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探討早年聽覺經驗對於中腦聽神經元突觸發育之影響與其分子調控機轉

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中文摘要

大腦從出生後仍具有可塑性。例如，聽覺皮層與中腦聽神經元之反應特性會因早年的聽覺環境而改變，而且其神經反應特性改變的結果會因受到環境聲音種類與開始接受此種聲音刺激的時間而有所不同。在文獻中已知以種因細胞活性改變而引起的神經可塑性，不僅會表現在反應特性上也會呈現在神經元的型態與神經迴路連結上。關於此種活性誘發的神經型態與迴路變化的假說有兩種，一種室認為活性會調控新突觸的形成，第二種則是認為活性會影響已形成的細胞突觸減少的速度或是其穩定性。在聽覺系統的研究中，不論是在動物實驗或是離體的單細胞或組織培養的實驗中均證實，若減少訊號傳遞而造成細胞活化機會減低的狀況下，其樹突的密度會降低，突觸的形態結構也會改變。至於，是否當增加聲音訊號輸入時，是否能引起神經元樹突密度，分布範圍，與突觸形態上有所改變則仍不清楚。因此本實驗的目的是要探討早年聽覺經驗除了可造成過去結果已知的中腦聽神經元反應特性改變外，是否亦會改變神聽元的型態結構，樹突密度與分部範圍，以及突觸的結構等。而此一神經形態上的改變是否只侷限在訊號輸入強度改變的區域(isofrequency laminar) 具有活性的專一性；或是此一形態改變的範圍亦會呈現在其他的區域 (laminae beyond the isofrequency laminar)。最後更要探討MAP2與GAP-43是否亦參與此一早年聽覺經驗所誘發之神經形態之調控機制中。

實驗組的幼鼠從出生後的第二週到第五週將接受連續四週，每週七天，每天十小時(22:00-8:00)的純音(4 kHz, 65 dB SPL)刺激。控制組的幼鼠飼養在相同的環境下但是無純音的刺激。幼鼠在聲音刺激結束後，將以單細胞外記錄法確定單一神經元的反應特性，在最佳反應頻率為4 kHz旁以 juxtacellularly iontophoresis的方式注射 neurobiotin於該單一聽神經元旁。在最後一個細胞注射的八小時後，將動物再度麻醉經全身灌流福馬林固定腦組織，再經脫水，切片及標準的染色步驟後，將在顯微鏡下檢查與評估神聽元形態變化情形。

實驗結果初步發現，再經過連續四週的聲音刺激後，中腦4kHz的聽神經元的突觸數目有明顯的增加，其樹突橫跨的範圍亦有增加的趨勢。電生理的實驗結果發現在經過幼年期的長期聲音刺激後，聽神經元的反應特性會變得較為複雜，且有較高比例的聽神經原僅對複雜聲反應。實驗結果初步證明，幼年期的長時間聲音刺激，不僅會造成聽神經元的反應特性改變，聽神經元的細胞型態亦會因為細胞活性的長期改變而在樹突的橫跨範圍與突觸數目上有所改變。

關鍵詞:

神經可塑性、聽覺中腦、樹突、突觸、長期純音刺激

Abstract:

The brain is till plastic after birth. Previous studies showed that characteristics of the central auditory neurons, particularly the cortical and collicular neurons can be

altered after the neonatal sound exposure. Furthermore, the above function changes are highly dependent on the type of stimuli and the onset of the sound exposure. Activities driven neural changes are not showed in terms of the functions but also reflected on neural structures. In the auditory system, both the in vivo and in vitro experiments showed that the synaptic density and morphology are decreased and altered by reducing the input activities. Whether the increased activities, particularly during the neonatal stages, have similar effects on altering the neural structures is still undetermined. Furthermore, if the experience driven plastic changes on the neural structures are restrict on the local areas with the input specificity or globally revealed in the whole relays also remains unclearly. The aim of the study in the first year is to determine whether the neonatal sound exposure have effects on remodeling the dendritic or axonal morphology of the midbrain.

The experimental animals were exposed to a mild sound with frequency and intensity at 4 kHz and 65 dB SPL from week-2 to week-5. Control animals were raised in the same environment without sound exposure. After the end of the sound exposure, animals were first characterized by using extracellular single unit recording technique following the end of the sound exposure. Then, neurons with best frequencies at 4 kHzwere were labeled by juxtacellularly injected with neurobiotin. Eight hours after final injection, the animals will be first anesthetized and perfused with the normal saline, followed by 4% papraformaldehye and standard staining procedures.

In comparison with the control group, neurons around 4 kHz iso-frequency laminae were with a larger dendritic field after neonatal sound exposure. In addition, these neurons were showed an increased complexity in dendritic morphology, specifically an increase in the density of dendritic spines following early sound exposure. The electro-physiological findings revealed that the response characteristics of 4-kHz were complex after long-term neonatal sound exposure. The results suggested that the increased activities, which are driven by neonatal sound exposure, not only changed neurons' responsiveness but also altered the neurons' morphology.

Keywords:
Neural plasticity, auditory midbrain, dendritic spine, sound exposure

Background:

The neural circuitries in the brain are not fixed after the birth. Though the experience dependent neural plasticity can be observed from early neonatal to adult, the plasticity is still most rapid and robust during the neonatal stage. The experience-driven neural plasticity has been studied in the auditory system especially

at the level of the auditory cortical area. For example, the tonotopic maps of the auditory cortex is replaced by retinotopic maps in the congenital deaf human subjects or animals with neonatal ablation of the cochleae (Kujala and Näätänen, 2000; Kral, 2007). Experiments also demonstrate the size of the auditory map is highly regulated by input activities. For example, the size of the auditory maps is significantly reduced after reducing or eliminating the sound inputs by sound deprivation procedures whereas an expanded auditory maps were revealed in functional images of the musicians, which characterized by functional magnetic resonance imaging (fMRI) techniques (Pantev et al., 2003). Recently, a series of studies from Merzenich's group suggested that early tone exposure produced functional changes in the auditory cortex. Specifically, they found an accelerated emergence and expansion of isofrequency areas representing the priming tone, and a delayed development in the overall tonotopicity and the appearance of broader-than normal receptive fields (Zhang et al., 2001, 2002). Zhou et al. (2008) also reported the plastic changes in the auditory cortex also dependent on the temporal structure of the input signals. Specifically, the over-representation of the priming frequency in cortical map was revealed in rats exposed to a pure tone bursts whereas the distorted cortical map with the large-than-normal areas tuned to high frequencies and the smaller-than-normal areas tuned to low frequencies was observed in those rats exposed to a continuous pure tone. However, the above results still could not rule out the possibilities that changes of the cortical organization were partially reflected what already altered at the lower auditory relays. The neonatal sound exposure altered tuning characteristics of auditory neurons have been shown at the midbrain level. For instance, comparing with the naïve control rats, the number of neurons with best frequency (BF) around the priming frequency was significantly increased after exposing to a continuous pure tone during the first month after birth (Poon and Chen, 1992).

In the auditory system, both the sensory deprivation by removing the cochlea and the increased activities by directly stimulating the input fibers or increased the sound stimulation in the environment showed that activities would modify the number of dendritic spines. For example, the cochlear removal in adult or young rats reduced the density of dendritic profiles of the medial superior olivary nucleus (MSO, Russell and Moore, 1999). The similar reduced activities induced changes on the dendrites also observed in the auditory cortex. The spine counts revealed that neonatally deafened rabbits had 38.7% fewer spines along their basal dendrites but no differences between experimental and control rabbits were found in terms of soma cross-sectional area, total number of basal dendrites, total number of dendritic branches and total basal dendritic length (McMullen and Glaser, 1988). On the other hand, the combined methods using the single-cell electroporation, live cell imaging, in vitro

deafferentation, pharmacology, and electrophysiological stimulation showed that local alterations in synaptic input would affect dendritic branch structure in Nucleus Laminaris (NL). They suggested that balanced activation of inputs of NL dendrites is required for maintaining the relative amount of dendritic surface area allotted to each input (Sorensen and Rubel, 2006). However, the above results were either from experiments of sound deprivation or manipulating the input activities in acute preparations. It is still unclear that the over-activities can also alter the neuronal morphology especially on the dendritic field and the number of dendritic spines. Therefore, the first aim of this project is to determine whether the sound exposure not only alter the neural responses but also remodel the dendritic or axonal morphology of the midbrain.

Materials and Methods

Animals: The experimental animals were exposed to tone for 4 weeks from week-2 to -5 in the sound-treated chamber. The control animals were raised in the same sound-treated chamber but without the exposing tone.

Sound exposures:

All the experimental animals will be exposed to a 4 kHz continuous tone on a half-day schedule during the night (22:00 to 8:00 hr). The 4 kHz will be chosen since it is located in the low frequency region of the rat audiogram (Kelly and Mastron, 1977). In previous studies on rats, tones with such spectral property showed to be effective in producing a clustering effect on tuning characteristics (Poon and Chen, 1992). Sound was presented to animals through a free-field speaker (Pioneer SP77) placed at the ceiling of the sound-treated chamber. A moderate intensity level of 65 dB SPL was chosen and confirmed by the measurement with a calibration microphone (B&K 4149) placed at the position of animals. During the week following after the termination of sound exposure, rats were studied in terms of the extracellular single unit recording combined with juxtacellular neurobiotin injection.

Surgical procedures:

Rats were first injected with atropine (0.05 mg/kg SC) at 15 min before anesthetized with Zoletil 50 (Virbac Laboratories, 15 mg/kg, i.m.). Under anesthesia, rats were mounted onto a head holder. For the recording in the IC, the skull overlaying on one side of occipital lobe will be first surgically exposed and drilled opened. To access the midbrain, the dura will be resected. A small stainless steel post will cemented onto frontal skull for subsequent fixation to a special head holder. Rectal temperature was controlled at 38±0.5°C by a thermal pad.

Electrophysiology

Free-field acoustic stimuli will be delivered to the animal inside a sound-treated

chamber (1.5x1.5x2.5 m3) through a speaker (Pioneer SP-77) placed 70 cm in the horizontal plane, 30 degree in azimuth, contralateral to the IC being studied electro-physiologically. Unit responses were first detected using glass micropipettes of fine tips filled with a 2% neurobiotin in 1.0 M potassium chloride. The tip of the glass electrode was placed on the surface of the exposed occipital lobe and it were advanced by a remote controlled stepping micro-drive (Narishige). The neural signal will be first recorded with a pre-amplifier (A-M 1600), and then filtered (300Hz-3 kHz), amplified and monitored on-line using an oscilloscope and an audio system. Spike signals will be conditioned with a level discriminator into 0.5 msec wide rectangular pulses and their times of occurrence will be stored into a computer (HP Pavilion) for offline analysis using a computer interface (Tucker Davis Technology, system III, RZ5). Auditory units will be either identified by their spontaneous activities or time-locked responses to an acoustic click (0.1 msec pulse, 90 dB SPL) usually with latencies between 7-20 msec (details see Poon et al., 1992). After the identification and isolation of an auditory unit, frequency responses to a steady tone were determined audio-visually for their best frequencies (BF) and minimum threshold (MT, Chiu and Poon, 2000). Then, their tuning characteristics were further studied in terms of response area (RA, Chiu et al., 1998).

Injection of tracers

Neurobiotin: Once a unit with specific BF (4 kHz) is identified and characterized, an iontophoretic injection of neurobiotin was placed (2 μA positive pulsed current, 7 s on, 7 s off, for 2 min, Miller et al., 2005) at that location.

Histology

Once the electrophysiological recording and neurobiotin injections were completed, animals were given a lethal dose of Zoletil 50 (Virbac Laboratories) and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed from the scalp and rinsed in 30% sucrose in 0.1 M phosphate buffered saline solution (PBS) overnight, and the next day 40 μm sections will be cut on a freezing microtome and placed into 0.1 M PBS for histology. Tissue will be soaked in 0.3% Triton X-100 in 0.1 M PBS for 30 min followed by three PBS rinses. Tissue will be then soaked in an avidin/biotin solution (ABC, Vector Laboratories, Burlingame, CA, USA) for 1.5 h followed by one rinse in 0.1 M PBS and two rinses in 0.1 M phosphate buffer. To visualize the neurobiotin injections, sections will be reacted with a 0.05% 3,3 diaminobenzidine tetrahydrochloride solution (Sigma, with 0.003% hydrogen peroxide in 0.1 M phosphate buffer). Sections will be mounted on chrome-alum slides, air-dried, dehydrated in ethanol and xylene, and then coverslipped for subsequent microscopic analysis.

Data analysis

All sections will be analyzed using light microscopy. Digital photomicrographs will be taken using a CoolSnap digital camera mounted on a Nikon microscope (Nikon E400). Pictures will be digitally adjusted for color, brightness, or contrast at the time that the photograph was taken, but no further digital adjustments will be made to the photograph of the tissue. High magnification (100X) composite images were created by compiling photographic stacks using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Locations of labeled cells and fibers were manually digitized (SummaSketch III, Xu et al., 1990) and reconstructed with software specially developed to visualize their 3D patterns of distribution.

Results and Discussions:

The neonatal sound exposure altered the BF-MT distribution by over-represented units tuned to the priming frequency (Fig. 1). In addition, the neurons' tuning selectivity and response properties were changed after 4 weeks neonatal sound exposure. Specifically, frequency tunings became broadened at regions around the priming frequency (mean Q10 dropped, $p<0.05$, Student's t-test; Fig. 2B). The effects of sound exposure further extended on the higher frequency regions ($p<0.05$; Student's t-test 2C). Furthermore, most units with BF around the priming frequency showed complex response area and responded selectively to frequency modulated (FM) tones with carrier frequency around the priming frequency.

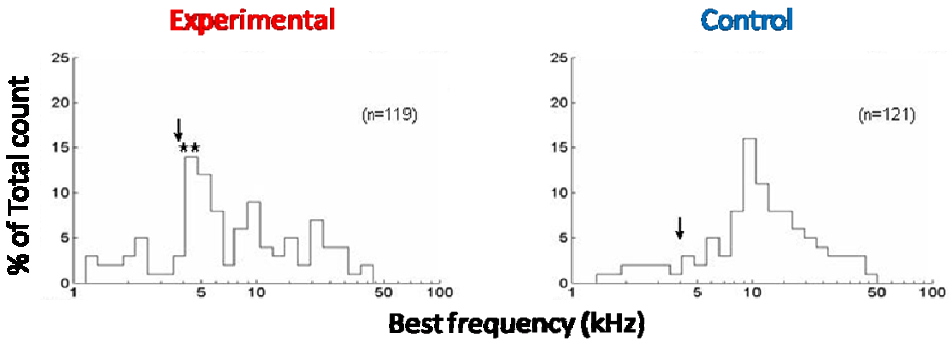

Figure 1: BF histograms of IC neurons that recorded from rats following exposure to a 4 kHz pure tone during the week-2 to -5 and their corresponding control groups. Note the prominent peak in the histogram around 4 kHz (arrows) was found in the experimental animals (**: $p< 0.01$, Chi-square test).

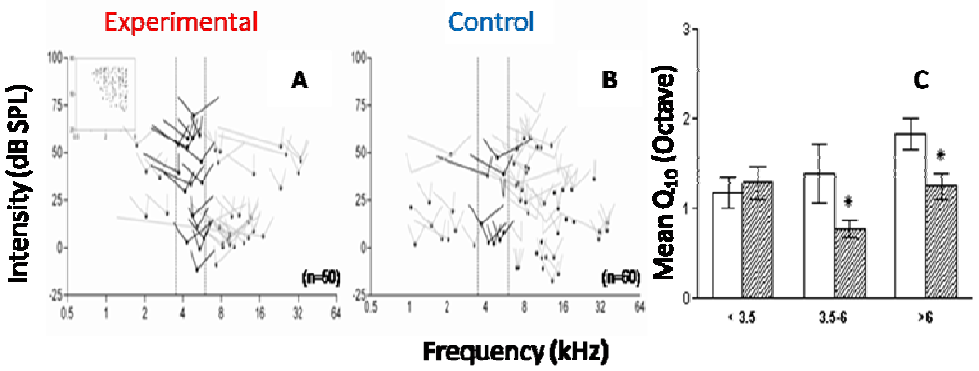

Figure 2: Effects of sound exposures on the frequency tuning of IC units. (A, B): graphic representation of the frequency tuning of individual units in the exposed and control groups: each 'V-shape' symbol is the approximation of the tip of tuning curve (details see Materials and methods: data analyses). Vertical dashed lines mark 3.5 and 6 kHz, or the gross frequency bounds of the exposing tone region; tuning curves of units within this region is highlighted in thick lines. (C): bandwidths of frequency tuning as expressed in Q10 for IC units in the control (open-bars) and exposed group (shaded-bars) according to different frequency regions. Note the broadening of frequency tuning in experimental groups. Insets: representative dot raster of a single unit with BF between 3.5 and 6 kHz.

The over-activities driven plastic changes also revealed on the neurons' morphology. Neurons were showed an increased complexity in dendritic morphology, specifically an increase in the density of dendritic spines following early sound exposure (Fig. 3, 4). Furthermore, Neurons around 4 kHz iso-frequency laminae were with a larger dendritic field after neonatal sound exposure (Fig. 5).

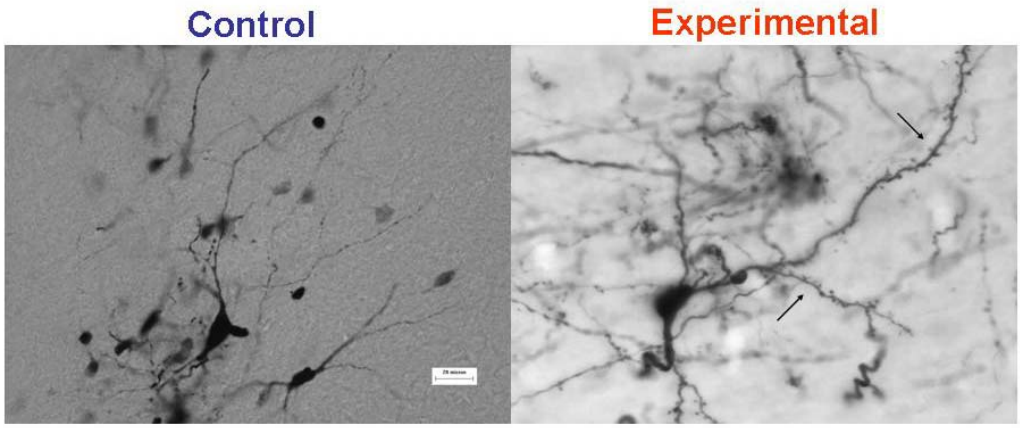

Figure 3: Increased dendritic spines shown in frozen section (40 μm) stained with neurobiotin.

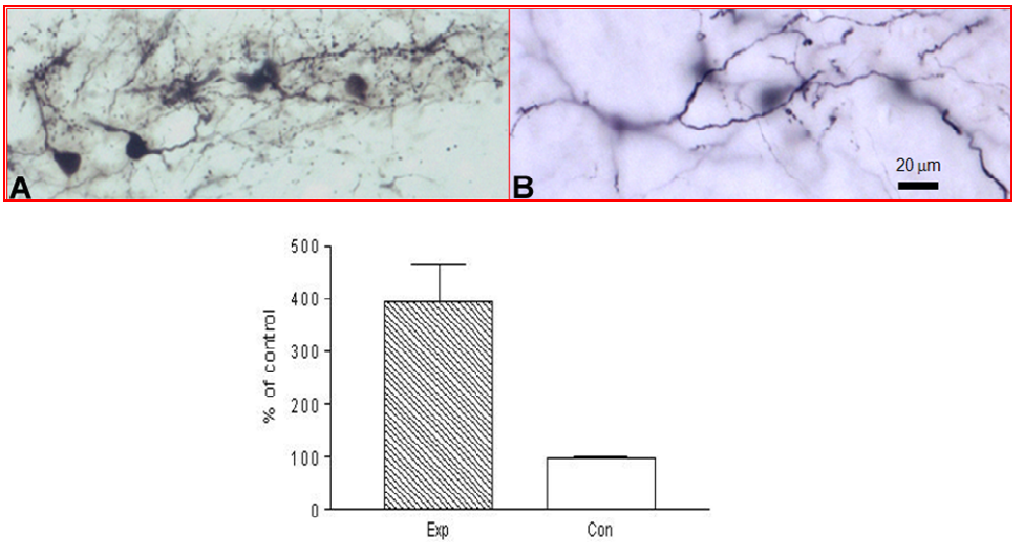

Figure 4: High power computer-enhanced digital photomicrographs showed labeling of boutons along the 4 kHz isofrequency laminar of the neurobiotin labeled region in the sound exposed (A) and control rats (B). The dendritic spines' density (number of spines per unit length) showed increment after week-2 sound exposure (C).

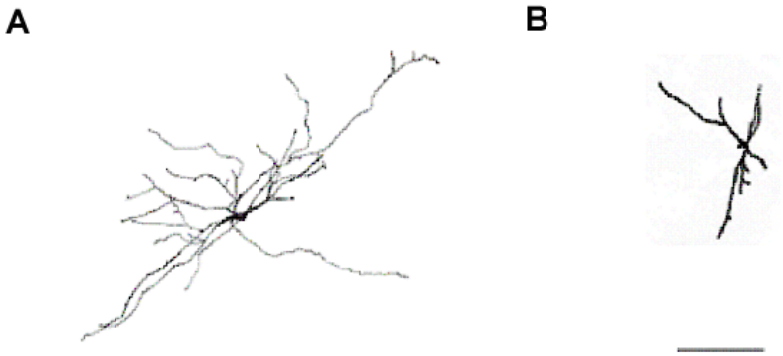

Figure 5: Neurobiotin-labeled cells at the 4 kHz laminae of experimental IC showing larger dendritic field (scale bar=125 µm).

The BF-MT distribution showed the number of neurons with BF at priming tone was increased following neonatal sound exposure is consistent with previous finding (Poon and Chen, 1992). Our results further revealed that effects of sound exposure on changing the tuning characteristics and complex sensitive. The sound exposure not only altered the responses properties of the midbrain neurons but also reshaped the neuronal morphology. We speculated that the increased dendritic spines and dendritic field probably related to neonatal sound exposure increased the FM sensitivities of the midbrain neurons, which had demonstrated are with larger dendritic fields, more

dendritic branching and more dendritic spines than the mixed FM or insensitive cells (Poon et al., 1992). However, the above hypothesized needs to be further confirmed by increasing the sample size and analyzed the histology data in details.
It is still unknown to what extend the effects on sound exposure enhanced on the higher frequency regions also observed on the reshape the neural morphology at the frequency regions. To confirm this issue, the further studies need to juxtacellularly injected with neurobiotin at those units with BF at 8 and 32 kHz. The results can also determine the effects of sound exposure on neuronal morphology will be varied across different frequency laminae. The underlying mechanisms of the activities induced structure plasticity following the sound exposure will be studied in the coming year. The experiment will assess the changes of MAP2 and/or GAP-43 expressions along with the changes at the synapses.

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