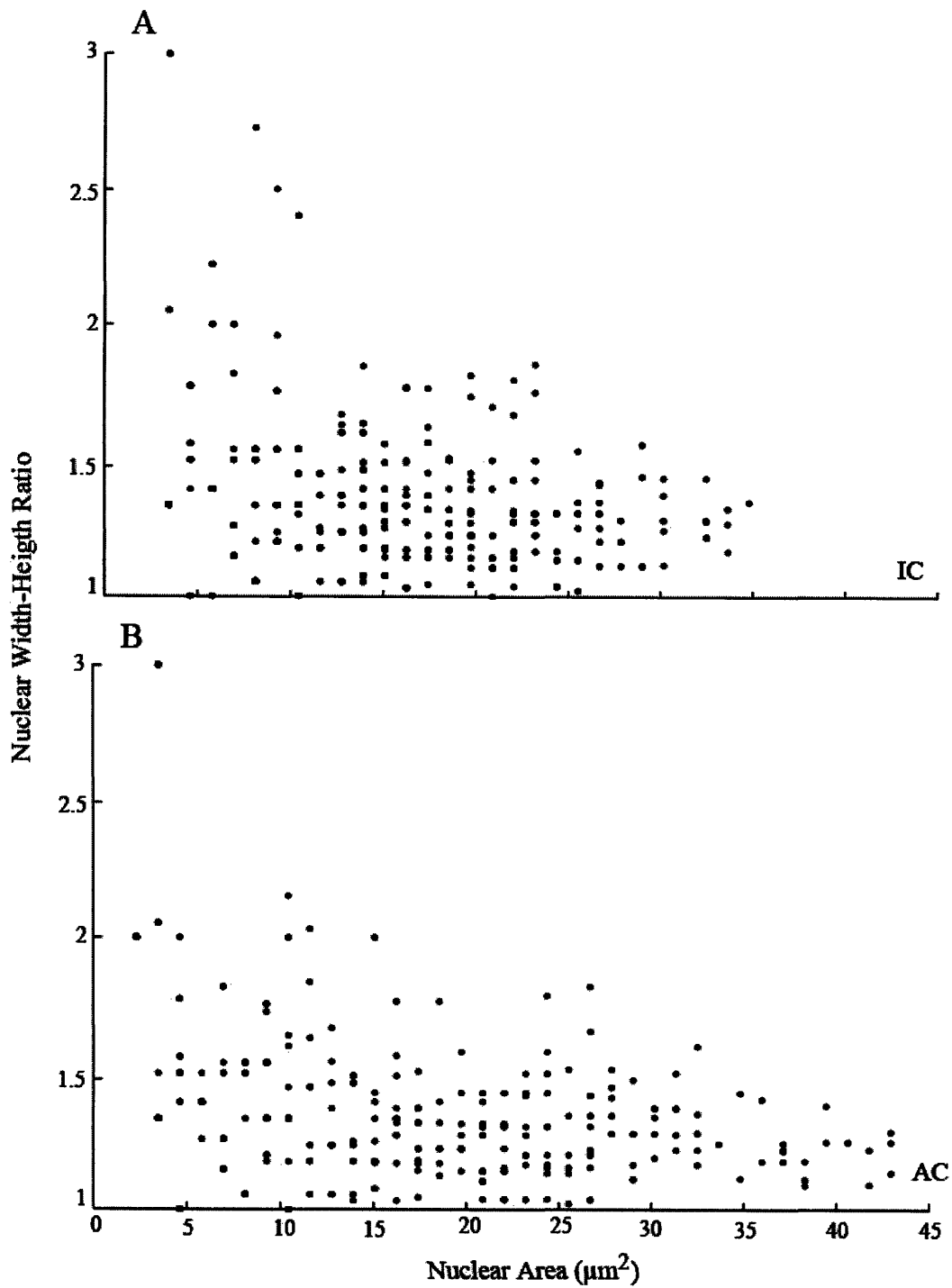


Figure 2.12. Counts of medium and large labeled cells (nucleus area  $\geq 25 \mu\text{m}^2$ ) in DCN, AVCN, IC and AC at low (14 or 24) and high (74) sound level. Counts are plotted as percent of the total number of labeled cells. The remaining labeled cells have nucleus area less than  $25 \mu\text{m}^2$ .



Appendix Figure 2.1. Nuclear width-height ratio (A) and darkness of labeling (B) as a function of nuclear size of labeled neurons. Each dot represents one labeled cell. Data from DCN of mouse stimulated with 12 kHz tone burst at 74 dB SPL. Darkness of labeling was normalized relative to the darkest cell.



Appendix Figure 2.2. Nuclear width-height ratio in IC (A) and AC (B) as a function of nuclear size of labeled neurons. Same animal as in appendix figure 1 (12 kHz, 74 dB SPL).

## **Chapter 3: Effects of auditory fear conditioning on Fos labeling in the auditory pathway, posterior intralaminar nucleus and amygdala**

### **INTRODUCTION**

Learning and memory have been studied with many models in controlled laboratory studies. Fear conditioning is a simple form of associative learning that has been widely studied and that is particularly important for survival (LeDoux, 2000; Maren, 2001). In fear conditioning an animal learns that a neutral stimulus, the conditioned stimulus (CS, e.g. a tone), predicts an aversive event, the unconditioned stimulus (US, e.g. a shock). The animal quickly develops a conditioned response (CR), e.g. freezing, to the CS that was previously elicited by the US only. The CR, in addition to freezing, consists of other behavioral and physiological responses such as increased heart rate and blood pressure, and release of hormones (Blanchard et al, 1969; Fendt et al, 1999; Maren, 2001).

Fear conditioning is critically dependent on the following structures: (1) the CS pathway, which for auditory fear conditioning is the auditory pathway, (2) the amygdala, and (3) brainstem nuclei that produce the CR (Blanchard et al, 1972; LeDoux et al, 1984; LeDoux et al, 1988; Romansky et al, 1992; Campeau et al, 1995; Muller et al, 1997). The exact role of some of these critical brain regions in auditory fear conditioning is still not clearly understood and further research is required (LeDoux, 2000; Paré et al, 2004). The amygdala is required for acquisition of auditory fear conditioning and it is thought to be an important place of CS-US convergence and plasticity (LeDoux et al, 1990a; Muller et al, 1997; Quirk et al, 1995; LeDoux et al, 1990b; Bailey et al, 1999). But several studies suggest that it is not the place of long-term storage of fear memories (Wilensky et al, 1999; LeDoux, 2000; Maren, 2001). The posterior intralaminar nucleus (PIN) is an important input pathway to the amygdala, receives convergent auditory and



somatosensory inputs, and could also play a role in CS-US plasticity (LeDoux et al, 1987; LeDoux et al, 1990b; Cruikshank et al, 1992). The auditory pathway reaches the amygdala from both the auditory thalamus and the auditory cortex. Either pathway by itself can lead to the development of auditory fear conditioning (LeDoux et al, 1990b; Romanski et al, 1993). The auditory cortex might play a larger role in conditioning to complex paradigms (Jarrell et al, 1987). Following conditioning, several physiological changes have been observed in the auditory pathway including increased evoked potentials, increased metabolic activity, and electrophysiological changes such as receptive field plasticity and increased representation of the CS (Gonzalez-Lima and Scheich, 1984; Bakin and Weinberger, 1990; Gao and Suga, 1998; Weinberger, 2004; Kilgard and Merzenich, 1998). CS-specific receptive field plasticity has been observed in auditory cortex (Bakin et al, 1990), in medial geniculate nucleus (Edeline et al, 1992), and inferior colliculus (Gao et al, 1998). In the auditory cortex, this receptive field plasticity is associative, rapidly induced, highly specific, and retained in the long term. This evidence suggests that this form of plasticity could constitute some form of physiological memory being stored in the sensory pathway (Weinberger, 1998). In addition, a few studies have shown that this increased representation of the CS frequency in the auditory cortex correlates with an improvement in auditory perception (Recanzone et al, 1993; Rutkowski et al, 2005).

The present study aims to give insight into the role of the amygdala, PIN and auditory pathway for learning and storage of auditory fear conditioning. Electrophysiologic techniques do not allow study of neural responses of many nuclei simultaneously in one animal. In addition, most electrophysiology studies require the use of anesthetics, which might be a hindering factor in understanding higher cognitive functions like learning and memory. Using Fos labeling to assess neuronal activation has several advantages including a high degree of spatial resolution (individual neurons can be distinguished), the ability to study different nuclei of the same animal, and the use of awake animals.

*c-Fos* is the best known immediate early gene. This family of genes is rapidly induced following stimulation and their expression does not depend on new protein synthesis (Morgan and Curran, 1989; Sheng and Greenberg, 1990). The gene *c-fos* encodes the transcription factor Fos. After the Fos protein has been produced in the

cytoplasm it migrates to the nucleus where it forms heterodimeric transcription complexes with members of the Jun family that can function as activators or repressors depending on the identity of the heterodimer (Morgan and Curran, 1989; Sheng and Greenberg, 1990). *In vitro*, *c-fos* expression can be induced by a variety of extracellular signals including neurotransmitters, growth factors and depolarization (Morgan and Curran, 1989; Sheng and Greenberg, 1990; Herdegen and Leah, 1998). *In vivo*, acoustic stimulation leads to production of Fos in auditory neurons in the dorsal cochlear nucleus (DCN), ventral cochlear nucleus, superior olivary complex, nuclei of the lateral lemniscus, inferior colliculus (IC), and auditory cortex (AC) (Ehret and Fischer, 1991; Friauf, 1992; Friauf, 1994; Rouiller et al, 1992; Sato et al, 1993; Adams, 1995; Brown and Liu, 1995; Scheich and Zuschratter, 1995; Iversen, 2001; Keilmann and Herdegen; 1997; Kandiel et al, 1999; Santos et al, 2004). Other studies have shown that fear conditioning leads to an increase in *c-fos* expression in the amygdala (Campeau et al, 1991; Beck et al, 1995; Milanovic et al, 1998; Radulovic et al 1998). In contrast, to stimulus-induced *c-fos* expression, the basal level of expression of this gene in most of the nervous system is very low (Herdegen et al, 1995).

The objective of this study is to help clarify the role of critical nuclei in auditory fear conditioning. In particular we were interested in studying areas that receive convergent auditory and somatosensory input (amygdala and PIN) and the auditory pathway (DCN, IC, AC). To achieve this aim we studied the pattern of neural activation in these nuclei during acquisition of auditory fear conditioning and following a memory test. Furthermore, we investigated if early stage sensory nuclei like the cochlear nucleus show conditioning-induced changes.

## MATERIAL & METHODS

### Animals

CBA/J male adult (3-6 months old) mice (n=53) from Jackson Labs (Bar Harbor, ME) were used in these experiments. This particular strain has been used as a standard in auditory research (Willot, 2001). It has no history of hearing loss (Henry et al, 1980), or

learning deficits (Bolivar et al, 2001). The animals were housed in the animal facility of Massachusetts Eye and Ear Infirmary, 3-5 per cage, on a 12h-light/12h-dark cycle, with food and water *ad libitum*, until the afternoon before the training session. On the afternoon before training, each mouse was put into an individual cage, with food and water, inside a sound attenuating box.

## **Behavioral training**

### Training session

On the morning of the training session, the mice were moved to and trained in a Coulbourn test cage (H10-11M-TC), inside an isolation cubicle (Coulbourn; H10-24A). Each animal was trained separately. Animals were awake and freely moving inside the training cage. The Coulbourn cage had a floor of metal rods connected to a Coulbourn shocker (H13-15). The conditioning box was thoroughly cleaned with 95% alcohol before each animal was trained. The conditioned stimulus (CS) was a 12 kHz tone burst (50 msec duration; 5 msec rise-fall time; repetition rate of 10/sec), 20 sec duration, 45-60 dB SPL. The CS was generated with a personal computer connected to an amplifier (Crown D-75) and an attenuator and delivered through a Radio Shack tweeter (#40-1310B) on top of the test cage. The unconditioned stimulus (US) was a scrambled footshock, 2 sec long, and 0.7 mA, and its delivery was controlled by the same computer that generated the CS. Five experimental conditions were used (Fig.3.1): (1) Unstimulated (n=3 mice); (2) Shock Only (n=3 mice); (3) Tone Only (n=7 mice); (4) Tone and Shock, Paired (Conditioning) (n=7 mice); (5) Tone and Shock, Unpaired (n=7 mice). The Conditioning paradigm consisted of 36 tone-shock pairings (average inter-stimulus time interval (ISI) of 3 minutes) with the shock being delivered immediately at the offset of the tone. The total duration of the training session was ~1h 45min. All other experimental groups received the same number of tones, shocks, or tones and shocks explicitly unpaired (i.e. shock never occurs when the tone is on) as the conditioning group, except for unstimulated animals, which were put into the training cage for the same amount of time but received no stimulation. The behavior of mice during the experiment was recorded with a digital camera (Sony DCRTRV460). 3-4 animals were

trained on each experiment day (at least one Tone Only, one Conditioning and one Unpaired) and their brains were processed together.

### Memory test

In this study, we wanted to compare the pattern of stimulus-induced gene expression at the time when the animal is learning with a time when long-term memory has been established and most consolidation processes have already taken place. Therefore, we decided to assess long-term memory in a test 4 days after training.

A group of animals that received either the Conditioning (n=5 mice) or the Unpaired paradigm (n=5 mice) was not immediately sacrificed at the end of the training session. They were moved back into Mass. Eye and Ear animal facility in individual cages. 3 days later, in the afternoon, they were moved into a sound attenuating box as before. On the following day (4 days after the training session), they were put into the conditioning box again. They were allowed ~2 mins to habituate to the box and then were given a series of tone presentations (12 kHz tone bursts; 45 dB SPL; 20 sec duration; avg. ISI=3 min; 36 repetitions) without any shocks being given (=Tone Only paradigm). A group of animals (n=5 mice) that had not received any previous behavioral training was given this same stimulation paradigm. The behavior of the animals was assessed as for the training session. As before, 3-4 animals were trained on each experimental day (at least one Tone Only (no previous training), one previously conditioned and one previously stimulated with the Unpaired paradigm) and their brains processed together.

### Scopolamine injections

Acetylcholine is known to be an important modulator of learning and memory processes (Gold, 2003; Kilgard et al, 1998; Weinberger, 2003). Consequently, we decided to inject a group of animals with scopolamine (an anti-cholinergic drug) before training to investigate a possible role of acetylcholine in conditioning induced Fos changes.

A group of animals was injected with scopolamine (intraperitoneally; 1mg/Kg) 10 minutes before training with the Conditioning (n=4 mice), Unpaired (n=2) and Tone Only (n=2) paradigms. One series of animals (1 Tone Only, 1 Conditioning, 1 Unpaired) was

sacrificed immediately after training. The rest of the animals were sacrificed following the memory test. Other steps of the procedure were the same as before. To determine possible effects of stress-induced injections, 3 animals received saline injections and were stimulated with the Tone Only paradigm.

### **Recording and analysis of freezing behavior**

The behavior of the animal during the experiment was recorded with a digital camera (Sony DCRTRV460). The camera was inside the isolation cubicle next to the conditioning box was. In addition, the behavior of the animal could be monitored in real time through a firewire connection to a personal computer. The behavior of the animal was recorded on tape. Later on, this tape was played back and a series of digital videos were acquired (trials: 1, 2, 3, 4, 5, 9, 13, 17, 21, 25, 29, 33) starting 10 secs immediately before the tone and ending at tone offset (total duration=30 secs). Freezing was assessed once every second during the duration of the tone (=20 secs) and in the 10 secs that preceded it by comparing successive frames acquired from the digital video. Freezing on the 10 secs preceding the tone was used as a measurement of contextual freezing. Contextual freezing for Unstimulated and Shock Only cases was assessed at around the time that the tone presentations would normally occur. Freezing measurements are expressed as percent time the animal spend freezing of the total time of the measurement interval (10 secs for contextual freezing; 20 secs for tone freezing). The algorithm to analyze consecutive frames and score freezing was created with Matlab. The main steps of this algorithm consisted of: (1) subtracting two consecutive images; (2) distinguishing between real pixel changes and noise (by converting the difference image into a binary image; 0=noise; 1=significant change; threshold determined empirically); (3) determining the minimal pixel change that corresponds to movement other than respiration and classifying values above that minimal as not freezing (=0) and values below as freezing (=1). Periods of immobility of less than 2 seconds were not counted as freezing. The scoring of freezing with our algorithm was in excellent agreement with manual observations of freezing behavior.

In addition, a few animals (Unstimulated: n=1; Shock Only; n=2; Conditioning: n=4; Unpaired: n=3) were put into a new box (also called new context) at the end of training.

The CS tone was played for 30 secs and freezing was scored by an observer once every 3 seconds while the tone was on.

### **Anesthesia/Perfusion/Fixation**

1 hr and 45 mins after the onset of the training session or the memory test, mice were anesthetized with an intramuscular injection of ketamine (~600 mg/kg) and put into their home cage inside an isolation box. 15 minutes later the animals were perfused with saline (with 0.1% of NaNO<sub>2</sub>) followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer; pH 7.3). The brains of mice were left in fixative for about 2.5 hrs, after which they were immersed in 30% sucrose (in phosphate buffered saline or PBS) overnight.

In order to have an idea of the time course of conditioning-induced changes in Fos labeling we compared the pattern of Fos labeling in animals sacrificed 2 hrs after the onset of training with animals sacrificed 3 hrs after the onset of training. Animals in the later group were kept in silence for 1 hr after the end of training. We did not observe any significant differences in Fos labeling between the two groups. Therefore, in the remaining experiments animals were sacrificed 2 hrs after the onset of stimulation as previously done in other Fos studies in the lab.

### **Histology**

On the morning of the following day brains were cut with a freezing microtome, in 80 µm sections, into PBS. The sections were then processed free-floating, with the following solutions (plus interspersed PBS washes): 10% methanol in distilled water with 3% H<sub>2</sub>O<sub>2</sub> (1 hr); blocking solution of 2% RIA grade bovine serum albumin (BSA) in PBS with 0.03% Triton-X 100 (1 hr); primary antibody solution (polyclonal anti-Fos antibody; Santa Cruz Biotechnology, sc-52) of 1:3000 dilution in 1% BSA in PBS (overnight); 0.1% glutaraldehyde in PBS (5 mins); secondary antibody solution (biotinylated donkey anti-rabbit antibody; Jackson ImmunoResearch) of 1:200 dilution, 1% BSA in PBS (1 hour); avidin-biotin-horseradish peroxidases (ABC kit; Vector Labs) (1 hour); 0.15 g diaminobenzidine in 300 ml 0.1 phosphate buffer (pH 7.3), with 0.01% H<sub>2</sub>O<sub>2</sub> (until Fos labeling became visible). Sections were then washed in PB (20 mins), mounted on subbed slides, dehydrated, and coverslipped with permount. Since the primary antibody

used was a polyclonal antibody (it recognizes more than one epitope) we named this reactivity as Fos-like immunoreactivity (FLI). For simplicity we will call FLI as Fos labeling.

### **Cell counts and data analysis**

Tissue microphotographs were acquired with a Hamamatsu CCD camera on a Nikon microscope. These microphotographs were processed (background subtraction and normalization of gray scale) and thresholded using the program Metamorph. The threshold was set to be above background darkness and to detect medium-to-dark labeled cells but not lightly labeled cells (set at ~60% of darkness scale). One series of animals processed together (1 Shock Only; 1 Tone Only; 1 Conditioning; 1 Unpaired) had very darkly labeled cells and an abnormally high number of labeled cells. This may have been due to some mistake done during processing, like the use of more concentrated primary or secondary antibody solutions. For this series we increased the threshold for counting cells (equally for all animals).

A region of interest was drawn manually and the number of labeled cells inside this region of interest was automatically counted. First we looked at Nissl stained material to define the borders of the regions of interest. We used the protruding and easily distinguishable medial geniculate nucleus (MGN) as an anatomical landmark to identify the location of PIN. We started counting labeling in the PIN in the second or third section after the appearance of MGN and restricted our counts to posterior-intermediate sections where MGN is in contact with the ventricular space. In more rostral sections labeling in the adjacent subparafascicular nucleus could not be clearly separated from labeling in PIN. Counts of the BLA complex include the lateral amygdala (LA) and the basolateral amygdala (BLA). Not much labeling was found in LA. Counts of amygdaloid nuclei were restricted to intermediate sections of amygdala, where both BLA and CE could be seen and clearly distinguished (its contours are surrounded by the external capsule). Counts of the entirety of PIN, BLA and CE were not qualitatively different from counts restricted to the chosen regions of interest. DCN could be clearly separated from the adjacent inferior cerebellar peduncle and granule cell lamina by its contrast. In IC counts were restricted to layers III and IV of dorsal cortex and central IC. The central gray was

clearly distinguished from the central nucleus of the IC by its contrast and this landmark was used to delineate the medio-ventral border of the region of interest. The ventro-lateral border of the region of interest was positioned at ~ 100  $\mu\text{m}$  of the free edge of IC in order to exclude the lateral nucleus and layers I-II of dorsal cortex. The most caudal (1-2 sections) and rostral (1-2 sections) of DCN and IC were excluded from analysis because labeled cell density in these regions was slightly different from more intermediate sections. The remaining intermediate sections used amounted to 3-5 for each side in DCN and 4-6 for each side in IC. In a few cases counts of the entire rostro-caudal extent of these nuclei were done, and no qualitative differences were found between these counts and counts restricted to intermediate sections. Auditory cortex counts were restricted to primary auditory cortex (AI). Counts of AI labeling were restricted to sections where the medial geniculate was seen and the hippocampus spread over the entire dorso-caudal axis. The region of interest was ~300  $\mu\text{m}$  wide along the rostro-caudal axis and 600  $\mu\text{m}$  high along the dorso-ventral axis in order to exclude the surrounding ventral and dorsal auditory cortices. The region of interest was placed at a distance of ~ 100  $\mu\text{m}$  from the rhinal fissure. Counts included all cortical layers.

In animals stimulated with 12 kHz tones a band of labeling was consistently seen in a ventral location in DCN and dorsolateral location in IC. The position of this band of labeling is consistent with the known tonotopic map of the mouse (Stiebler et al, 1985; Mulle et al, 2004). A contour was drawn around this tonotopic band of labeling. Labeled cell density in the tonotopic (CS) band was determined by dividing the number of labeled cells in the band by the area of the band. The spread of the tonotopic (CS) band of labeling was measured by determining the distance between the low frequency edge and high frequency edge of the band of labeling.

The data were analyzed and plotted using Matlab, Excel and Adobe Illustrator. For statistical analysis we used the Kruskal-Wallis test (a non-parametric test adequate for analysis of small populations).



## RESULTS

### 1. Behavior: Freezing at the end of Training

Animals were trained with one of five different paradigms: Unstimulated; Shock Only; Tone Only; Conditioning; and Unpaired (Fig.3.1). The Conditioning paradigm consisted of 36 tone-shock pairings with the shock being delivered immediately at the offset of the tone. All other experimental groups received the same number of tones, shocks, or tones and shocks explicitly unpaired as the conditioning group, except for unstimulated animals, which were put into the training cage for the same amount of time but received no stimulation. To obtain a measurement of freezing to tone for Unstimulated animals and Shock Only cases, a few of these animals were put into a new box at the end of training, stimulated with the CS tone and their freezing behavior was scored by an observer. In all cases, freezing is plotted as the percent of time spent freezing of the total time of measurement interval.

Unstimulated animals and animals trained with the Tone Only paradigm showed virtually no freezing to either tone or context (Fig.3.2). As expected, all animals that received shocks developed some level of freezing to context. There was no statistically significant difference between the level of contextual freezing for Shock Only, Conditioning and Unpaired groups. The conditioning paradigm resulted in significantly higher levels of freezing to the tone at the end of training (Kruskal-Wallis test;  $p < 0.05$ ). To have a clearer measure of conditioning to the tone, a few animals were put into a new box at the end of training and presented with the CS tone. Only conditioned animals froze to the CS tone in the new box (Appendix Fig.3.1). Overall, animals that received the Unpaired paradigm, did not have a statistically significant difference in freezing behavior to tone versus context at the end of training. But, two populations of animals could be identified: one group of animals (about  $\frac{1}{2}$  of total) developed some level of inhibitory conditioning (less freezing to the tone than to the context) (Appendix Fig. 3.2A). Another group of animals (about  $\frac{1}{2}$  of total) had similar levels of freezing to tone and context at the end of training (Appendix Fig. 3.2B).

## **2. Fos labeling in nuclei of auditory-somatosensory convergence (PIN, BLA and CE) following Training**

In Fig.3.3, Fos labeling can be seen in PIN, BLA, and CE of 3 animals stimulated with one of three paradigms (Shock Only, Tone Only and Conditioning). Every darkly labeled dot corresponds to one Fos labeled neuron. Counts of Fos labeling for these nuclei for all animals are presented in Fig.3.4. A differential pattern of Fos production was observed in PIN, BLA and CE for the different training paradigms. Unstimulated animals show very low numbers of Fos labeled neurons in all 3 nuclei. Tone Only animals also show low numbers of labeled neurons in PIN and CE. All animals that receive shocks show high numbers of Fos labeled neurons in the PIN, but animals that received tones and shocks (Conditioning and Unpaired groups) have more Fos labeled neurons in this nucleus than Shock Only cases. A similar pattern of labeling is observed in CE where conditioning and Unpaired cases have the highest numbers of Fos labeled neurons. Shock Only cases have more Fos labeling than Tone Only and Unstimulated cases but less than Conditioning and Unpaired. Interestingly, the pattern of labeling is similar for all animals that received shocks with most labeling being found in the medial division and the amygdalo-striatal edge of CE. Conditioning and Unpaired groups also show high levels of Fos labeling in other nuclei known to play a role in learning and memory processes (Posterior Thalamus, Suprageniculate Nucleus, Basomedial Amygdala, Perirhinal Cortex). Animals that received tones (Tone Only, Conditioning and Unpaired paradigms) had more Fos labeling in BLA than animals that just received the shock. Only Unpaired animals showed a significant increase of Fos labeling in BLA when compared to Tone Only controls.

We also investigated the hypothesis that the magnitude of change of Fos labeling could be correlated with the level of freezing behavior to the tone. The change in Fos labeling for each conditioned animal was normalized relative to its Tone Only control (animals stimulated on the same day and processed together). For each conditioned animal, we normalized the amount of freezing to the tone at the end of the experiment by subtracting the amount of baseline freezing. Baseline freezing was defined as the average of contextual freezing at trials 5, 9, 13, 17, 21, 25, 29, and 33. Conditioned animals that show more freezing to the tone above baseline have a bigger increase in Fos labeling in PIN (corr=0.93; \*p<0.05) (Fig. 3.5). We do not see a correlation between freezing and

Fos labeling in BLA or CE. In addition, we do not see such a correlation for animals in the Unpaired group.

### **3. Fos labeling in the Auditory Pathway (DCN, IC, AC) following Training**

The pattern of Fos labeling in IC for animals stimulated with either Shock Only, or Tone Only or Conditioning can be seen in Fig.3.3. Counts of Fos labeling for DCN, IC and AC for all animals are presented in Fig.3.4. Unstimulated animals had virtually no Fos labeling in the dorsal cochlear nucleus (DCN) and very low numbers in inferior colliculus (IC) and auditory cortex (AC). Animals stimulated with the Shock Only paradigm also show low, but slightly higher, numbers of Fos labeled neurons in these auditory nuclei. In animals stimulated with tones (Tone Only, Conditioning and Unpaired paradigms) we observe a band/cluster of labeling in DCN, IC and AC in the 12 kHz region consistent with the known tonotopic map of the mouse (Stiebler et al, 1985; Stiebler et al, 1997; Muller et al, 2004) (IC: Fig.3.3F, 3.3I). In addition, there is also some labeling in areas containing higher best frequency (BF) neurons. In animals stimulated with tones, Fos labeling could also be observed in ventral cochlear nucleus, medial nuclei of trapezoid body, and secondary auditory cortical areas.

Conditioned animals showed a significant increase in the number of Fos labeled neurons in DCN (33% increase), IC (78% increase) and AC (70% increase), when compared to animals that just received the tone (Kruskal-Wallis test;  $p < 0.05$ ) (Fig. 3.4). Animals that received the Unpaired paradigm also showed a tendency (not statistically significant) to have higher numbers of Fos labeled neurons in DCN, IC and AC than TO cases. Unpaired cases that froze more to context than to tone at the end of training (inhibitory conditioning) tended to have the bigger increases in Fos labeling of this group, in IC and AC, relative to Tone Only controls (although smaller than Conditioning increases) (appendix fig. 3.2). Unpaired animals with similar levels of freezing to tone and context tended to have either no or small changes (increase or decrease) in Fos labeling in IC and AC when compared to Tone Only cases.

We measured the spread of the band of labeling and the density of labeling in animals stimulated with tones (Tone Only, Conditioning and Unpaired) (Fig.3.6). Measurements of the spread of labeling and labeled cell density in Conditioning and Unpaired cases

were normalized as percent change relative to Tone Only cases. We observed that in conditioned animals labeling spreads further, to higher CF regions, in both DCN (13.9% wider band) and IC (18.4% wider band). The same trend is observed in the Unpaired group. In addition, the labeled cell density in the 12 kHz band in Conditioning animals is higher than in Tone Only and Unpaired animals, in particular in the IC (65.5% increase).

As in PIN, conditioned animals that developed more freezing to tone tended to have bigger relative increases in Fos labeling in DCN, IC and AC (Appendix Fig.3.3). This correlation was not observed in Unpaired cases.

#### **4. Behavior: Freezing at the Memory Test**

A memory test was performed 4 days after the Training Session in a group of animals that were trained with the Conditioning or Unpaired paradigms. Animals were given ~2 minutes to habituate to the conditioning box before any stimulus was presented. After this period none of the animals showed contextual freezing. Then the conditioning tone was presented without any shocks being given (like Tone Only paradigm). Conditioned animals showed freezing to the tone in the initial trials, which was extinguished after the first 4-6 trials. Unpaired animals showed virtually no freezing to the tone. Freezing at trial 2 to the tone was chosen as a representative memory test (Fig.3.7).

#### **5. Fos labeling in PIN, BLA, CE, DCN, IC and AC following the Memory Test**

Conditioning and Unpaired animals sacrificed after the memory test did not show a difference in the number of Fos labeled neurons when compared to Tone Only controls in amygdala (BLA and CE) and auditory nuclei (DCN, IC, AC) (Fig.3.8), despite the fact that conditioned animals clearly froze to the tone in the memory test. In fact, in the AC, we observed that both Conditioning and Unpaired animals tended to have less Fos labeling than Tone Only animals. Interestingly, conditioned animals showed slightly higher numbers of Fos labeled neurons in the PIN relative to Tone Only or Unpaired cases (Fig. 3.8 and appendix Fig.3.4). Overall, the number of Fos labeled neurons in PIN, BLA and CE following the memory test was greatly reduced in comparison to Fos levels in Shock Only, Conditioning, and Unpaired animals following the training session.

## **6. Effects of anticholinergic drug Scopolamine**

To test if the observed conditioning induced changes in Fos labeling could be cholinergic dependent we injected animals with an anti-cholinergic drug (scopolamine) before training. Animals injected with scopolamine (10 minutes before training; intraperitoneal injections; 1mg/Kg) showed almost no freezing in the first 75 minutes of the training session. Towards the end of training animals developed some freezing behavior to tone and context although less than uninjected animals (Appendix Fig 3.5). In addition, animals that received scopolamine injections before training had greatly reduced levels of freezing at the memory test. In contrast, no changes were observed in Fos labeling for this group of animals relative to uninjected conditioned controls (Appendix Fig.3.6). Animals injected with scopolamine before training with the Unpaired paradigm also showed reduced levels of freezing. To test for possible injection-induced effects, a group of animals was injected with saline before training with the Tone Only paradigm (data not shown). This group of animals did not develop freezing to either tone or context. In addition, Fos labeling in saline-injected animals in the areas studied was not different from uninjected Tone Only controls. Furthermore, injections of scopolamine before training with the Tone Only paradigm did not induce freezing behavior nor had an effect on Fos labeling (data not shown). Finally, one group of animals (1 Tone Only, 1 Conditioning, 1 Unpaired) was injected with scopolamine before training and immediately sacrificed at the end of the training session (data not shown). Despite the reduced levels of freezing of the conditioning + scopolamine case, Fos in IC, AC, PIN and CE was still increased relative to the Tone Only control. Overall, these results suggest that conditioning induced changes in Fos labeling are not acetylcholine dependent.

## **DISCUSSION**

In agreement with previous studies, we showed that conditioning results in increased Fos labeling in amygdaloid nuclei (Campeau et al, 1991; Beck et al, 1995; Milanovic et al, 1998; Radulovic et al, 1998; Holahan et al, 2004). To our knowledge, the present

study showed for the first time that animals that receive shocks have high levels of Fos labeling in PIN, supporting the idea that this nucleus is particularly important for processing of somatosensory USs (Cruikshank et al, 1992). Furthermore, and in agreement with previous electrophysiology and metabolic studies, this work showed that auditory fear conditioning leads to an increase of auditory neuronal responses to the CS, as measured with Fos labeling (Gonzalez-Lima et al, 1984; Bakin et al, 1990; Gao et al, 1998; Kilgard et al, 1998; Weinberger, 2004). This conditioning-induced increase in Fos expression could play a role in the formation and stabilization of long-term memory (Clayton, 2000; Guzowsky, 2002).

#### Behavior and effects of control paradigms

Freezing is a widely used and reliable behavioral metric of auditory fear conditioning (LeDoux, 2000; Maren, 2001). In our experiments, animals clearly became conditioned as they consistently developed high levels of freezing to the tone at the end of training. In addition, this paradigm produced long-term memory as conditioned animals also significantly froze to tone in a memory test, 4 days after training.

The Unpaired paradigm led to two different behavioral effects. About half of the animals had similar levels of freezing to tone and context at the end of the experiment while others developed some amount of inhibitory conditioning to the CS. We chose an explicitly unpaired paradigm because truly random paradigms lead to the formation of some degree of excitatory conditioning between CS and US (Rescorla, 2000). Explicitly unpaired paradigms can lead to the development of inhibitory conditioning, particularly if a large number of trials are given (Rescorla, 1967; Weinberger, 2004). In our case, the possibility of development of some level of inhibitory conditioning was regarded as preferable because we would still be allowed to compare very clearly distinct learning situations (excitatory conditioning versus inhibitory conditioning). In contrast, the truly random control might not allow for a clearly distinctive comparison with the conditioned group (lesser degree of excitatory conditioning versus higher degree of excitatory conditioning) and we could expect that it could lead to similar effects physiologically, in particular in the CS (auditory) pathway. We were surprised to observe that in fact the animals that developed inhibitory conditioning tended to have the bigger increases in Fos

labeling in IC and AC in the Unpaired group (although still smaller than conditioning induced changes), while animals with similar levels of freezing to tone and context at the end of training had little or small changes in Fos labeling in IC and AC relative to Tone Only controls. Thus, it is possible that Fos expression is increased not only during excitatory conditioning but also to some degree when the animal clearly learns that the tone predicts the absence of the shock. Further experiments would be needed to test this hypothesis. Alternatively, the increase in Fos labeling that we observe in DCN, IC and AC for Unpaired cases could be due to a more general increase in neuronal excitability. In electrophysiology studies, similar paradigms (sensitization) have been shown to induce general changes in neuronal excitability in Medial Geniculate Nucleus and Auditory Cortex (Bakin et al, 1990; Edeline et al, 1992). Another possibility is that the presence of the shock itself is responsible for this small increase of Fos labeling in DCN, IC and AC in the Unpaired group, as we have observed that shock alone leads to a slight increase in the number of Fos neurons in DCN, IC and AC, and there are direct projections from the somatosensory system to these auditory nuclei (Coleman et al, 1987; Li et al, 1997; Fu et al, 2003). In addition, stimulation with shocks leads to activation of the reticular system, which has been shown to increase metabolism in auditory nuclei (Gonzalez-Lima et al, 1984). Nevertheless, the number of Fos labeled neurons in DCN, IC and AC for Shock Only cases is low, similar to Unstimulated cases. Thus, we can conclude that somatosensory input alone does not evoke much Fos labeling in these 3 auditory nuclei. In contrast, all animals stimulated with tones showed a clear band of labeling in the 12 kHz region (CS frequency).

Anatomical, electrophysiological and lesion studies suggest that PIN plays a role in auditory-somatosensory convergence

Our results strongly support the hypothesis that cells in the PIN play an important role in the processing of shock information because only animals that received shocks (Shock Only, Conditioning and Unpaired paradigms) had Fos labeling in this nucleus. Our results further suggest that this nucleus is part of the CS-US convergence pathway since animals that received tones and shocks (Conditioning and Unpaired paradigm) had more Fos labeling than animals that just received the shocks. Furthermore, for individual animals

the magnitude of Fos labeling increase in this nucleus correlated nicely with the strength of the conditioned response measured. The PIN receives both auditory and somatosensory input (LeDoux et al, 1987), projects to the amygdala (LeDoux et al, 1990b), and contains units responsive to both auditory and somatosensory stimuli (Bordi et al, 1994). PIN stimulation paired with an auditory CS is an effective US substitute, thus supporting the idea that this nucleus is part of the US pathway and maybe a site of CS-US convergence (Cruikshank et al, 1992). Electrolytic lesions of the PIN impair fear conditioning due to a disruption of somatosensory processing but this effect seems to be due not just to a lesion of local cell bodies but also to fibers passing through this area (Lanuza et al, 2004).

#### The role of amygdala (BLA and CE) in acquisition and retention of auditory fear conditioning: insights from Fos labeling studies

Many lesion, electrophysiology, pharmacology and genetic studies have showed that the amygdala plays a fundamental role in the acquisition of fear conditioning (LeDoux, 2000; Maren, 2001). But the role of different amygdaloid nuclei in fear conditioning is still unclear (Paré et al, 2004). It was previously believed that the basolateral complex (BLA) was the site of CS-US convergence and plasticity, and that the central amygdala (CE) played a more passive role during acquisition of fear conditioning. Different lines of evidence have suggested that CE has in fact a more active role and could be an important site of CS-US convergence and plasticity during acquisition of fear conditioning (Killcross et al, 1997; Collins et al, 1999; Paré et al, 2004). Increased Fos production in the amygdala correlates with acquisition of fear conditioning, indicating that this gene might be part of the genetic machinery necessary for acquisition and retention of fear memories (Campeau et al, 1991; Beck et al, 1995; Milanovic et al, 1998; Radulovic et al, 1998; Holahan et al, 2004). In this study, we observed that tone activated the most neurons in BLA, while shock activated the most neurons in CE, thus suggesting a differential role of these two amygdaloid nuclei in auditory fear conditioning. Interestingly, animals trained with paradigms that involved “more” learning (Conditioning and Unpaired), and stimulated with tones and shocks, had the highest numbers of Fos labeling in either BLA or CE, in agreement with the idea that the



amygdala is important for learning and a locus of CS-US convergence. Further support comes from previous Fos studies, in agreement with ours, showing that tone-cued fear conditioning evokes high levels of Fos labeling in BLA, while context-shock, shock-context and tone-shock evoke high levels of Fos labeling in CE (Milanovic et al, 1998; Radulovic et al, 1998; Holahan et al, 2004). In contrast, one study (Rosen et al, 1998) observed that animals trained with shocks did not show a difference in Fos labeling in CE when compared to animals stimulated with context only. This discrepancy might be due to the fact that in this later study they measured *in situ c-fos* mRNA, while the other studies used an antibody to detect the Fos protein (same as our experiments). In addition, the handling procedure of the animals for the two experiments was different. Overall, these results support the current thinking that CE plays an important active role during acquisition of auditory fear conditioning. CE might be particularly important for processing shock information, while BLA might play a bigger role in sensory processing.

We observed that Fos production in conditioned animals is not different from Tone Only controls 4 days after training in a memory test. These results are in agreement with a previous study that showed no changes in Fos labeling in context-conditioned animals 5 days after training following a memory test (Milanovic et al, 1998). Interestingly, other studies show that Fos labeling is increased in BLA and CE in context-conditioned animals tested (put into conditioned context) shortly after training (up to 2 days) (Campeau et al, 1991; Beck et al, 1995; Milanovic et al, 1998; Hall et al, 2001; Scicli et al, 2004; Holahan et al, 2004). This increase in Fos labeling in BLA and CE following a short-term memory test was not observed in animals that had been context-sensitized or context-conditioned but tested in a different context. Thus, Fos production correlates with behavioral training during acquisition but not during long-term retention.

#### Conditioning induced changes in the auditory pathway: comparison with electrophysiology and metabolic activity studies

We showed that conditioning induced changes, as measured by an increase in the expression of the IEG *c-fos* are seen in auditory nuclei even at early stages of this pathway such as cochlear nucleus. A few other studies have showed that auditory fear

conditioning can lead to physiological changes in DCN (e.g. increase in the number of units responding to the auditory CS and increased metabolic activity) (Gonzalez-Lima et al, 1984; Woody et al, 1992; McIntosh et al, 1993), but the idea that early stages of sensory processing can be plastic in adulthood is still an early one requiring further evidence. Interestingly, the magnitude of change in Fos labeling in DCN was smaller than in IC and AC, suggesting a diminished preparedness of this nucleus for plasticity.

Lesions (pre- and post-training) of the IC prevent the animal from developing and expressing associative conditioned responses thus demonstrating that this structure is necessary for auditory fear conditioning (LeDoux et al, 1984; Heldt et al, 2003). In addition, auditory fear conditioning leads to increased metabolic activity in IC (Gonzalez-Lima et al, 1984; McIntosh et al, 1993) and to best frequency shifts of collicular neurons towards the CS frequency (Gao et al, 1998; Gao et al, 2000; Ji et al, 2001). All these studies support the idea that the IC is not just a passive relay of sensory information but that somehow plays a role in learning and memory processes. Our results further support this idea, since only in conditioned animals did we observe a significant increase in Fos labeling in IC. It is of interest to note that this increase in Fos labeling in conditioned animals in the 12 kHz region (CS frequency) of this nucleus was due to more cells being recruited from that area (as measured by an increase in labeled cell density) and from neighboring higher CF areas (as measured by the total spread of the tonotopic band). Gao and Suga observed that neurons up to 15kHz above the CS frequency are retuned towards the CS (Gao et al, 1998). Previous work by the authors (Santos et al, 2004; Santos, 2006) demonstrated that increasing the Sound Pressure Level (SPL) of an auditory stimulus leads to these same phenomena: increase in labeled cell density of the tonotopic band; and spreading of labeling towards higher CF regions. In DCN and IC auditory fear conditioning mimics an increase in SPL of about 10 dB as measured with Fos labeling. In the AC, a clear tonotopic band cannot be defined but we observed that increasing SPL leads to a more widespread pattern of labeling in this nucleus, and this same effect was seen in the pattern of cortical labeling of conditioned animals.

Several studies have shown that conditioning leads to electrophysiological and metabolic changes in AC (Gonzalez-Lima et al, 1984; Bakin et al, 1990; Quirk et al, 1997; Kilgard et al, 1998; Gao et al, 2000; Weinberger, 2004). Our results are consistent

with all these studies that show that conditioning leads to increased levels of CS-induced neural activity in AC.

The fact that only the Conditioning (and not the Unpaired) paradigm induces a significant increase in Fos labeling in auditory nuclei strongly suggests that the observed changes are associative. Moreover, this increase in Fos labeling is seen in the CS region. The observation that the strength of the conditioned response correlates with the magnitude of increase in Fos labeling in PIN and AC further supports the idea that the observed changes are associative. Nevertheless, in the present study the Fos labeling response to frequencies other than the CS was not tested and the possibility that all or part of the observed changes are due to sensitization cannot be ruled out. Different studies have shown that conditioning induces some degree of general threshold shift in auditory neurons (Bakin et al, 1990; Edeline et al, 1992). For example, in the medial division of geniculate nucleus and auditory cortex about 25-30% of the observed conditioning-induced changes consist of general increases in the responsiveness of neurons. The observation that scopolamine-injected conditioned animals had impaired learning but still showed an increase in Fos labeling suggests that at least part of the observed changes may be non associative. Nevertheless the results from the scopolamine experiments are preliminary as only a few animals were used. Furthermore, the animals did develop some freezing to the tone at the end of training.

In contrast to the observation that receptive field plasticity in IC and AC is retained for a long-time (up to 2 months in A1) (Suga et al, 2003; Weinberger, 2004), we observed that conditioning induced changes in Fos labeling are only seen following acquisition, but completely disappear when the animals are tested 4 days after training.

*c-Fos* and other IEGs seem to play an important role in the stabilization of newly formed memories: this study supports that idea

Many other studies have shown a correlation between behavioral learning and IEG expression, including *c-fos*, in structures critical for learning (Clayton, 1998; Guzowski, 2002). Furthermore, disruption of IEGs expression, with injections of antisense oligodeoxynucleotides (ODNs) into critical regions in the brain before training or knocking out this gene in CNS, impairs the formation of long-term memory with no

effect on acquisition or short-term memory (Lamprecht et al, 1996; Mileusnic et al, 1996; Grimm et al, 1997; Morrow et al, 1999; Guzowski, 2002; Fleischmann et al, 2003; Countryman et al, 2005; Yasoshima et al, 2006). All these studies suggest that Fos and other IEGs help stabilize newly formed memories (Clayton, 1998; Guzowsky, 2002).

In our, and other studies we have observed that Fos expression is increased following acquisition of auditory fear conditioning but not during a long-term memory test, despite the fact that animals showed retention of fear memories, as measured behaviorally. These results are in agreement with the proposed function of Fos as a stabilizer of newly formed memories following learning.

### Summary

In summary, we showed that different learning paradigms lead to a differential pattern of Fos expression in CS, and CS-US convergence pathways. This differential gene expression presumably reflects differences in the processing and storage of information relating to auditory fear conditioning.

A model where *c-fos* plays a role in stabilizing long-term memory changes following learning would account for the observed dissociation between gene expression during learning versus retention and between gene expression versus behavior at retention. Furthermore, it is in agreement with the current thinking that the amygdala is necessary for acquisition but not retention of fear memories (Wilensky et al, 1999; LeDoux et al, 2000).

Considering the strong evidence suggesting that Fos is important for the stabilization of newly formed memories, this study suggests that learning-induced plasticity about auditory CSs could be stored in the auditory pathway and that more complex associative information could be stored in the amygdala, particularly in CE.

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**DAY 1: Training Session**

**DAY 5: Memory Test**

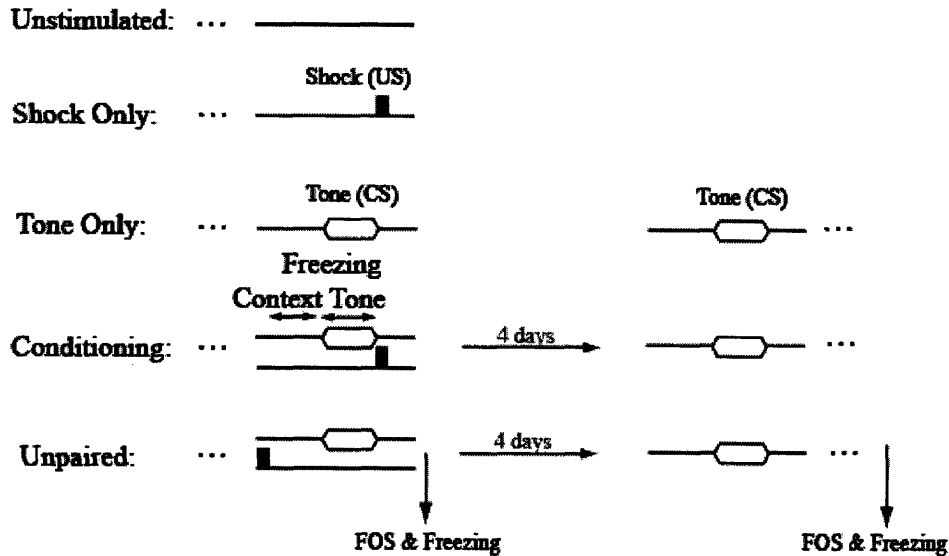


Figure 3.1. Diagram illustrating experimental design and paradigms. Animals were trained with one of five different paradigms: Unstimulated; Shock Only; Tone Only; Conditioning or Unpaired. The conditioned stimulus was a 12 kHz tone, 20 sec duration, 45-60 dB SPL. The unconditioned stimulus was a 2 sec scrambled footshock, 0.7 mA. The training session consisted of 36 tone and/or shock presentations, with an average interstimulus interval of 3 minutes. Freezing was measured in the 10 secs preceding the tone (=contextual freezing) and in the 20 secs when the tone was on. A group of animals trained with the Conditioning or Unpaired paradigm was not immediately sacrificed after training. 4 days after the training session, this group of animals was put into the conditioning box and presented with the tone (like Tone Only paradigm; no shocks were given). This was called the memory test. At least one untrained animal was stimulated with the same Tone Only paradigm and sacrificed at the same time as previously trained animals. Freezing was assessed as before.

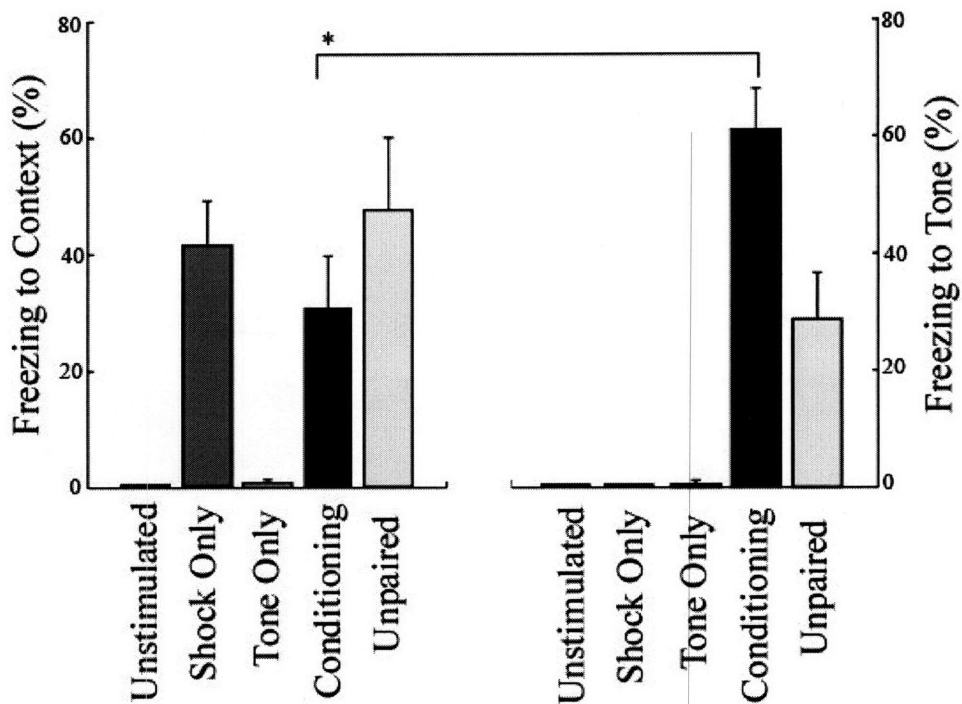


Figure 3.2. Freezing behavior (mean  $\pm$  s.e.) assessed at trial 33 of the training session for all paradigms (Unstimulated:  $n=3$ ; Shock Only:  $n=3$ ; Tone Only:  $n=12$ ; Conditioning:  $n=12$ ; Unpaired:  $n=11$ ). Freezing to context for unstimulated animals was measured at about the same time that trial 33 would occur. Data regarding freezing to tone for Unstimulated and Shock Only animals was obtained by putting animals at the end of training in a new box followed by a brief presentation of the conditioned tone. Only conditioned animals significantly froze more to tone than to context. In this and subsequent figures, \* indicates a significant difference with  $p<0.05$  and \*\* a significant difference with  $p<0.01$  using the Kruskal-Wallis test.

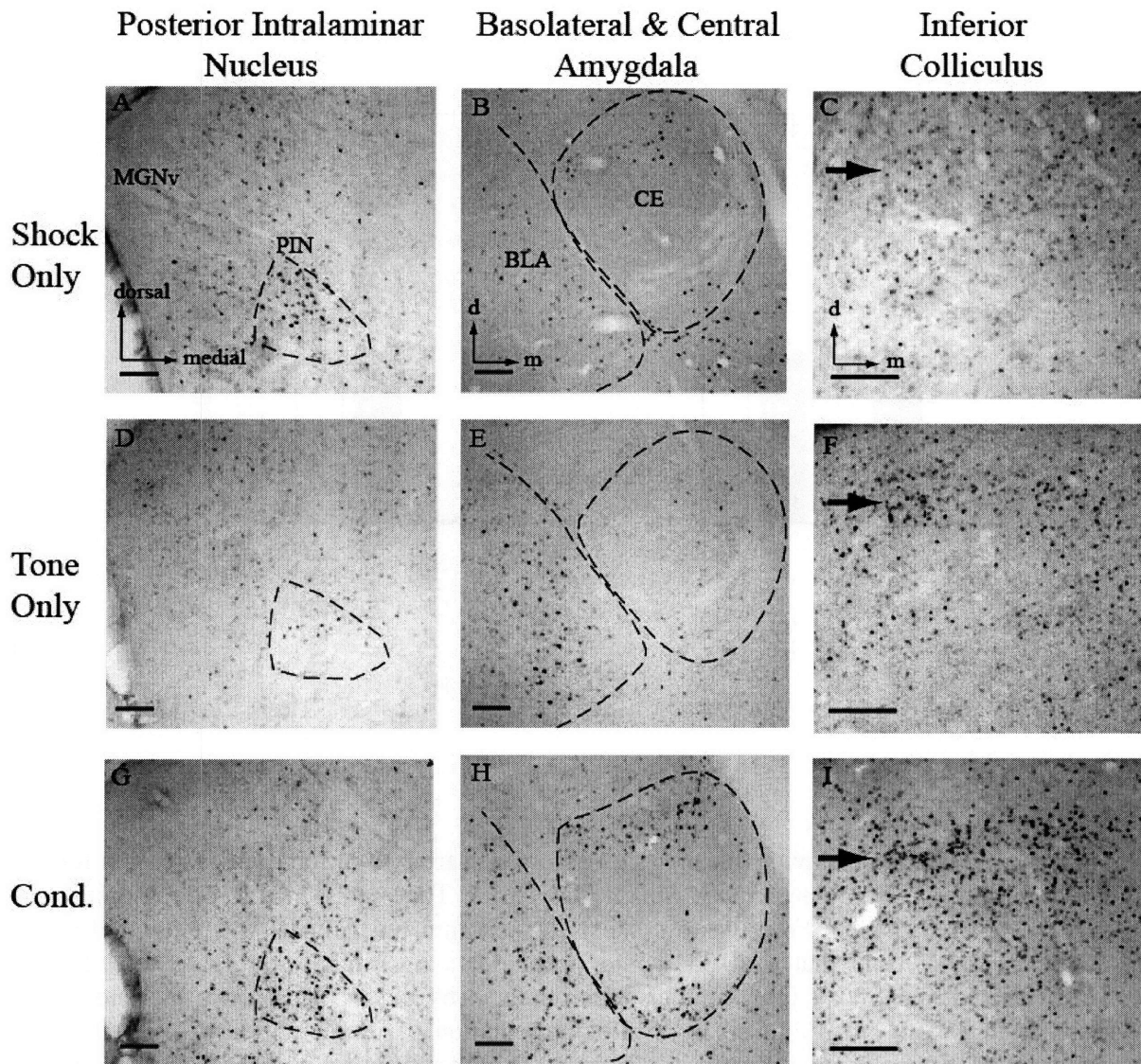


Figure 3.3. Representative photomicrographs showing Fos labeling in PIN (A, D, G), amygdala (BLA and CE) (B, E, H) and central nucleus of IC (C, F, I) for 3 different paradigms (Shock only: A-C; Tone Only: D-F; and Conditioning: G-I). Arrows in figures C, F and I indicate the approximate expected location of the 12 kHz region in IC. All animals sacrificed after training. Scale bar 100  $\mu$ m.

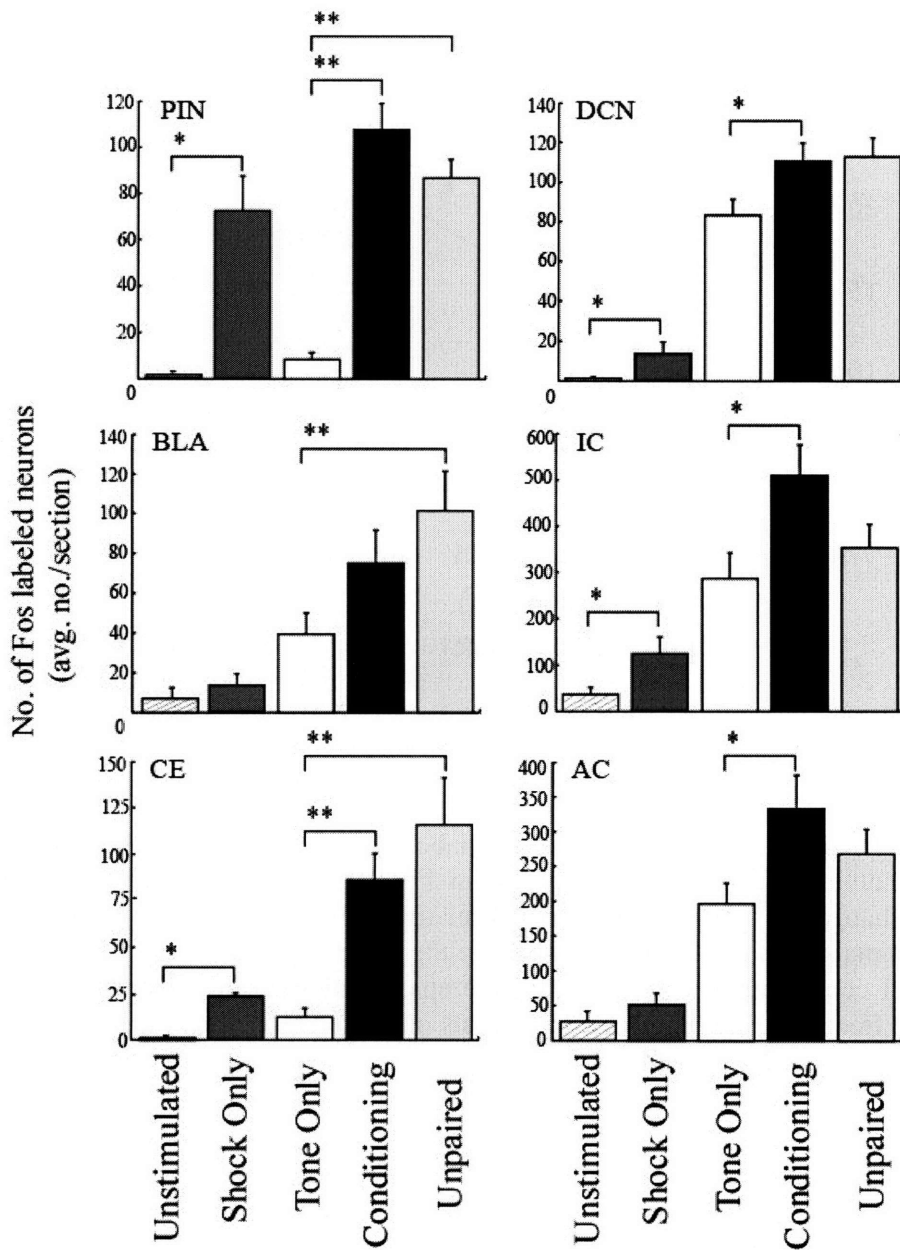


Figure 3.4. Counts (mean  $\pm$  s.e.) of Fos labeled neurons in PIN, amygdala (BLA and CE) and auditory nuclei (DCN, IC, and AC) from animals trained with the different paradigms (Unstimulated: n=3; Shock Only: n=3; Tone Only: n=7; Conditioning: n=7; Unpaired n=7).



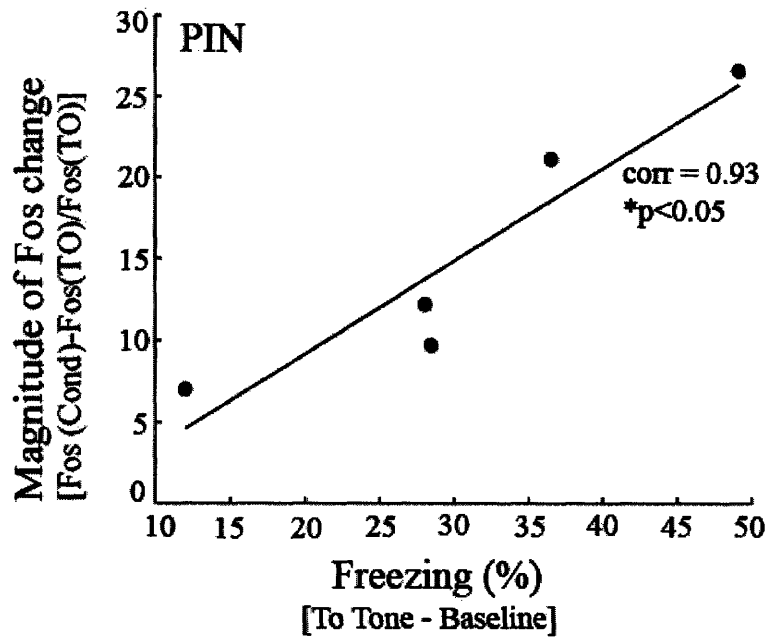


Figure 3.5. Correlation between increase in Fos labeling and strength of conditioning to the tone for individual animals. The increase in Fos labeling was normalized as the magnitude of change in the conditioned animal relative to the Tone Only control of each experiment (animals stimulated and sacrificed on the same day and processed together). The strength of conditioning was defined as the amount of freezing to tone at trial 33 above baseline freezing. Baseline freezing consists of the average of contextual freezing at trials 5, 9, 13, 17, 21, 25, 29, and 33.

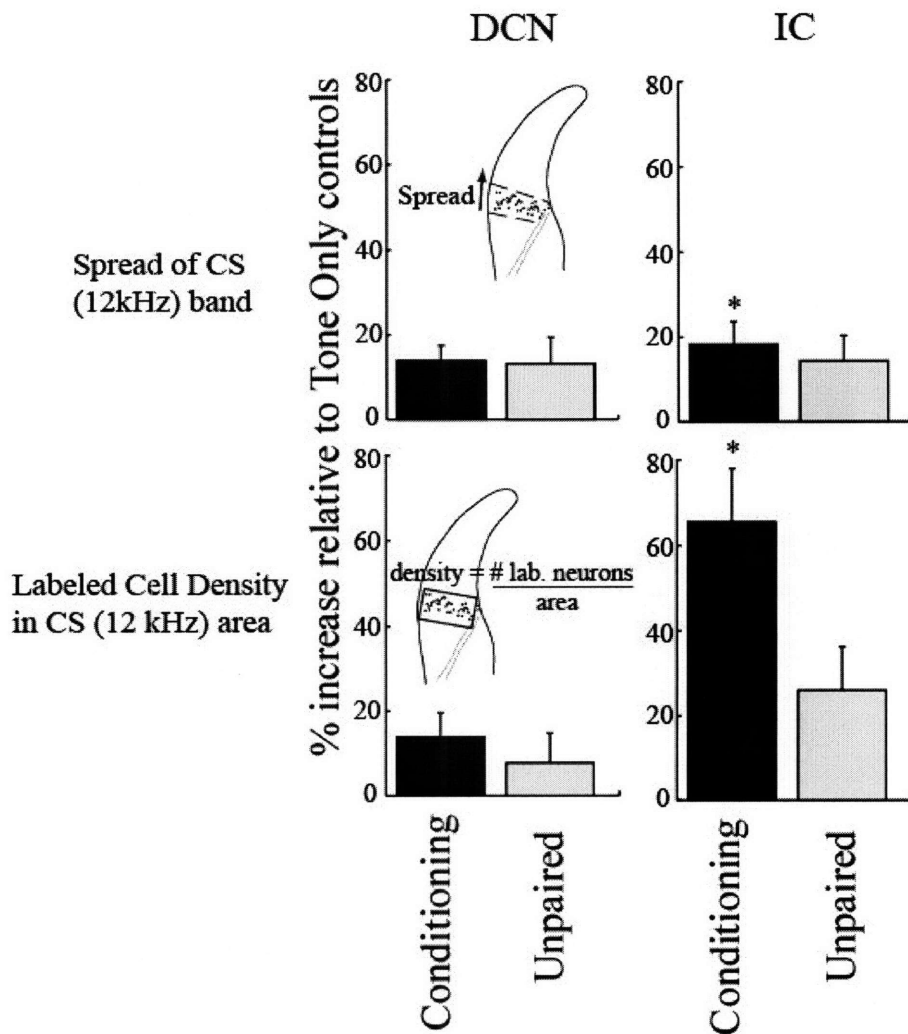


Figure 3.6. Normalized spread and labeled cell density (mean  $\pm$  s.e.) in the 12 kHz (CS) band in DCN and IC of Conditioning and Unpaired cases. Measurements of the spread of the band of labeling and labeled cell density were done in Tone Only, Conditioning and Unpaired animals. The spread and density of labeling in Conditioning and Unpaired groups was normalized and plotted as percent increase relative to the same measurements in Tone Only controls. Only in IC of conditioned animals were the observed increases in spread and density of labeling statistically different from Tone Only measurements.

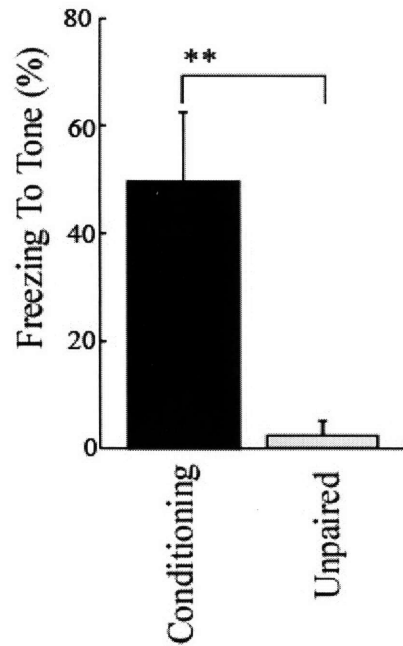


Figure 3.7. Freezing behavior (mean  $\pm$  s.e.) at the memory test (trial 2). Animals were put into the conditioning box again and presented the conditioned tone (like Tone Only paradigm). Animals had been previously trained (4 days before) with Conditioning (n=5) or Unpaired (n=5) paradigms. Only conditioned animals showed high levels of freezing to tone.

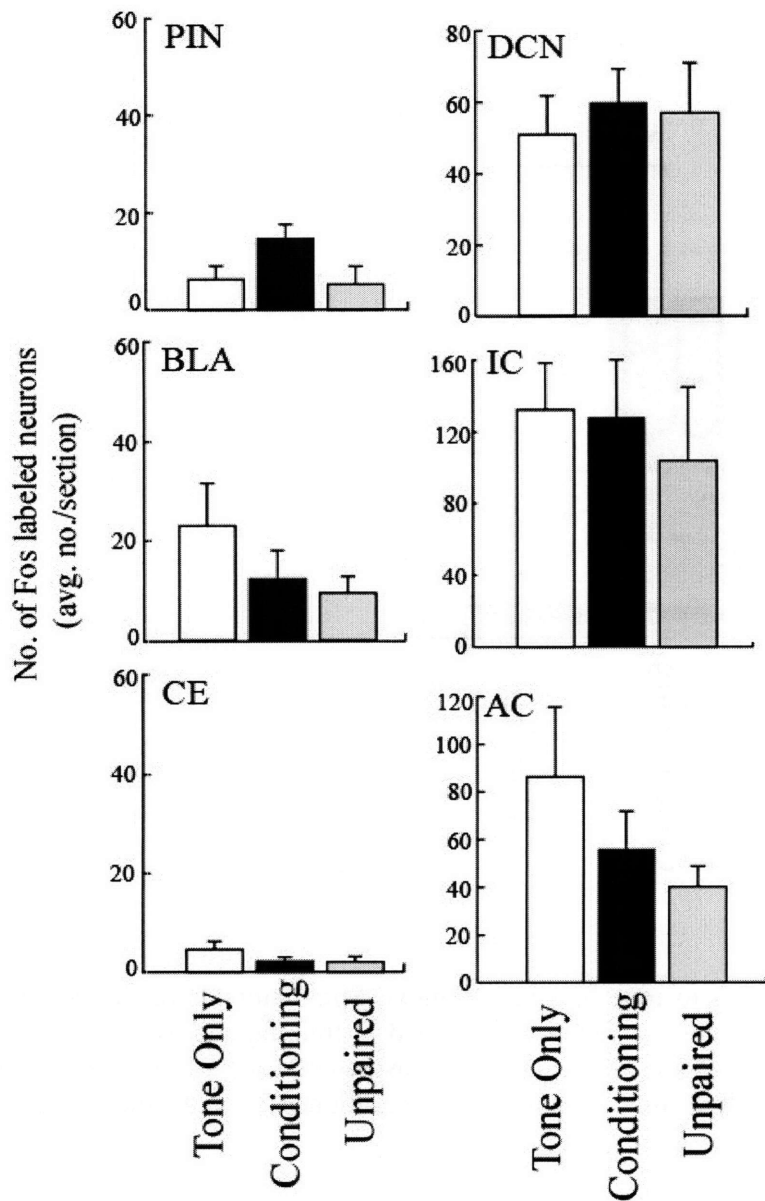
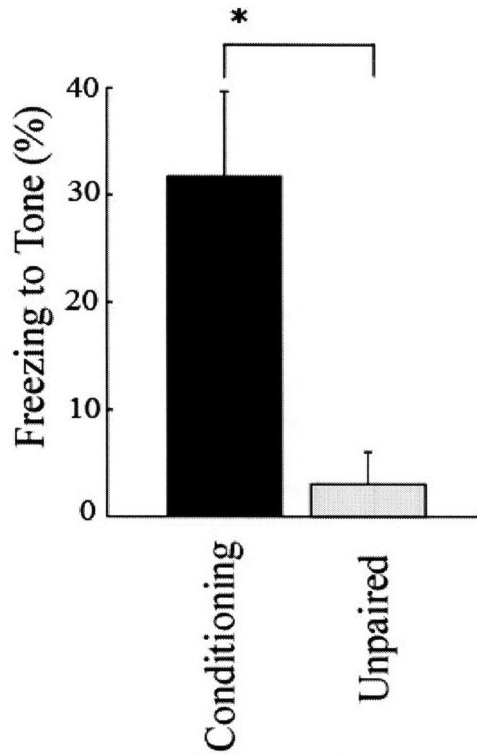
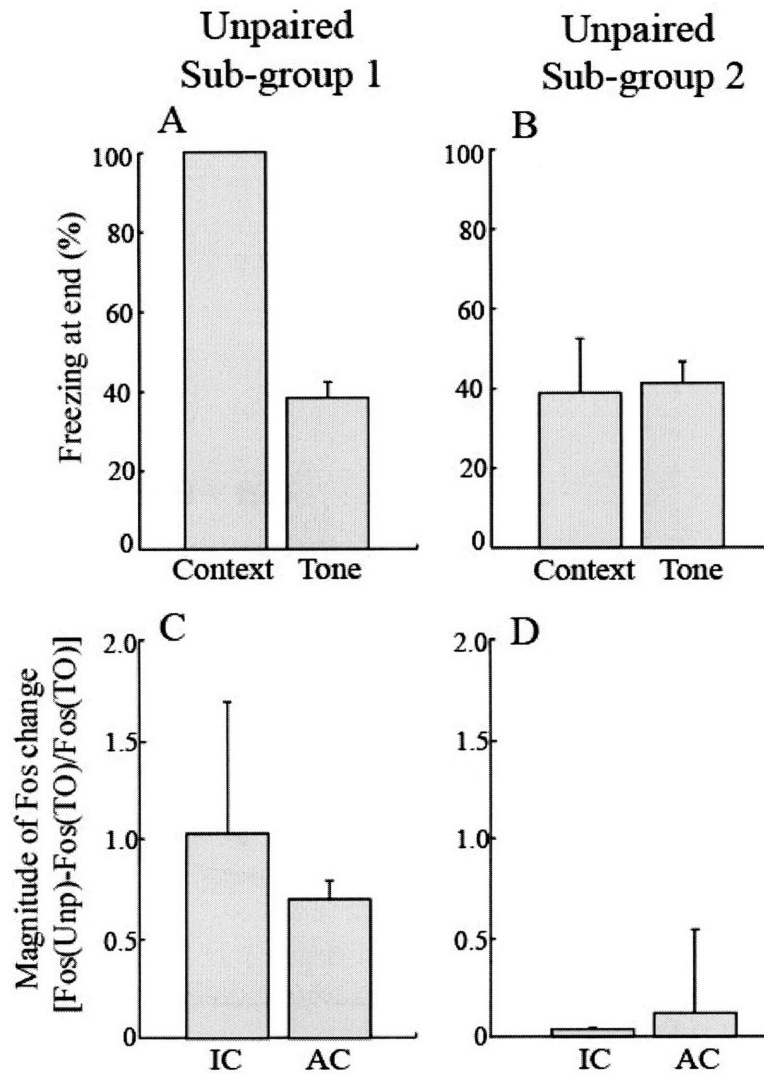


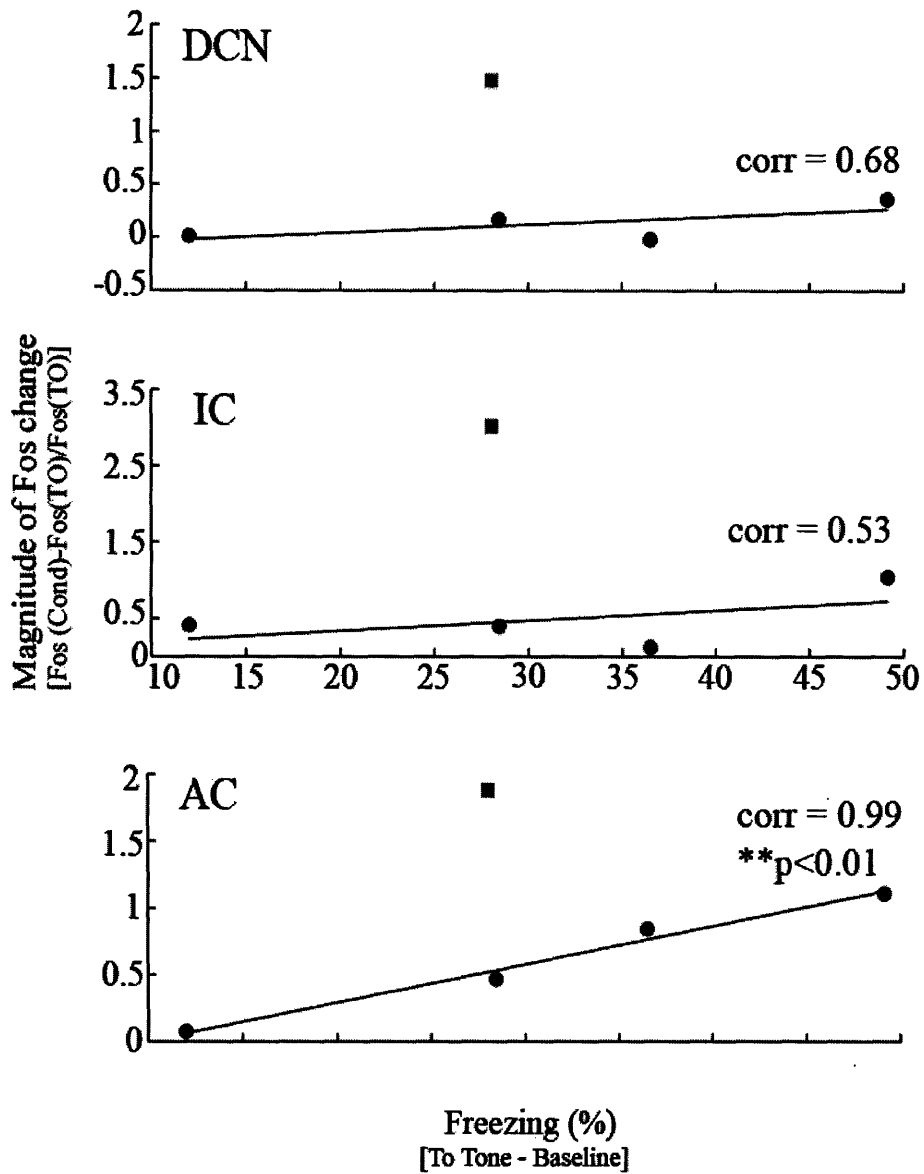
Figure 3.8. Counts of Fos labeled neurons (mean  $\pm$  s.e.) from animals sacrificed at the end of the memory test (see legend of fig. 3.7 for details) (Conditioning: n=5; Unpaired: n=5). Tone Only animals did not receive any previous training. At least one untrained Tone Only case was stimulated on the same day and processed together with previously trained animals. No statistical difference in Fos labeling was observed between untrained and previously trained animals.



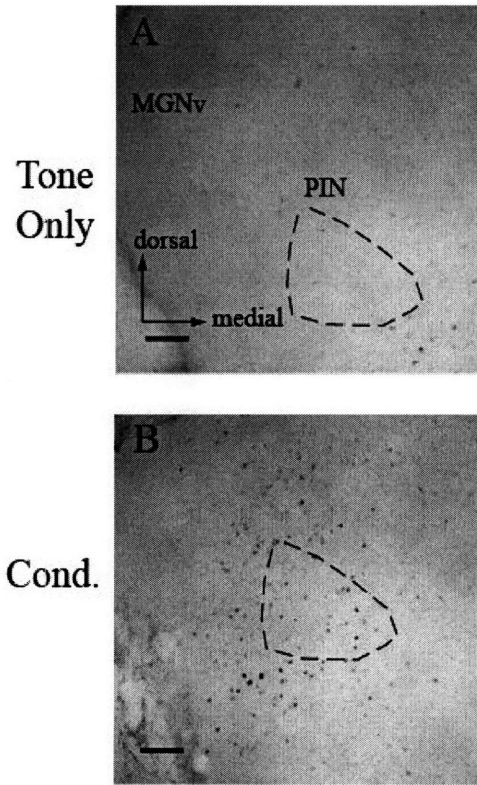
Appendix Figure 3.1. Freezing to tone in a new context. Some animals (Conditioning: n=4; Unpaired: n=3) were put into a new box at the end of training and were presented with the conditioned tone for 30 secs. Freezing was measured and scored by an observer once every 3 seconds while the tone was on. Only conditioned animals showed high levels of freezing to the tone.



Appendix Figure 3.2. Cases stimulated with the Unpaired paradigm were separated into two groups: sub-group 1 (n=2), animals that developed inhibitory conditioning (less freezing to tone than to context) (A); and, sub-group 2 (n=3), animals showing similar levels of freezing to tone and context (B). A, B: Freezing (mean  $\pm$  s.e.) to context and tone at trial 33 of the training session. C, D: Magnitude of Fos change (mean  $\pm$  s.e.) in IC and AC of sub-groups 1 (C) and 2 (D). See figs. 3.5 for details on this metric. Only animals from which we measured freezing behavior and Fos during training were included in this analysis.

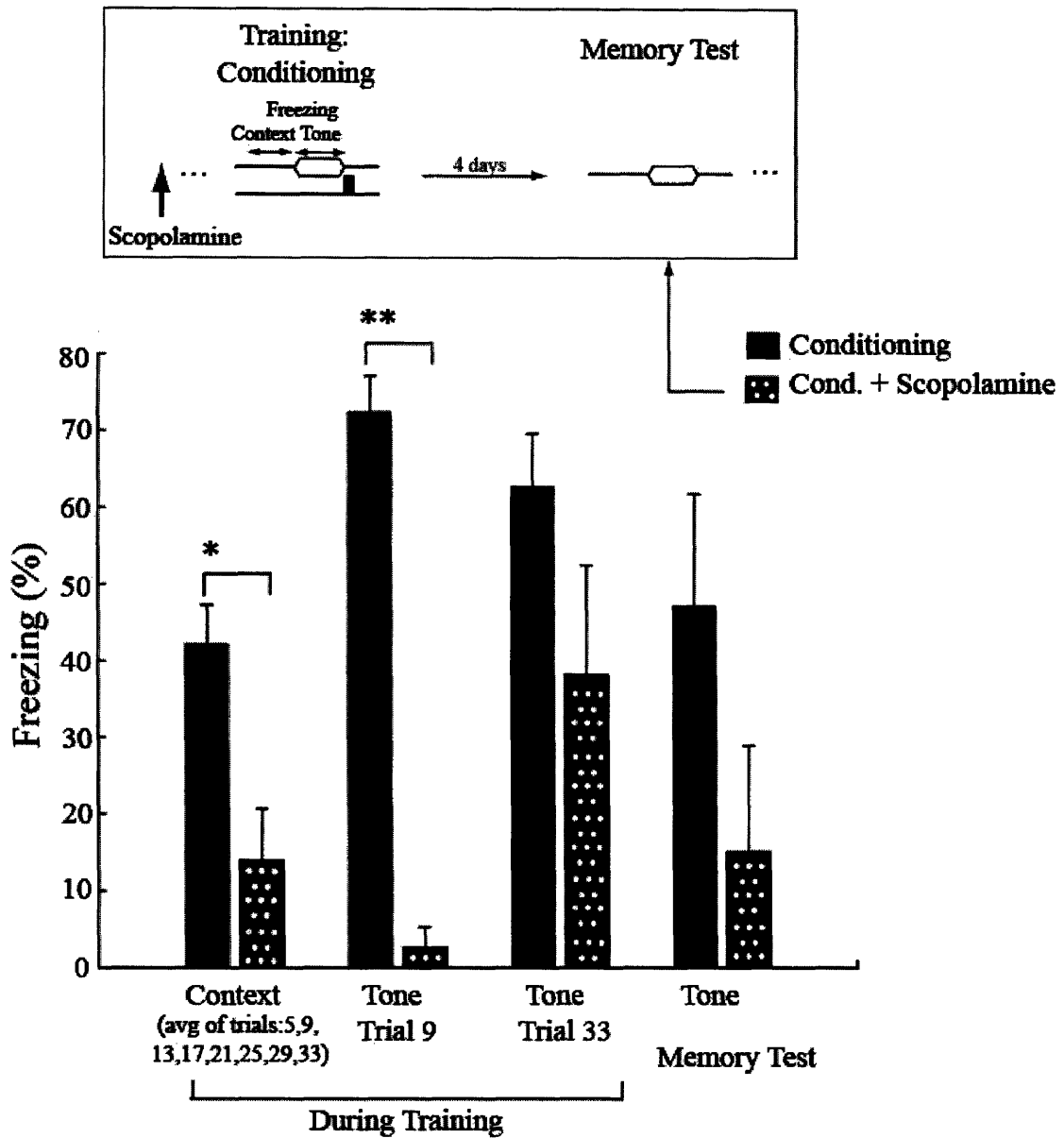


Appendix Figure 3.3. Correlation between increase in Fos labeling in auditory nuclei and strength of conditioning to the tone for individual animals. See figure 3.6 for description of metrics. One particular Tone Only control had considerable lower numbers of Fos labeling in all 3 auditory nuclei in comparison to all other Tone Only cases. The relative magnitude of change in labeling in the conditioned animal normalized to this Tone Only case was consequently much higher than all other cases and we labeled this case as an outlier (square). The outlier in the data was excluded from curve fitting and calculation of the correlation coefficient.

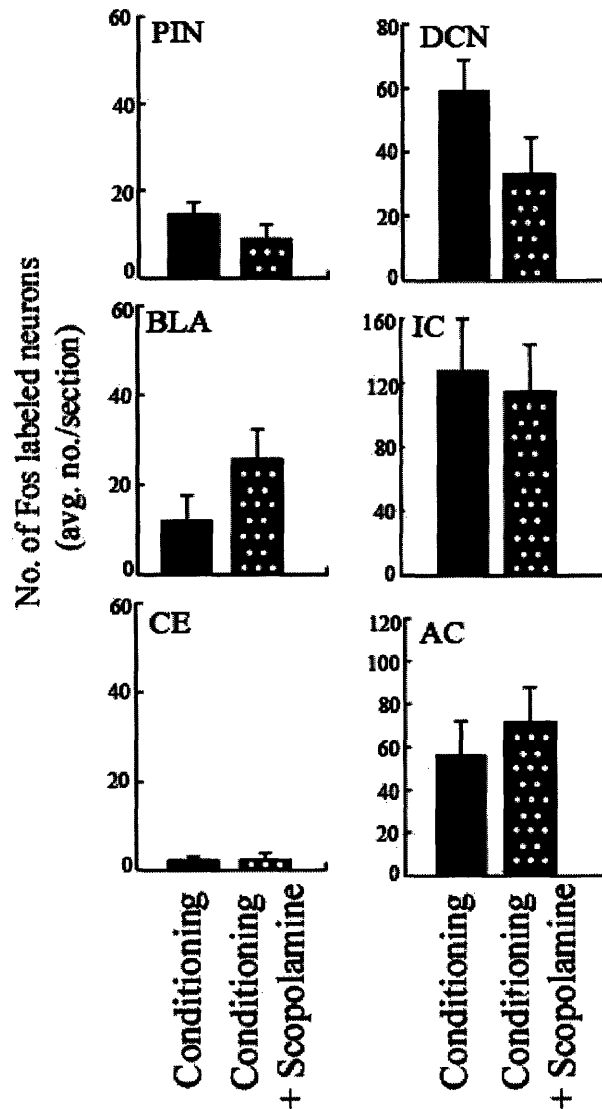


Appendix Figure 3.4. Representative photomicrographs showing Fos labeling in PIN of animals sacrificed following the memory test. See figs 3.7 and 3.8 for details. Scale bar, 100  $\mu$ m.





Appendix Figure 3.5. Comparison of freezing behavior between animals that were injected with scopolamine before training with the Conditioning paradigm (n=4) and uninjected conditioned controls (n=12). Scopolamine was injected 10 mins before training (intraperitoneal; 1 mg/Kg).



Appendix Figure 3.6. Counts (mean  $\pm$  s.e.) of Fos labeled neurons from animals sacrificed at the end of the memory test (see legend of fig. 3.7 for details) and previously trained with the Conditioning paradigm. A group of animals received scopolamine injections (see appendix fig. 3.5 for details) before training (Conditioning:  $n=5$ ; Conditioning + Scopolamine:  $n=3$ ). No differences were observed in Fos labeling between animals injected with scopolamine and uninjected animals.

## **Chapter 4: The Big Picture: Summary and Conclusions**

### **I. Chapter 2: Summary of results and implications for intensity coding**

In Chapter 2 we showed that the spatial pattern of neural activation as measured with Fos labeling is a function of sound level. The main findings of this study were that:

- Spread of neural activation towards higher CF regions (but not lower frequency regions) is observed as a function of sound level at all levels of the auditory pathway.
- Labeled cell density increases in a particular frequency area with increasing sound level. More high threshold neurons seem to be recruited at high sound levels.
- Labeling spreads in directions other than the tonotopic axis. Units with differing thresholds might be topographically organized in a threshold map at all levels of the auditory pathway.

In summary, particular areas seem to be selectively activated by sound at moderate-to-high levels and we propose that this spatial information contributes to intensity coding. Perceptual studies support the possibility that spread of excitation contributes to intensity coding as the presence of a high frequency noise masker impairs intensity discrimination to a tone at high sound levels (Moore et al, 1974; Carlyon et al, 1984). There are no perceptual studies relating the possible contribution of threshold maps for intensity coding. We propose the following experiment to test for the significance of this mechanism for behavior: Place lesions in the most caudal and rostral sections of IC and test intensity discrimination at high levels.

### **II. Chapter 3: Summary of results and implications for understanding the role of different brain regions for acquisition and storage of auditory fear memories**

In chapter 3 we studied the pattern of neural activation as measured with Fos labeling in critical areas of the brain during acquisition of fear conditioning and following a

memory test. In addition, we compared Fos labeling with a behavioral index of auditory fear conditioning (freezing). The main findings of this study were:

- Shocks (the unconditioned stimulus) strongly evoke Fos labeling in posterior intralaminar nucleus (PIN).
- Tones + Shocks (Conditioning or Unpaired paradigms) strongly evoke Fos labeling in central amygdala (CE) during behavioral training.
- Conditioned animals show an increase in Fos in auditory nuclei during behavioral training.
- Following a memory test there no differences are seen in Fos labeling in the studied regions between animals that received previous training and untrained animals. In contrast, animals trained with the Conditioning paradigm develop long-term auditory fear memories as measured with behavior (freezing).

In the paragraphs below we will discuss the implications of these results for understanding the role of these critical brain regions (PIN, amygdala, and auditory pathway) for acquisition and storage of auditory fear memories.

#### What is the role of PIN?

The exact role of PIN for auditory fear conditioning has been a subject of controversy. This nucleus is thought to be a place of CS (tone)-US (shock) convergence and it might be particularly important for US processing as has been shown by:

- Anatomical studies: receives auditory and somatosensory input and projects to the amygdala (LeDoux et al, 1987; LeDoux et al, 1990)
- Electrophysiology studies: contains units responsive to tones and shocks (Bordi et al, 1994)
- Lesion studies: Lesions of PIN impair fear conditioning if the US is a shock but have no effect if the US is a loud noise (Lanuza et al, 2004).
- Manipulation studies: PIN stimulation paired with an auditory CS is an effective US substitute to produce conditioning (Cruikshank et al, 1992).

We observed that Tone Only did not evoke Fos labeling in PIN. In contrast, all paradigms with shocks strongly induced Fos labeling in this nucleus. These results strongly support the idea that PIN is particularly important for processing

somatosensory USs. The fact that the magnitude of Fos increase correlated positively with the strength of conditioning is consistent with the idea that PIN is involved in tone-shock convergence and associativity. Nevertheless, the number of Fos labeled cells was not much different for Shock Only, Conditioned and Unpaired animals. Therefore, these results suggest that PIN may not be critical for the association of tone and shock.

#### What is the locus of CS-US convergence and plasticity?

Traditionally, most models of auditory fear conditioning viewed the basolateral amygdala (BLA) as the most important site of CS-US convergence and CE as more of a passive relay. This model has been disputed by the fact that: (1) BLA does not project directly to the neurons in CE that are involved in the production of conditioned responses (Krettek et al, 1978; LeDoux et al, 1988); and (2) CE also receives convergent auditory and somatosensory input (LeDoux et al, 1987; Linke et al, 2000; Bernard et al, 1996). In light of these findings, a new model of auditory fear conditioning proposes that CE is an important site of CS-US convergence and plasticity (Pare et al, 2004). We observed that Tone Only evokes more Fos labeling in BLA than Shock Only suggesting that BLA plays a bigger role in the processing of auditory/sensory information. In CE, the paradigms where shock and tone were presented (Conditioning and Unpaired) elicited the most labeling. These results support the idea that CE and not BLA is the critical site of CS-US convergence and plasticity.

#### The role of the auditory pathway

The amygdala, although critical for the acquisition of auditory fear conditioning, does not seem to be the place of storage of auditory fear memories (Wilensky et al, 1999; LeDoux, 2000). Alternatively, the auditory cortex, has been proposed as the site of storage of auditory fear memories (Weinberger, 1998). Conditioning induced receptive field plasticity in the auditory cortex is associative, frequency-specific, rapidly induced and long lasting suggesting that this form of plasticity constitutes some form of physiological memory being stored in the auditory pathway. Furthermore, perceptual studies support the idea that this auditory plasticity has an impact in behavior. It has been shown that

increased representation of a tone following behavioral training correlates with an improvement in auditory perception (Recanzone et al, 1993; Rutkowski et al, 2005).

Only conditioned animals (and not Unpaired) showed an increase of Fos production in auditory nuclei. In addition, the observed increase in Fos labeling was seen in the CS region and correlated with the strength of the conditioned response. All these observations suggest that the conditioning-induced Fos changes seen in DCN, IC and AC are associative. The gene *c-fos* seems to be important for the stabilization of newly formed memories (see section III). In light of our findings and of the putative role of Fos, these results support the idea that auditory fear conditioning memories may be stored in the auditory pathway.

Two models have been proposed to explain conditioning-induced plasticity in the auditory pathway (Weinberger, 1998; Suga et al, 2003; Weinberger, 2004). Two important points of divergence between the models are: (1) the role of amygdala; and (2) the role of primary auditory cortex (A1). (1) Suga's model postulates that the amygdala is the main site of plasticity between CS and US while Weinberger's model postulates that CS-US plasticity is a distributed function that involves the medial geniculate-PIN complex and parts of the amygdala. Our results suggest that CE is more important for CS-US plasticity than PIN, therefore they support Suga's model in this issue. (2) Relative to the role of A1 in auditory fear conditioning, Suga's model proposes that A1 is the active site of plasticity and responsible for inducing subcortical plastic changes. Weinberger's model postulates that A1 is not necessary for the induction of subcortical plasticity but is an important and active site of plasticity. The present study does not provide insight relating this issue. But, our results suggest that both IC and AC might be important sites of plasticity.

Both models agree that strengthening of auditory cortical plasticity depends on acetylcholine modulation via the nucleus basalis. In the present study, it was observed that injections of scopolamine, an anticholinergic drug, before training had no effect in the observed upregulation of Fos in the auditory pathway following conditioning. A small number of animals was used in these experiments. In addition, the scopolamine injections were systemic and had some general effects on behavior (increased motility; possible induction of an altered state of consciousness). Therefore, we cannot conclude that the

observed upregulation of Fos production in the auditory cortex is not acetylcholine dependent.

### **III. Implications for the understanding of Fos labeling**

#### What is the function of *c-fos*

There is considerable evidence supporting the idea that Fos plays an important role in the stabilization of newly formed memories. Two types of studies have provided evidence supporting this hypothesis:

- Correlative studies that show a correlation between increased Fos production in critical brain nuclei and behavioral learning during acquisition (Campeau et al, 1991; Beck et al, 1995; Milanovic et al, 1998; Radulovic et al, 1998; Holahan et al, 2004).
- Expression disruption studies (with injections of antisense oligonucleotides or by knocking out the gene) showing that disruption of Fos production results in impaired long-term memory (but no effects are seen in acquisition or short-term memory) (Lamprecht et al, 1996; Mileusnic et al, 1996; Grimm et al, 1997; Morrow et al, 1999; Guzowski, 2002; Fleischmann et al, 2003; Countryman et al, 2005; Yasoshima et al, 2006).

A model where the role of *c-fos* is to stabilize cellular changes following learning would explain the following results of this study: (1) Fos labeling increases during learning but not following the long-term memory test; and, (2) despite the fact that the animal clearly developed long-term fear memories (shown by behavior) there is no change in gene expression following the long-term memory test in the areas where we quantified Fos labeling. The proposed model of *c-fos* function can explain these results, if we assume that any learning that is happening at the memory test (e.g. extinction) involves other brain structures. Many studies support the idea that extinction of memories is a process involving higher cognitive nuclei such as the frontal cortex (LeDoux, 2000). Therefore, the results presented in chapter 3 are consistent with the current model of Fos function and support it.

If we assume that the role of Fos is to stabilize newly formed memories, we might question the appropriateness of Fos labeling as a marker of neuronal activation to study more 'basic' processes such as intensity coding. First, it is important to note that many of functional Fos studies mentioned above suggest that this gene acts as a general modulator of memory stabilization and is not per se the inducer of specific cascades of cellular plasticity. Therefore Fos might represent the 'probability' of change and not change itself. Second, the overall pattern of Fos labeling follows important functional principles of auditory physiology. In the present thesis, it was shown that the pattern of Fos labeling is dependent on sound level and that the observed level-dependent effects are consistent with data from electrophysiology studies (Kim et al, 1979; Shofner et al, 1985; Stiebler et al, 1986; Kim et al, 1991; Taberner et al, 2005). Furthermore, it has been consistently observed that the pattern of tone-evoked Fos labeling follows the known topographic map of frequency of auditory nuclei (Stiebler et al, 1985; Stiebler et al, 1997; Ehret et al, 1991; Friauf, 1994; Iversen, 2001; Müller et al, 2004). Acoustic stimulation results in Fos labeling in only a relatively small percentage of neurons (up to ~25%), but the similarities between tone-evoked Fos labeling and electrophysiology responses suggest that the population of labeled neurons is representative of the overall population of auditory neurons. Therefore, Fos labeling seems to be a good tool to study the pattern of neuronal activation in large regions of the brain for many different research questions.

#### Fos labeling versus electrophysiology in the auditory pathway: Puzzling differences and proposed explanation

Some aspects of tone-evoked Fos labeling in our study were surprising and could not have been predicted by electrophysiology studies: (1) acoustic stimuli evoke more Fos labeling in DCN than AVCN; (2) There is a preponderance in Fos labeling of the DCN-IC pathway over the AVCN-SOC-IC pathway; (3) There is almost no Fos labeling in the medial geniculate nucleus. Some of these observations have been documented in previous Fos studies (Friauf, 1992; Rouiller et al, 1992; Adams, 1995; Brown et al, 1995). In light of the proposed Fos function, it is possible that these differences in Fos labeling might reflect differences in the ability of different nuclei to change in response to stimuli in the environment. We tend to think that sensory pathways are relatively rigid but in fact



several lines of evidence suggest otherwise. Changes in the responses of auditory neurons have been observed following:

- Associative learning: e.g. auditory fear conditioning leads to increased responses to the tone used as conditioned stimulus (e.g. Gonzalez-Lima et al, 1984; Bakin et al, 1990; Kilgard et al, 1998).
- Non-associate learning: e.g. repeated presentation of a tone results in a decrement of the response to the repeated stimulus. This phenomenon is called auditory habituation (Westenberg et al, 1976; Condon et al, 1991; Iversen, 2001).
- Injury: e.g. frequency maps get reorganized following cochlear lesions. The frequency regions that are deprived of sensory input are occupied by expanded representations of adjacent frequencies (Robertson et al, 1989; Irvine et al, 2000).
- Adaptive changes as a function of the environment: e.g. neurons can change their rate-level functions to pure tones, by shifting them to higher sound levels, if the tones are presented in a constant level of background noise (Gibson et al, 1985; Rees et al, 1988).

Similar effects have been observed in other sensory pathways. All these experiments strongly suggest that sensory pathways are highly adaptive and show that the responses of neurons change not only due to associative processes (like auditory fear conditioning) but also just as a consequence of simple exposure to stimuli (like auditory habituation). A plausible hypothesis is that Fos expression in the auditory pathway following acoustic stimulation represents the ‘possibility of change’. Our hypothesis is that even simple exposure to a tone will lead to small changes in the responses of neurons, some of these changes become stable and long-term, and Fos expression contributes to the stabilization of those small changes. Such a model of Fos function, would imply that DCN and IC have a greater potential for change and plasticity than AVCN. Consistent with this idea is the observation that DCN and IC contain highly complex circuitry and have many neurons with complex responses (Rhode et al, 1986; Ehret et al, 1988; Pollack et al, 2003; Oertel et al, 2004). Furthermore, such a model would suggest that the medial geniculate nucleus is not a locus of plasticity. This idea is supported by the observation that conditioning-induced plasticity in the ventral division of the medial geniculate

nucleus is transient and not long-lasting (Edeline et al, 1991). In contrast, conditioning induced plasticity in the auditory cortex can last up to 8 weeks (Bakin et al, 1990).

In summary, we propose that just exposing an animal to a tone can lead to changes in the responses of auditory neurons and that the gene *c-fos* helps stabilize these putative changes. Therefore, we propose that the differential pattern of tone-evoked Fos labeling in auditory nuclei represents the ability of specific nuclei to be plastic.

#### Population of Fos labeled neurons

The population of Fos labeled neurons in the auditory pathway is heterogenous in terms of:

- Size: In our study we observed that labeled cell nuclei could vary in size from 70  $\mu\text{m}^2$  to less than 20  $\mu\text{m}^2$ . At least 80% of labeled cells are small with a nucleus area smaller than 25  $\mu\text{m}^2$ . This variability in nucleus size of labeled cells has been observed in other studies (Adams, 1995; Yang et al, 2005). Although most Fos labeled cells are small neurons, not all small neurons are Fos positive.
- Number of perisomatic endings: Adams (1995) observed that labeled cells can have from only a few to many perisomatic endings. The majority of cells have few perisomatic endings, but not all cells with few perisomatic endings are Fos positive.
- Cell type: Almost all cell types in cochlear nucleus can have Fos labeling (Yang et al, 2005). The largest neurons represented less than 1% of the total Fos labeled population.
- Neurotransmitter: Co-labeling studies have shown that Fos positive cells can be Acetylcholine esterase (AChE) or L-glutamic acid decarboxylase (GAD) positive (Adams, 1995; Gleich et al, 1995).

These results suggest that Fos positive cells do not share a particular anatomical characteristic. Instead, Fos positive neurons could share a specific physiological characteristic. In this study, we observed that the pattern of Fos labeling in the cochlear nucleus was similar to the pattern of innervation of low and medium spontaneous rate fibers. Furthermore, it has been shown that rind areas of AVCN contain a much higher proportion of units with low spontaneous rate than core AVCN (Goshal et al, 1996). Most

AVCN labeling was seen in the rind. *In vitro* studies of Fos induction using neurons that had no spontaneous activity show that maximal Fos expression is produced by short bursts of activity repeated at short intervals. This study suggests that Fos induction depends on the occurrence of a cellular event distinctly above background (Fields et al, 1997). Moreover, areas with high levels of neuronal activity such as the visual cortex do not show significant Fos expression (Kovacs, 1998). We propose that Fos labeling is produced as a consequence of neural activity in cells that have low levels of spontaneous activity.

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