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Original Article

Assessment of Synaptic Plasticity via Long-Term Potentiation in Young Mice on the Day after Acoustic Trauma: Implications for Tinnitus

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OBJECTIVE: This experimental study evaluated the pathophysiological association of long-term potentiation (LTP)-mediated synaptic plasticity in tinnitus in 30 BALB/c mice.

MATERIALS and METHODS: Baseline hearing levels and tinnitus perception were examined with startle reflex time and gap detection time measurements using an acoustic stimulus of a 6-kHz pure tone at 90 dB sound pressure level (SPL) on post-natal day 16. The acoustic trauma group was exposed to 6-kHz pure tone at 120 dB SPL on post-natal day 16. On post-natal day 17, the acoustic trauma group underwent re-measurements of hearing levels and tinnitus perception using an acoustic stimulus of 6-kHz pure tone at 100 dB SPL. Fifteen tinnitus-induced and fifteen control subjects were sacrificed on post-natal day 17, and LTP in the dorsal cochlear nuclei of each animal was examined.

RESULTS: With respect to gap detection time, there were no statistically significant between-group differences; however, there was a statistically significant difference between the pre- and post-trauma period in the acoustic trauma group. Moreover, LTP was significantly higher in the acoustic trauma group than in the control group.

CONCLUSION: The results suggest that LTP underlies tinnitus pathogenesis.

KEYWORDS: Tinnitus, noise induced, neuronal plasticity, long-term potentiation, cochlear nucleus, acoustic trauma

INTRODUCTION

Recent investigations suggested that the upper auditory pathways and areas were re-organized during tinnitus. Among the various areas, the spontaneous hyperactivity of the dorsal cochlear nucleus (DCN) was considered to play a critical role in tinnitus [1].

In humans, an acute acoustic trauma is generally immediately followed by an acute tinnitus [2]. Many studies have recently indicated that exposing animals to loud sound can cause them to develop tinnitus [3, 4].

This study aims in developing an acute tinnitus after an acoustic trauma in young mice and in investigating the role of synaptic plasticity via long-term potentiation (LTP) in tinnitus pathophysiology.

MATERIALS and METHODS

Animals

In this study, 16-day-old BALB/c mice, which were produced by the Experimental Researches Centre of the Fırat University, were used. The study conformed to the principles of the Declaration of Helsinki. The study received the Local Animal Ethics Committee Approval (Dated 09.12.2010 and Numbered 118) from the Fırat University.

The mice were divided into two groups: 15 were assigned to an acoustic trauma group (whose tinnitus was certain according to the behavioral tests) and the other 15 were assigned as the control group. During non-experimental hours, the mice were kept with

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their mothers in family-sized cages at 22±1°C under a 12-h day and 12-h night cycle, which was created by artificial lighting. The mice were breastfed during non-experimental hours.

The mice underwent behavioral assessments on post-natal day 16, were acoustically traumatized on post-natal day 16 after behavioral assessments, and were sacrificed on post-natal day 17. DCN of each animal was then electrophysiologically examined.

Behavioral Assessments

All behavioral assessments were performed in a startle chamber with dimensions of 30×30×40 cm, which was placed in a sound-attenuated chamber with outside dimensions of 75×90×60 cm, which was designed to absorb 50 dB sound pressure level (SPL) or lower tones. The sound-attenuated chamber was located in a room with background noise of <50 dB SPL. The walls of the sound-attenuated chamber were 18-mm-wide slats. The slats were completely coated with 2-cm-wide and 10-kg/m^3-dense white styrofoam. The inside layer was coated with 3-cm-wide and 40-kg/m^3-dense rock wool, and the rock wool layer was covered with a 3-mm-wide flexible plywood. No space was left between the layers, and the cover was fixed so that it could be completely opened and closed. Three light emitting diode bulbs were fixed on the ceiling of the chamber for optimum lightening.

A 15-cm-diameter free-field piezoelectric loudspeaker was fixed on the upper side of the startle chamber. During the startle reflex time (SRT) and gap detection time (GDT) measurements, 6-kHz pure tones at 90 and 100 dB SPL were generated using an Interacoustic AC 40 Audiometer (Interacoustics A/S, Middelfart, Denmark). The output signal of the Interacoustic AC 40 Audiometer was amplified using a custom-made amplifier to obtain a 6-kHz pure tone at 120 dB SPL for the acoustic trauma protocol. The intensity of all the acoustic stimuli was calibrated using a Brüel and Kjaer sound level meter. The tone level was determined by adding the estimated tinnitus threshold (25 dB SPL) and the auditory threshold of the experimental animals as formerly described in the literature for this specific age group [5, 6].

The sound-attenuated chamber, startle chamber, and high definition (HD) camera were supplied by Inter Medkom Electronics San. Tic. (İstanbul, Turkey). The testing system was provided by the Department of Ear, Nose, and Throat-affiliated Unit of Hearing, Speech, and Balance at the Dokuz Eylül University School of Medicine.

Each mouse underwent individual behavioral assessments. The experiments were performed between 09.00 A.M. and 01.00 P.M. During non-experimental hours, the mice were returned to their mothers for feeding and rest.

The experiments were performed on post-natal days 16 and 17. During the tests, the mice were randomly exposed to 6-kHz pure tone at 90 dB SPL. The tone intensity was 100 dB on post-natal day 17 in the acoustic trauma group that were previously exposed to the acoustic trauma.

Both SR and GD were measured 12 times for each animal. Four SRT and GDT values were recorded in every session. At the end of the three periods, 12 results were obtained from each mouse. The moment when SR was observed was recorded and defined as SRT. SRT was used to determine the hearing level of the animals. There was no background noise during SRT.

The tone-off period before the sound stimuli was called the "gap." The time when SR was observed was identified after the gap as GDT. The upper limit for the mice to recognize the gap and react (demonstrate SR) was 60 s. If SR was not observed in 60 s, which indicated non-perception of the gap, GDT value was recorded as "60 s" in the statistical analysis. GDT was used to signify tinnitus. SRT and GDT values were measured. The arithmetical means of these 12 results were used in the statistical analyses.

During the experiment, a HD camera was used to monitor the mice. The camera was fixed on the bottom side of the acoustic chamber and was controlled by an external computer. To avoid operator errors, latencies were measured from the HD camera records after the experiments were performed.

Acoustic Trauma Procedure

After the SRT and GDT measurements on post-natal day 16, each mouse in the acoustic trauma group was individually exposed to 6-kHz pure tone at 120 dB SPL for 5 h from 07.00 P.M. to 12.00 P.M. without anesthesia. Acoustic trauma was applied by the piezoelectric loudspeaker as described above, which was fixed on the startle chamber inside the sound-attenuated chamber. After acoustic trauma, the procedure was completed and each animal was returned to its mother.

Brain Slices

A total of 30 mice was decapitated on post-natal day 17, and coronal slices were prepared from both DCN [7]. After decapitation, the head was placed in normal physiological saline. After the brain was extracted from the skull, it was sectioned in the coronal plane on the mid-collicular level, and the rostral surface of the caudal side was glued to a teflon block with cyanoacrylate adhesive (superglue). Slices (180-µm wide) were removed with an oscillating tissue slicer (Frederick Haer; New Brunswick, ME, USA) and placed in recently prepared and oxygenated physiological saline at 33°C. The sections were then moved to another laboratory division, and the oxygenated saline was perfused at 5 ml/min for electrophysiological recordings. Fusiform cells in the sections were visualized with a differential interference optical microscope (Axioscope FS, Zeiss, Carl Zeiss Microscopy LLC; NY, USA) and a 63× water-immersion objective [8].

Solutions

The normal physiological saline was formulated with 130 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 20 mM $NaHCO_3$, 3 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 mM glucose, with 95% O_2 and 5% CO_2 and saturated water of 7.4 pH (25–31°C). The external solution that was used in the current clamp (CC) measurements was formulated with 138 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, with a pH of 7.4. The external solution that was used in the voltage clamp (VC) measurements was formulated with 135 mM tetraethylammonium chloride, 10 mM CsCl, 10 mM 4-aminopyridine, 5 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, with a pH of 7.4 [8].

To minimize errors during the measurements, low-resistant pipettes (3–4 MW) were used. The pipettes were made of borosilicate glass with a 1.2-mm external diameter. They were filled with a solution comprising 110 mM potassium gluconate, 9 mM HEPES, 9 mM ethylene glycol tetraacetic acid, 4.5 mM MgCl2, 14 mM phosphocreatine (Tris salt), 4 mM ATP (Na salt), 0.3 mM GTP (Tris salt); pH was adjusted to 7.4 with KOH. All the chemicals used in the study were obtained from Sigma-Aldrich Co. LLC. (St Louis, USA) [8].

Electrophysiological Protocol

The CC and VC recordings were obtained by the standard whole-cell patch-clamp (WCPC) method using an Axopatch 200 Amplifier (Axon Instruments; Foster City, CA, USA). The data were filtered with a 5–10-kHz low-frequency filter. The CC and VC data were sampled in 10–40 kHz and were digitized using the Digidata 1320 Interface (Axon Instruments; Foster City, CA, USA). The data were recorded in a graphic recorder and transferred to an IBM (IBM Corporations; NY, USA) personal computer. The generation of the stimuli, data recording, and analyses of the digitized data were performed using a pClamp software (version 8.03, Axon Instruments; Foster City, CA, USA). A high-resistance seal (91 GW) was used in the WCPC method. All recordings were compensated for a junction potential of −12 mV. Excitatory post-synaptic currents (EPSCs) were recorded every 30 s [8].

During the LTP protocol, fusiform cells were depolarized from −80 mV to −30 mV, while a 100-Hz 1-s duration stimulus was administered twice to the molecular layer at an interval of 20 s. The EPSC amplitudes were monitored at −80 mV and 0.1 Hz [8].

Ten baseline EPSCs were obtained from each fusiform cell in the first 5 min of the recordings. After the high-frequency stimulation (HFS), the recording continued for an additional 20 min. The arithmetic averages of the baseline EPSCs and post-HFS EPSCs between the 5.5 and 10th, 10.5 and 15th, and 15.5 and 20th min were calculated. An increase of ≥10% of post-HFS 5.5th- and 20th-min period according to the baseline EPSCs was referred to LTP generation [7]. LTP recordings were stable during the experiment.

Statistical Analyses

Numerical data were presented as the mean±standard deviation. A p value of <0.05 was considered statistically significant. The Mann–Whitney U test was used to compare the GDT values of the pre-trauma acoustic trauma and control groups, and the Wilcoxon test was used to compare the effect of the acoustic trauma on pre- and post-trauma GDT values. Pearson's Chi-square test was applied to compare LTP prevalence in the control and acoustic trauma groups. The statistical analyses were performed with Statistical Package for the Social Sciences 160 software (IBM Corporations; NY, USA) for Windows 7 (IBM Corporations; NY, USA).

RESULTS

Table 1 presents the mean of the 12 pre-trauma GDT values in the control and acoustic trauma groups. In the control group, the lowest and highest GDT values were 3 and 23 s, respectively. In the acoustic trauma group, the minimum GDT value was 2 s, and the maximum GDT value was 21 s. The Mann–Whitney U test of the GDT values in the control and acoustic trauma groups revealed no meaningful differences. The mean pre-trauma SRT value in the acoustic trauma group was 1.5222±0.19787 s, while that in the control group was 1.9222±0.28776 s.

In the acoustic trauma group, the minimum post-trauma GDT value was 11 s, and the maximum post-trauma GDT value was 60 s. The Wilcoxon test of the pre- and post-trauma GDT values in the acoustic trauma group (Table 2) revealed an apparent increase in the post-trauma measurements. The mean SRT score was 1.5111±0.25756 s in the post-trauma acoustic trauma group.

The results of Pearson's Chi-square test of LTP prevalence revealed a significantly high rate in the acoustic trauma group (Table 3).

The rate of EPSC alteration from baseline that was observed in mice with LTP demonstrating the change between the post-HFS at 5.5 and 10th, 10.5 and 15th, and 15.5 and 20th min are presented in Table 4 (a negative value indicates a decrease, vice versa). As observed in Table 4, all the recordings were stable in both the groups. LTP in a fusiform cell is shown as a graph in mouse number six in the acoustic trauma group in Figure 1.

DISCUSSION

Behavioral tests have demonstrated that the same agents that cause tinnitus in humans can produce tinnitus in animals [9]. In this study, acoustic trauma was used to generate tinnitus in mice. Furthermore, tinnitus generated by a trauma has a similar etiology to that observed in humans with an acute tinnitus [4].

The pitch in tinnitus that was induced by acoustic trauma was 1–1.5 octaves higher than that of the tone. Acoustic trauma sometimes

Table 1. Mean gap detection time (GDT) values in the acoustic trauma and control groups

Parameter	Groups	Measured value (s)	Standard deviation	p
Mean GDT (s)	Acoustic trauma (pre-trauma)	9.58	1.65	0.604
	Control	10.45	2.87	

GDT: gap detection time; s: seconds

Table 2. Mean gap detection time (GDT) values in the acoustic trauma group, before and after acoustic trauma

Parameter	Groups	Measured value (s)	Standard deviation	p
Mean GDT (s)	Pre-trauma AT	9.58	1.65	0.001*
	Post-trauma AT	36.89	5.08	

GDT: gap detection time; s: seconds; AT: acoustic trauma

Table 3. The long-term potentiation (LTP) prevalence and number of mice with LTP was observed at both the groups

Groups	Number of LTP (–) Mice	Number of LTP (+) Mice	Ratio of the LTP	p
Acoustic Trauma	2	13	86.7%	0.000*
Control	13	2	13.3%	

AT: acoustic trauma; LTP: long-term potentiation

Table 4. The rate of excitatory post-synaptic current (EPSC) alteration from baseline in the mice with given numbers with the long-term potentiation (LTP) was observed, respectively, between post- high frequency stimulation (HFS) 5.5 and 10th, 10.5 and 15th, and 15.5 and 20th min (Negative values indicated decrease, vice versa)

Animals (Group and number of mice	% ESPC alteration (post-HFS, 5.5 and 10th min)	% ESPC alteration (post-HFS, 10.5 and 15th min)	% ESPC alteration (post-HFS, 15.5 and 20th min)
AT 1	37	25	17
AT 3	128	88	104
AT 4	39	31	44
AT 5	73	72	51
AT 6	97	72	54
AT 7	31	29	32
AT 8	30	47	13
AT 10	40	52	48
AT 11	121	128	107
AT 12	38	24	29
AT 13	48	52	35
AT 14	62	65	54
AT 15	44	50	47
C 4	19	14	23
C 5	21	25	24

AT: acoustic trauma; C: control; EPSC: excitatory post: synaptic current; LTP: long-term potentiation; HFS: high-frequency stimulation; min: minutes

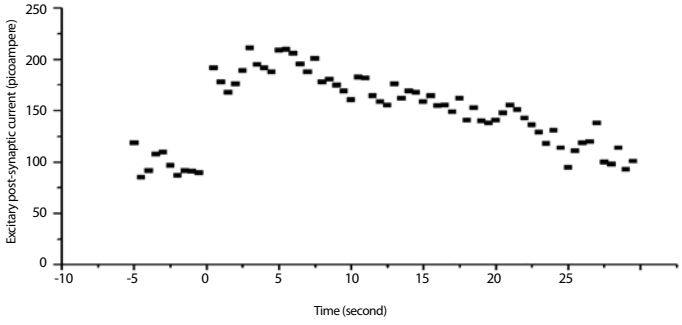

Figure 1. The long-term potentiation (LTP) in a fusiform cell is shown as a graph in mouse number six at the acoustic trauma group. "y" axis refers to excitatory post-synaptic current (EPSC) (pA values), while "x" axis shows the duration of the experiment. High-frequency stimulation was applied to evoke LTP at the point "0"

shifts the auditory threshold by producing a pitch of 0.5 octaves higher than the tone. Such a shift is particularly likely to occur when pure tones are used to produce acoustic trauma. Previous studies demonstrated that acoustic trauma of a long duration affects auditory thresholds in the same way as trauma of a short duration but that the long duration generates relatively stable tinnitus [4].

Behavioral observations can be used to determine hearing levels and tinnitus in laboratory animals. SRT and GDT are often preferred for this purpose, particularly when it is difficult or impossible to perform objective tests as in infants (younger than post-natal one month) and very young animals with small body shapes or ears. These two methods have already been described in detail in the literature. As generally accepted, the magnitude and peak latency of the SR response are the basic parameters of this method [10, 11]. In this study, SRT was used to determine the subjective auditory threshold of the mice, and GDT was employed to detect tinnitus induction. The magnitude of SR could not be evaluated in this study because of technical in capabilities. Therefore, this study was mainly focused on latency results.

The similar GDT values of the groups revealed that tinnitus perception of both the groups was initially similar. After exposure to acoustic trauma, there was a small shift of 10 dB in the auditory threshold of the acoustic trauma group, a finding that is compatible with the literature [4, 12]. The very significant increase in the GDT values after acoustic trauma indicates tinnitus induction. Binaural acoustic exposure can impair temporal processing, and this could be reflected in the latency to respond to an acoustic stimulus. This is, of course, the most significant and challenging aspect of any experiment investigating acoustic trauma-induced tinnitus [13]. However, this study revealed that the SRT values remained stable after the tone was increased to 100 dB SPL, whereas the GDT values were significantly elevated. This result implicated induction tinnitus. The fact that LTP was statistically higher in the acoustic trauma group than in the control group suggests that synaptic plasticity was associated with tinnitus.

There are four main plasticity theories related to tinnitus: injury-induced, temporal, stimulus-dependent, and modulator plasticity [14]. Because of the direct and indirect connections of DCN with the brainstem somatosensory nuclei, cortex, limbic system, and forebrain, they are believed to play a critical role in all plasticity theories, despite some doubts regarding injury-induced plasticity. Tinnitus can be observed without DCN hyperactivity during hearing loss, hair cell injury, or acoustic nerve injury, although activity-dependent hyperactivity, such as LTP, was reported in cases of acoustic nerve excitotoxicity [14].

Many studies identified a significant increase in spontaneous burst frequencies in DCN in cases of tinnitus; others highlighted the multi-layer and organized structure of DCN that facilitated synaptic plasticity induction, such as LTP and long-term depression. LTP can be evoked by an appropriate HFS in fusiform cells because of their cellular and synaptic characteristics [7, 15, 16].

Synaptic plasticity is classified into long and short term, according to the duration of re-modeling [17]. Bliss and Lømo [18] first demonstrated LTP in the rabbit hippocampus. The essential components of LTP and the requirement for synaptic specialization (e.g., N-methyl D-aspartate receptors) have been discussed in detail elsewhere [17]. LTP can be monitored with electrophysiological systems. Among these, the WCPC method is the most commonly used [8].

Fusiform cells are located at the intermediate layer of DCN and are essentially responsible for locating sound. Because of the variable conductance of these cells, they are capable of generating different kinds of response. The manner in which fusiform cells are evoked depends on the actual stimulus, and they can even be potentiated. The apical dendrites of fusiform cells are in contact with parallel fibers and direct synapses in the auditory fibers. They are characterized by spontaneous, long-lasting, and slow onset EPSPs, and they are principally

responsible for establishing a balance between excitation and inhibition. Consequently, they act as synaptic modulators in DCN [19, 20]. Earlier studies described the receptor subtypes and synaptic organization of fusiform cells in detail [7].

Kaltenbach and McCaslin [21] were the first to describe DCN as a tinnitus-synaptic plasticity association center based on a study of hamsters. Similar studies concluded that acoustic overexposure was responsible for DCN hyperactivation. This hyperactivity was first considered to be spontaneous, but later experiments described stimuli-evoked responses [14, 22, 23]. Clinical data on the relationship between synaptic plasticity and tinnitus in humans are insufficient. However, according to a trial by Soussi and Otto [24], the intensity of tinnitus significantly regressed in six of seven patients after electrical stimulation of their DCN. Fujino and Oertel [7] reported that intense electrical stimulation had stable long-lasting neuronal effects on DCN and generated LTP in 11 of 18 fusiform cells using the WCPC method; Tzounopoulus et al. [16] reported similar results. NMDA and calcium channel blockers have been used in an attempt to treat tinnitus in animal models [22].

The role of synaptic plasticity via LTP in DCN of tinnitus remains hypothetical. Brozoski et al. [3] indicated that despite acting as a trigger, DCN does not act as a simple feed-forward source of tinnitus. They suggested that they affect other steps of the auditory system and modulate the experience of tinnitus. Many studies of tinnitus have attributed synaptic plasticity to DCN hyperactivation [14-16]. However, given the long-term effects of LTP on neurons and transcription and translation pathways, with consequent genetic alterations, the study of LTP is more suitable for shedding light regarding the role of synaptic plasticity in tinnitus [17].

The lack of accurate data on the association of tinnitus with LTP is mostly because previous experiments failed to demonstrate whether animals with LTP in their DCN had tinnitus and whether they were related to the absence of non-tinnitus-induced control group [7].

In this study, the mice were tested 1 day after acoustic trauma. We used LTP-mediated spontaneous hyperactivity and synaptic plasticity as a substrate for tinnitus. Finlayson and Kaltenbach [25], using hamsters, examined the effects of intense single tone exposure on spontaneous activity in DCN. Recordings were conducted 5-6 days after the exposure. Pilati et al. [26] examined fusiform cells by the WCPC method 3-4 days after acoustic trauma, but they used juvenile rats. Moreover, some studies preferred to use juvenile animals at postnatal or very early ages to lead a much more severe alteration on neuronal plasticity [27] and acoustic startle reflex [28]. This study used the WCPC method of Fujino and Oertel [7]. The method was only applicable in mice up to the age of postnatal day 17-18 in the aforementioned study. As a technical crux, WCPC recordings become extremely difficult and inconvenient in aged animals particularly because of the age-related brain tissue stiffness. In this study, according to our experimental experience and technical equipment, the WCPC recordings were only applicable up to the age of postnatal day 17 in BALB/C mice. As a general consideration, the sooner the subjects are exposed to a stimulant (e.g., acoustic trauma), the better the synaptic plasticity can be formed. LTP as a substrate for tinnitus was detectable 1 day after acoustic trauma in this study.

In this study, tinnitus was successfully induced by acoustic trauma, and tinnitus strongly facilitated LTP. This evidence confirms that the mechanism that generates tinnitus signals corporates with a long-term plasticity signal. In other words, LTP-dependent synaptic plasticity underlies tinnitus pathogenesis in the mice. Thus, tinnitus can be considered as a plastic re-modeling of the upper auditory system. Technical deficiencies, such as the lack of morphometric methods (immunohistochemistry), and a third complementary subject group for measuring synaptic density are the limitations of our study. However, we hope that the results of this study will guide and provide future experiments.

In light of our results, new treatment algorithms that focused on synaptic plasticity can be attempted in tinnitus. New therapeutic approaches that are tailored to the re-organization of synaptic plasticity may also be effective and successful. However, the association of synaptic plasticity with tinnitus pathogenesis needs to be further analyzed by conducting similar studies with a greater number of subjects and in vivo studies.

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