The experiment was performed according to proposal. The details of which are given below as the form of a manuscript. In addition, a number of publications have been generated and which bear the due acknowledgement of NSC:


Report:

**Plastic changes in the rat midbrain following tone exposure: a combined electrophysiological and Fos-immunohistochemical study**

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Abstract

Acoustic experience during neonatal period can shape responses of central auditory neurons, but details are not clear. In this study, we explored the ‘critical’ period and details of the plastic changes in the rat midbrain. Rat pups were first primed with a pure tone (4 kHz, 65 dB SPL) in postnatal week-2 when their ear canals first opened. Changes in midbrain auditory neurons were determined in the following week using single unit electrophysiology and activity marking with Fos-immunohistochemistry. More cells in the inferior colliculus (IC) of the experimental group were found to tune towards the frequency of the priming tone and with elevated thresholds. Such changes were not obvious in rats receiving tone exposure during week-3, suggesting a ‘critical’ period in week-2. Elevated thresholds of single units to 4 kHz tone were further confirmed by the activity marking experiment, in that fewer cells in IC expressed Fos when briefly stimulated with the same priming tone. In addition, more units tuned around the priming tone appeared to respond to sounds with a time-varying feature such as frequency modulation (FM). This shift to FM sensitivity in the experimental group was also confirmed by the appearance of more Fos-positive cells following brief stimulation with a 4 kHz FM tone. We conclude that in the rat, a mild tone exposure during the postnatal week-2, likely the ‘critical’ period, was effective in altering response properties in central auditory neurons, including a preference towards FM sounds.
Introduction

Previous studies showed that the central nervous system is not hard-wired at birth, but remains plastic especially in the neonatal stage, especially during the ‘critical’ period (Gilbert and Wiesel, 1992; Weinberger et al., 1993, Kaas, 1991; review see Weinberger, 1995). Neonatal plasticity has the apparent advantage of adapting the developing brain to the postnatal environment. In the auditory system, such plastic changes could be important as they may be related to processing complex sounds including speech. Previous electrophysiology studies in rodents showed that response of central auditory neurons could be altered following neonatal sound exposure. For example, in young mice, after exposure to a broad-band sound (i.e., a click), frequency tuning of single cells at their inferior colliculus (IC) were broadened. In rats, over-exposure to a pure tone in a similar way moved the frequency tuning and response threshold of IC cells towards the frequency and intensity of the priming tone (Poon et al., 1990; Poon and Chen, 1992). These changes could be closely associated with the acoustic experience during a ‘critical’ period, likely around the time when the external auditory meatus first opens in these animals (about day 9 or during week-2 in rats). Since IC is an important center for processing time-varying signals, complex sensitivities to sounds may also be altered in addition to changes in simple response properties.

In this study, we explored the details of the plastic changes at IC following a neonatal tone exposure, in particular the presence of a ‘critical’ period was tested for. We adopted a combined approach of single unit electrophysiology and activity marking. The single unit recordings would reveal functional changes like complex response properties. The activity marking study would visualize more global changes in the whole IC, in particular the tonotopic structures which may be difficult to be revealed by single unit recordings.

An activity marker, Fos-protein, was used. Fos has been found to reveal tonotopic structures in the auditory system following brief stimulation with tones (Friauf, 1992 and 1995). It is a transcriptional factor induced by activating an immediate early gene, c-fos. The transcriptional level of Fos is low at quiescent neurons but it can be rapidly increased at with acoustic or electrical stimulation in the auditory system. Its functional role, though not completely clear, is considered to be related to among other things, compensation of the store of neurotransmitters. Results of Fos immunohistochemistry in the auditory brainstem are in general consistent with radioactive glucose-uptake experiments (Scheich and Zuschratter, 1995). This finding has been taken to reflect its application as an activity marker (ref??). The expression of Fos is localized to the level of individual nucleus of the cell (Sagar and Sharp, 1993; Sharp et al., 1993). Such high spatial resolution is desirable to determine regional
differences in neural activity of the brain, and hence fine tonotopic structures.

Results from both electrophysiology and Fos-immunohistochemistry showed that changes at the auditory midbrain of young rats are related to the acoustic experience in week-2, a time likely overlaps with the ‘critical’ period. The changes further included a response preference to complex sounds.

**Methods**

*Animals and sound exposure*

Young rats (Sprague Dawley strain) were raised with their mother in a sound-treated chamber (1.5x1.5x2.5 m³). Starting from postnatal week 2 (or on day 8), they were exposed to a pure tone (4 kHz, 65 dB SPL measured at the site of the animals) through a free-field speaker for one week on a half-day schedule (22:00 to 8:00 hr). The control group was raised in the same environment but without the priming tone.

To determine for the presence of a ‘critical’ period, experimental rats were exposed to the priming sound during the postnatal week-3 and they were again studied for electro-physiology and immunohistochemistry during week-4 and results were compared with the age-matched control.

*Electro-physiology*

During the week after tone exposure had stopped, the young rats (total n=40, including 20 experimental, 20 control) were anesthetized with urethane (Sigma, 1.5 g/kg, i.p.) and skull overlying one side of the auditory brainstem opened. The animal was mounted onto a special head holder and placed inside the sound-treated room (Industrial Acoustic Corporation). Free-field acoustic stimuli were delivered via a speaker placed 70 cm in the horizontal plane 30° in azimuth contra-lateral to the side of recording. This placed the sound source in the direction of the acoustic axis for maximal stimulus effects.

Single unit activity was recorded with micropipette electrode filled with 3M KCl solution with a fine tip (resistance: 30 - 80 MΩ), which was advanced into the brain using a stepping micro-drive (Narishige). Electrical signals were pre-amplified (Axonprobe-1A), amplified (Princeton Applied Research 113) and further filtered (0.1-3 kHz) to suppress noise. Spike signals (amplitudes >2 mV against a background of 0.5 mV) were conditioned with a level discriminator into 0.5 msec TTL pulses. Their times of occurrence were stored in a computer (Hewlett Packard E40 equipped with an ADDA interface Tucker Davies Technology DD1) for off-line analysis. After an auditory unit had been identified by its click-evoked response, the micro-drive stopped advancing, and the unit was characterized with a battery of acoustic stimuli: first, frequency responses to tone stimulation were assessed in terms of best frequency
(BF) and minimum threshold (MT). To further characterize the unit’s response area, a slow tone sweep was generated by controlling the frequency of an oscillator (Tektronix) with an exponential waveform delivered from the ADDA interface. The carrier frequency of the tone was set at the unit’s BF and the intensity systematically varied across 60 trials (at 0.5 - 1.0 dB attenuation/trial) to cover its response in the sub- and supra-threshold regions. Thus the response area (RA) in the frequency-intensity plane of the stimulus tone could be revealed by an area of increase in unit discharge. This method has been found to be efficient in characterizing RA of most IC cells (Chiu and Poon, 1997).

At the end of experiment, the final recording site was marked by electrophoretic injection of Chicago blue. The rat was perfused with saline via an intra-cardiac catheter and then with 10% formaldehyde for histological confirmation of recording sites.

**Immuno-histochemical study**

During the week after the tone exposure, experimental young rats (total n=40, 20 experimental, 20 control) were first transferred into the sound-treated room (Industrial Acoustic Corporation) for 8 hrs (6:30 to 15:30 hrs) to minimize background Fos expression. Sound was delivered through a free-field speaker to the animals for 30 minutes. The sound was either (a) a 4kHz, 65dB SPL pure tone or (b) a 4kHz FM tone that varied from 2 to 6 kHz in a pseudo-random fashion (for details of stimulus please see our previous work). The same FM signal had been found to be powerful in eliciting IC response around 4 kHz. Then 60 minutes after sound stimulation, animals were anesthetized with urethane (1.5 g/kg, i.p.) and perfused through an intra-cardiac catheter, first with normal saline then with 4% paraformaldehyde in phosphate buffered saline. The remaining procedures were conventional Fos-immunohistochemistry as described briefly below.

The brain was removed from the skull and put in the same fixative solution overnight, before transferred to 30% sucrose phosphate buffered saline for 2 days. Frozen sections (40 μm) were cut in the coronal plane and incubated in the following solutions, interspersed with phosphate buffered saline (PBS) rinses: (a) 1:4,000 dilution of c-fos antibody (rabbit polyclonal IgG, SC52, Santa Cruz Biotechnology) with normal goat serum (1:250) and Triton (0.02%) in PBS (or PBSX) for 2 days at 4 °C; (b) biotinylated anti-rabbit IgG (1:1,000, Vector) in PBSX (1.3%) for 4 hrs at room temperature; (c) avidin-biotin complex solution (1:400, Vector) in PBSX (1.3%) for 2 hrs at room temperature. Sections were washed 3 times in PBS and 0.1 M acetate buffer. Sections were finally reacted with glucose oxidase-nickel-diaminobenzidine (DAB) solution for 15 minutes. Sections were washed twice in
0.1M acetate buffer before mounted on glass slides.

**3D reconstruction of the Fos-positive stains in the IC**

Since the IC is a 3-dimensional structure, a special display program is implemented to visualize the distribution of the Fos-positive cells. All Fos-positive stains were first examined under microscope (Nikon E400) at high magnifications (100x to 400x) and only those appeared most darkly were considered positive. Their locations in the IC were marked on a hard copy of the field image grabbed by a digital camera interface (CoolSnap) at a lower magnification (20x, Fig. 1A). It required over 30 serial sections (or total thickness of 1.2 mm) visualizing the whole IC. The boundary of IC and the locations of Fos-positive stains were manually digitized (SummaSketch III, for details see Xu et al., 1990), and reconstructed with a software (written in Visual C) specially developed to visualize them.

----------(Insert Fig. 1 about here)----------

**Data analysis**

For the electro-physiology experiment, the number of neurons with BF within 3 to 6 kHz was counted and their proportion to the sampled population determined. The two-sample test for binomial proportions (test of independence) was used to assess the difference between the experimental and control groups. The mean MT of these neurons with BF around 4 kHz was further compared with the two-sample Student-t test. In both tests, a $p$ value of $<0.05$ was considered significant.

For the immuno-histochemistry experiment, the number of Fos-positive cells (or those darkly stained nuclei) was counted on each section of the IC at the area of interest. Their positions, in the case of forming an apparent band, were determined with respect to the distance from the dorso-lateral border of the IC and expressed as percentage in depth from the lateral border from the IC (Fig. ??). The overall conformation of the band was qualitatively compared across animals by viewing it at different angles.

**Results**

*Electro-physiology*

A total of 575 units (340 experimental, 235 control) were collected from the central and external nuclei. A striking difference in the distribution of BF and MT was found between the week-2 exposed groups and the control. Such differences were less apparent when tone exposure was delayed to week-3.

With sound exposure in week-2, a clustering of response characteristics of units was found closely related to the priming tone, in both frequency and intensity (i.e., at 4 kHz and 65 dB SPL). The clustering was also discernible in the histograms of either BF or MT in the experimental group. The difference in MT distribution was
significant \( (p < 0.01, \text{Student } t\text{-test}) \). In addition, there were more neurons at the 4 kHz bins in the experimental group (14.1\% versus 6.4\%) with a significant difference \( (p < 0.005, \text{Normal theory test}) \). Such apparent clustering in BF and MT was not found when the animals exposed during week-3. This time-dependent changes strongly indicated the involvement of a ‘critical’ period most likely in week-2.

In the week-2 experimental group, over half of units (52.9\%) with BF around 4 kHz responded to the slow tone sweep and among them over half (55.6\%) displayed a complex RA we called ‘multiple-frequency-band’ (MFB) RA. These units all responded to FM stimuli. In contrast, almost all units with BF around 4 kHz in the control group showed RA with single frequency band and they were not sensitive to FM tones.

\[\text{----------(Insert Fig. 2 about here)----------}\]

\textit{Fos immunohistochemistry}

Results of Fos-labeling in IC viewed with 3D display were in general consistent with those showed in single sections, although the conformation of the labeled cells was much easier to view. Differences between the control and experimental groups can be shown more clearly, in particular, when results across animals were compared. Striking differences in the IC were found between the week-2 experimental and control groups. In the experimental group, when a 4 kHz tone was again delivered briefly at 65 dB SPL, Fos-positive stains were found scattered throughout the whole IC but never forming a band-like structure like the control. In the control, the cluster of Fos-positive stains appeared in a band-like conformation along the presumable iso-frequency laminae, or specifically when viewed from the lateral aspect, having an elongated structure that shaped like a head with a tail. The conformation was in general consistent with the band-like structure as described in the literature.

Conversely, when induced with a 4 kHz FM tone of a similar level, a band of Fos-positive stain appeared around the similar depths. But more Fos-positive stains were found in the experimental group. Interestingly, two clusters of Fos-positive cells were observed in the central nucleus of IC. One cluster appeared at the dorsal part corresponding to the iso-frequency laminae of 4 kHz. Another cluster appeared at the central IC likely corresponding to iso-frequency laminae of 8-9 kHz. What accounted for the 8-9 kHz band was not clear at this point, but this was studied further by looking at the cochlear nucleus (see below).

For the week-3 groups, the Fos-expression was more similar in the experimental and control groups. Both the 4 kHz pure tone or the 4 kHz FM tone induced a band-like expression of Fos-positive stains in the 4 kHz frequency laminae (from \( \text{??-??} \mu \text{m in depth from the lateral border of IC} \)). FM stimulus induced more Fos-positive stains that appeared across a greater depth. These findings are consistent with the known
depths of these isofrequency laminae in the IC. The main difference on the Fos-positive bands between the week-3 experimental groups and the control was related to number of Fos-positive stains. The experimental IC showed slightly fewer stains in the 4 kHz band.

---(Insert Fig. 3 about here)---

**Fos expression at the cochlear nucleus**

To explore on the mysterious 8-9 kHz Fos-positive stains at the IC, the picture of Fos-immunohistochemistry was examined at the cochlear nucleus (CN). After induction by a 4 kHz tone, Fos-positive stains were found clustering into a clear band in both the dorsal (DCN) and ventral (VCN) divisions of the CN. They corresponded to the iso-frequency laminae of 4kHz as described in the literature (ref??). When induced by a 4 kHz FM tone, more Fos-positive stains could be found in both DCN and VCN, again in positions similar to those induced by pure tone though more dispersed.

---(Insert Fig. 4 about here)---

In the week-2 experimental group, Fos-positive stains were again found at similar locations of the DCN and VCN when induced with a 4 kHz pure tone although their numbers decreased somewhat compared with the control. When induced with the 4 kHz FM tone, there was little difference between the experimental and control groups. No signs for the presence of any 8-9 kHz bands were evident. Again, in the experimental group, CN appeared free from Fos-positive stains in the absence of tone stimulation even though the IC showed diffused labels of Fos.

---(Insert Fig. 5 about here)---

**Discussion**

In the rat, the external ear canals usually open around day 9 and the neural response to sounds appeared around day 10. It is likely that the auditory experience during this week is particularly important in shaping the response properties of cells in the central auditory system. Our finding of a ‘critical’ period in the midbrain is consistent with earlier studies, specifically, in that over-stimulation during the initial weeks after birth produced plastic changes in auditory responses. This over-stimulation could result in over-activity in the neurons at least at the beginning. How the over-activity could lead to plastic changes remains unclear at this stage. Perhaps by staining those IC neurons with altered responses with an intracellular dye or by intra-cellular recording of their responses to sound could reveal more details of the underlying mechanisms of plastic changes.

The importance of neonatal period as ‘critical’ is consistent with experiments in other sensory systems and in other animal species. Our preliminary results suggested
that such changes could last over a longer period of time with sound exposure for only one week. Since over a period up to 3 weeks after tone exposure in week-2, similar changes in response properties and Fos-immunoreactivity could still be observed (data not shown). It is not possible for us to determine the exact time course of the ‘critical’ period in this experiment. Since for technical reasons, we only tested two time windows (week-2 and -3). It is likely that the ‘critical’ period may extend beyond week-2, since we found small differences between the week-3 experimental group and the control. Our previous experiment using a longer exposure time (3 to 5 weeks after birth) appeared to have stronger effects on the clustering of BF. We cannot rule out the possibilities that the ‘critical’ period may also differ depending on the sensory stimulus used, the kind of response tested (e.g., we tested only BF, MT and FM sensitivity) and the level of the auditory system (e.g., IC or CN) examined.

In this study, a mild tone (65 dB SPL) was chosen to avoid damages to the peripheral organ. This could be important for inducing plastic changes in the central auditory system through over-activity. Since the exposing sound needs to be sufficiently soft to preserve the integrity of the peripheral system, even though the auditory thresholds of rat pups at 4 kHz during the neonatal stage tend to be higher than the adult. The normal-looking Fos-immunoreactivity at CN suggested that the cochlea inputs in the experimental groups were basically preserved. With exposing tones at 80 dB SPL, deprivation of BF around the exposing tone was reported in a similar experiment (Sun et al 1998). One must therefore be aware of the consequences in choosing loud sounds as the priming stimulus.

A shift of FM sensitivity towards the exposing tone in the week-2 experimental group suggested that the plastic changes at the IC could be associated with the primary functions that the cells normally serve, i.e., complex tone sensitivity. Furthermore those neurons with BF around 4kHz showed complex RA. It is likely that the exposing sounds reaching the ears of the rat pups could have been perceived somewhat differently. For instance, a pure tone could sound like FM if the animals had been moving a lot in the cage. In fact, we noticed that pups were usually rather mobile. Hence, it could explain for the shift towards FM sensitivity.

Alterations in response properties of IC cells were well correlated with activity changes as revealed by Fos-immunohistochemistry, again confirming the usefulness of Fos as an activity marker. A decreased Fos expression in the 4 kHz band in the week-2 experimental IC may not be solely related to the short-term adaptation to the 4 Hz tone. Since the observed changes in Fos-expression remained rather stable up to 3 weeks after the priming tone had terminated (data not shown). While Fos is thought to express well with a novel stimulus (Chen et al., 1996; Keilmann and Herdegen, 1996; Morgan et al., 1987; Curran and Morgan, 1995; Ikeda and Nakagawa, 1998), there is a
trend for the expression to decline with prolonged stimulation. For the stimulus
paradigm we used, the novelty effects for Fos likely became less marked due to the
relatively long interval (i.e., up to 1 week) between the termination of sound exposure
and the start of immunohistochemistry experiment. Our results on the week-3
experimental groups showed that IC neurons around 4 kHz could still express Fos
under brief stimulation. In our experimental rats of week-2, even CN failed to express
Fos to a brief 4 kHz tone. This suggested that in addition to a shift in FM sensitivity at
the IC, there could also be an elevation of thresholds at the CN or in the auditory
periphery. Whether or not such changes in the periphery could be related to cochlear
damages or to the auditory efferents remains to be explored.

When stimulated with a 4 kHz FM tone, IC of the week-2 experimental group
showed two Fos-positive bands. One corresponded to the 4 kHz in depth and another
around 8-9 kHz. The presence of 4 kHz band suggested that cells at 4 kHz were still
able to be activated by FM sounds and express Fos to brief stimulation even though
the expression to 4 kHz pure tone was basically absent. The extra band at 8-9 kHz
was unexpected. In addition to the two bands, there was a diffused appearance of Fos-
positive stains in response to a 4 kHz FM tone. Such diffused Fos-labels were found
in the experimental IC even without sound stimulation [please check if this statement
is true]. Furthermore the increase in Fos-positive stains in the IC was not paralleled by
an increase in Fos-expression at the CN, suggesting a central origin of the activity at
the IC. This finding suggested that the experimental animals could be suffering from a
mild form of tinnitus of central origin. The appearance of an extra Fos-band near 8-9
kHz in the IC is similar to Fos-expression of those rats, which tinnitus was supposed
to be induced by salicylate administration (Wu et. al, 2000). Similarly in the
salicylate-treated animals the abnormal Fos-expression at the IC is thought to be of
central origin as it occurs in the absence of activity changes in CN. Our preliminary
observation in electrophysiology that spontaneous activity in IC also appeared to be
elevated further supported such conjecture of tinnitus in the week-2 experimental
animals. Since the 8-9 kHz is also the part of audiogram where the hearing is known
to be most sensitive for this animal. It could be related to changes in the periphery and
the auditory efferent system. The finding that the 8-9 kHz band was absent in the
week-3 groups ruled out the possibility of the presence of spurious sounds in the
sound-room. If neonatal sound exposure during in the ‘critical’ period indeed induced
tinnitus, we will have a new animal model for tinnitus. However, the similarities and
differences between one with tone-exposure and the other with salicylate treatment
remain to be determined before it can be established as a tinnitus model.