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ORIGINAL ARTICLE

BDNF and Gad_{65} Responses of Primary Auditory Cortex and Inferior Colliculus to Noise Exposure and Cochlear Ablation

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Objective: To investigate Brain-derived neurotrophic factor (BDNF) and glutamate decarboxylase $(GAD)_{65}$ expressions of rat primary auditory cortex (PAC) and inferior colliculus (IC) in response to noise and cochlear ablation.

Materials and Methods: Of 20, 4 rats were assigned to control group. The remaining 16 rats were divided into noise and ablation groups. Each group was further stratified equally based on timing of sacrifice, 2nd hour and 28th day. The rats underwent stereotaxic surgery in order to obtain tissue samples from PAC and IC. Expression rates of BDNF exon IV and GAD_{65} genes were measured using Relative Quantification with Real Time Polymerase Chain Reaction.

Results: PCR showed that in noise group there was a reduction in GAD_{65} of PAC bilaterally and an elevation in GAD_{65} expression of IC contralaterally at 2nd hour. BDNF expression of PAC showed ipsilateral and contralateral elevations on the 28th day. Ipsilateral BDNF elevation in IC was observed at 2nd hour. Significant elevation of GAD_{65} expression of PAC was detected bilaterally on 28th day compared to 2nd hour levels while there was no change in BDNF in PAC and IC bilaterally. In ablation group, bilateral expressions of GAD_{65} in IC showed an elevation at 2nd hour. GAD_{65} expression in PAC was low bilaterally on 28th day. There was no significant change in BDNF levels in PAC bilaterally in both periods. There was an elevation only in contralateral IC expression of GAD_{65} on 28th day compared to 2nd hour.

Conclusion: Clear differences were detected in GAD_{65} expression of PAC and IC in response to permanent and temporary hearing loss. GAD_{65} expression in PAC is almost exactly opposite to GAD_{65} expression in IC. Whereas GAD_{65} expression in PAC appears to be linked to presence of hearing loss, its expression in IC might be linked to existence of excessive stimuli and permanency of hearing loss. This is also likely to be related to protective action of IC. Ablation did not cause BDNF exon IV expression. The results showed slow reaction of BDNF in PAC in response to acoustic trauma.

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Introduction

Temporary or permanent loss of cochlear sensitivity is believed to initiate some alterations in central auditory pathway. While the central auditory pathway responds to peripheral hearing loss in a certain way, molecular changes in the central auditory pathway is not clearly understood as to whether how it reacts to temporary or permanent conditions. Furthermore, it still remains obscure whether the central auditory pathway's response to different assaults such as noise exposure and cochlear ablation would be the same.

Synaptic inhibition is considered to be crucial to activity of the central auditory pathway. Activity pattern of the central auditory neurons is affected by alterations in the periphery. Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmittter involved in shaping responses of the central auditory neurons, especially primary auditory cortex (PAC) and inferior colliculus (IC). [1,2] Some changes in GABA in several areas of the central nervous system have been reported to occur following an injury. These areas where the changes were seen could be cerebral cortex, cochlear nuclei, superior olivary complex and/or IC. [3,4]

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Persistant elevation in GABA release was reported in inferior coliculus contralateral to the side where the ossicles had been removed. Two peaks of elevation in GABA release were noted on the 5th and 145th days of cochlear ablation. On the 59th day, the GABA release was near the normal.[5] There are two isoforms of glutamate decarboxylase (GAD) that synthesizes GABA from glutamate: GAD_{65} and GAD_{67}. Of those, GAD_{65} is more prevalent.[6]

Brain-derived neurotrophic factor (BDNF) has a key role in neuronal survival, differentiation and adaptive processes in nervous systems.[7] It supports the neuronal survival and encourage the growth and differentiation of new neurons and synapses.[8] Furthermore, BDNF signaling was shown to increase strength and number of GABAergic terminals.[9]

Bearing all these in mind, purpose of the study was to investigate changes in expressions of GAD_{65} and BDNF exon IV in PAC and IC of rats that had undergone noise exposure or cochlear ablation.

Materials and Methods

The study protocol was approved by the Local Ethic Committee of the Hospital (08/81 K-R). All experiments were performed in accordance with the principles of the European Communities Council Directive (86/609/EEC). The study included 20 Wistar Albino rats. All rats were examined under microscopic view and tested with Preyer's reflex and Auditory Brainstem Response test in order to confirm normal ear canal and eardrum, and normal hearing.

Out of 20 rats four were served as control for relative quantization. Based on hearing loss model the remaining sixteen rats were divided into 2 groups, as having 8 animals in each group, noise exposure and cochlear ablation groups. Then each group was further stratified based on timing of sacrifice, 2nd hour and 28th day Therefore, there were 4 rats in each subgroup. For noise exposure group, 113 dB SPL-white band noise from a custom made-generator was delivered to the right ear of noise group through an insert phone for 150 minutes. For cochlear ablation, a large fistula in cochlea was created in the right ears. Then, the cochlea was ablated. Once ABR has been completed at 2nd hour or on the 28th day, the rats underwent stereotaxic surgery in order to obtain tissue samples from PAC and IC.

ABR Methodology

Bioelectric signals amplified X 5,000 (Glonner Neurosys 2000, Krailling/ Germany) were recorded by a computer based-system with a data acquision system of 16 bit (Advantech PCL 816, Cincinnati/USA). All recordings were performed in a sound-proof and electromagnetically-insulated booth. After shaving the skull, needle electrodes were placed subcutaneously in nasion, vertex and mastoid regions. Utmost care was exerted on keeping inter-electrode impedance less than 5,000 Ω. A series of click stimuli was delivered to ears through an insert phone. Rate was 10/sec. The obtained bioelectric signals were band-pass filtered between 159 Hz and 3,000 Hz.

Tissue Sampling using Stereotaxic Surgery

Coordinates of PAC and IC were obtained from rat brain stereotaxic atlas.[10] Both regions were mapped into an A6 paper. The rat was placed in the surgical table in prone position with anterior teeth fixed to the table. Earbars were used to fix the ear canals in the horizontal axis. In order to determine site of PAC, the sites on the temporal bone 3.3 mm posterior and 6 mm lateral (to the right and left) to the bregma were marked. Additional sites corresponding to the PAC were marked. Burr holes on the determined sites were created on the bone using a drill placed on the stereotaxic surgery equipment. Methylene blue was used to mark PAC underneath the tempoparietal bone. PAC in rats is 3X2.5 mm and has a rectangular shape. It is 3 mm deep to the bone. After removal of this bone, the region corresponding to the PAC was removed.

IC is like a bud that measures 2 mm. It is located in brainstem. IC's on both sides were easily localized and removed.

Expression rates of BDNF exon IV and GAD_{65} genes were measured using RT-PCR.

Relative Quantification with Real Time Polymerase Chain Reaction (RT-PCR)

We isolated RNA from both right and left PAC's and right and left IC's. We isolated RNA from a total of 160 tissue materials. RNAs which were used as

reference samples for relative quantification metod were isolated from the control group. We performed the quantification of left and right IC's and PAC's individually. For the reference samples we used the same part of the control group. We performed the RT-PCR within 3 steps.

RNA Isolation

We stored the tissue samples at -80ºC before the isolation step. We stored the tissue samples no more than 1 week. We obeyed the RNA isolation kit instruction certainly (Norgen Biotek Corporation, Total RNA purificatin kit). We eluted the RNA at a 50 µl volume. We stored the RNAs at -80ºC.

cDNA Synthesis

We performed the cDNA Synthesis with oligo (dT) primers at a volume of 20 µl. We certainly obeyed the instruction of cDNA Synthesis kit instructions (RevertAid First Strand cDNA Synthesis Kit/Fermentas Life Science).

Relative Quantification

In the study we had 2 target genes; GAD_{65} and BDNF exon IV and 1 reference gene HPRT1. Except for BDNF exon IV primers the primers used in the study were designed with "Primer Blast" Software (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC= BlastHome).

Primers for the reference gene were HPRT1F (5'-TTG CTC GAG ATG TCA TGA AGG-3') and HPRT1R (5'-CAC ACA GAG GGC CAC AAT G-3'), and they amplify a 56 bp fragment. The primers for GAD65 were GAD65F (5'-CTG GAG GAA ATT TTG ACG CA-3') and GAD65R(5'-GAT GCC CTG TT TAA TCG CA-3') and they amplify a 61 bp fragment. Primers for the BDNF gene were described previously.[11].

The assay was performed using Roche Lightcycler 1.5 instrument and Roche Faststart SYBR Green Master kit. The reaction mixture consisted of 5 µl RNA, 7 µl water, 10 µl 2X Master Mix, a final concentration of 250 nM each primer with a total volme of 20 µl. The reaction mixture was incubated at 95 ºC for 10 minutes. It was then thermal-cycled for 45 cycles as follows; 95ºC for 10 s, 57 ºC for 15 second, 72 ºC for 15 s. After the reactions, melting curve analyses were also performed. The relative standard curves were performed by using serial dilutions. Lightcyler software 4.0 Version was used for preparing the standart curve.

Statistical Analysis

Levels of expressions are represented in the graphs relative to control group value of 1 and the values are presented in median± standard error of mean. Changes in BDNF and GAD_{65} expressions of each subgroup at 2^{nd} hour and on the 28^{th} day relative to control value of 1 were compared in a paired-wise manner using Mann-Whitney test in SPSS software (version 16).

Results

All rats including controls, noise and ablation groups had normal hearing before the interventions.

ABR confirmed temporary hearing loss of 50 dB nHL in the right ears of the noise group at 2nd hour followed by a recovery on the 28^{th} day. The left ears showed normal hearing at 2^{nd} hour and on the 28^{th} day.

ABR showed total hearing loss at 2^{nd} hour and on the 28^{th} day in the right ears of the ablation group. Left ears of that group showed normal hearing both at 2^{nd} hour and on the 28^{th} day.

Noise Group

GAD_{65} Expression in IC: Whereas ipsilateral upregulation in expression at 2^{nd} hour was not significant ($p>0.05$), contralateral upregulation was noteworthy relative to the control value ($p<0.05$). Ipsilateral and contralateral down-regulation on the 28^{th} day was not significant in comparison with neither control expression nor at 2^{nd} hour (p's>0.05) (Figure 1A).

GAD_{65} Expression in PAC: GAD_{65} expressions showed a significant down-regulation ipsilaterally and contralaterally at 2^{nd} hour (p's<0.05). Only a slight upregulation was noted in right and left PAC's on the 28^{th} day. Whereas this upregulation was not significant with reference to expression of control group (p's>0.05), it was significant with reference to only contralateral expression at 2nd hour ($p<0.05$) (Figure 1A).

BDNF exon IV Expression in IC: Whereas BDNF expression at 2nd hour did slightly change contralaterally ($p>0.05$), there was a significant change ipsilaterally ($p<0.05$) with reference to control expression. On the 28th day, there was a slight down-regulation in its expression ipsilaterally and contralaterally. These changes were not significant (p's>0.05) in comparison with neither control expression nor expression at 2nd hour (Figure 1B).

BDNF exon IV Expression in PAC: Contralateral BDNF expression showed a slight elevation at 2nd hour ($p>0.05$). The marked increase in ipsilateral median value of expression was not significant at 2nd hour ($p>0.05$). On the 28th day, a more than two-fold increase was observed ipsilaterally and contralaterally. Whereas these changes were significant in comparison with control values ($p<0.05$), they were significant with reference to the expression at 2nd hour (p's>0.05) (Figure 1B).

Ablation Group

GAD_{65} Expression in IC: Significant upregulation was noted at 2nd hour ipsilaterally and contralaterally (p's<0.05). Contralateral expression decreased on the 28th day. This change was not significant when compared to control expression, but significant in comparison with 2nd hour-expression. Ipsilateral expression maintained its elevated level on the 28th day (p's>0.05) (Figure 2A).

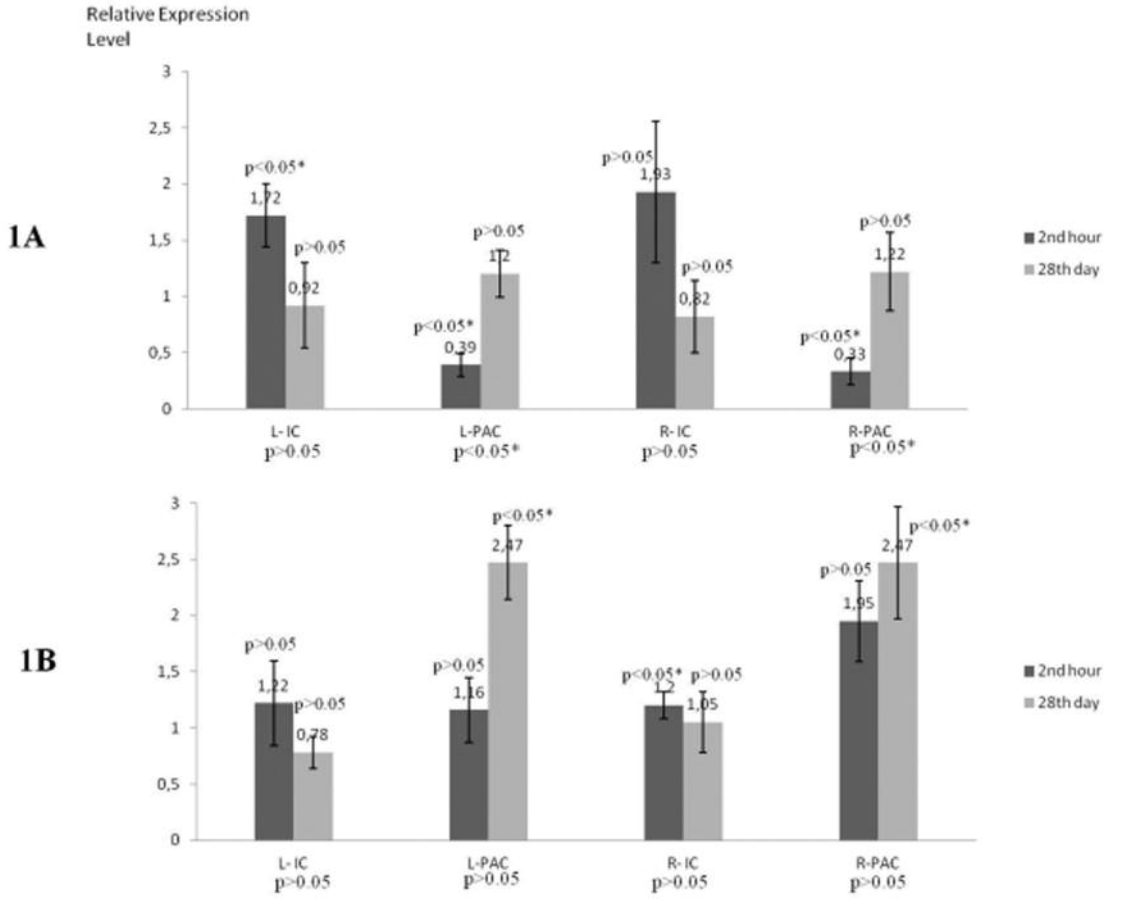

Figure 1. **A.** Relative quantitation of GAD_{65} expression (median± 75th percentile – 25th percentile) in primary auditory cortex (PAC) and inferior colliculus (IC) in noise group. **B.** Relative quantitation of BDNF exon IV expression (median±75th percentile – 25th percentile) in primary auditory cortex (PAC) and inferior colliculus (IC) in noise group. 1 in vertical scale represents normalized value of control group. P values above the columns represent comparison between control and group of interest. P values under x axis represent comparison between 2nd hour and 28th day values.

GAD_{65} Expression in PAC: A slight reduction in ipsilateral and contralateral expressions was observed at 2nd hour (p's>0.05 relative to control expression). A further down-regulation was noted on the 28th day ipsilaterally and contralaterally. This down-regulation was significant when compared to control expression (p's<0.05), but not significant with reference to 2nd hour-expression. (Figure 2A).

BDNF exon IV Expression in IC: BDNF expression was slightly down-regulated ipsi- and contralaterally at both time periods. Any paired comparison regarding these changes were not significant (p's>0.05) (Figure 2B).

BDNF exon IV Expression in PAC: Whereas ipsilateral BDNF expression showed significant down-regulation (p>0.05), contralateral expression was slightly down-regulated at 2nd hour (p>0.05). On the 28th day, a slight upregulation was noted (p's>0.05) (Figure 2B).

Tables 1 and 2 summarize changes in GAD_{65} and BDNF expressions at 2nd hour and on 28th day.

Discussion

Design of this study allowed us to observe differences in responses of ipsilateral and contralateral PAC's and IC's to permanent and temporary hearing loss models. While noise exposure model represented temporary hearing loss, cochlear ablation was a representative of permanent hearing loss in this study.

The results indicated that PAC's of both sides had significantly downregulated GAD_{65} expression in response to noise exposure at 2nd hour compared to unexposed controls. However, when the hearing

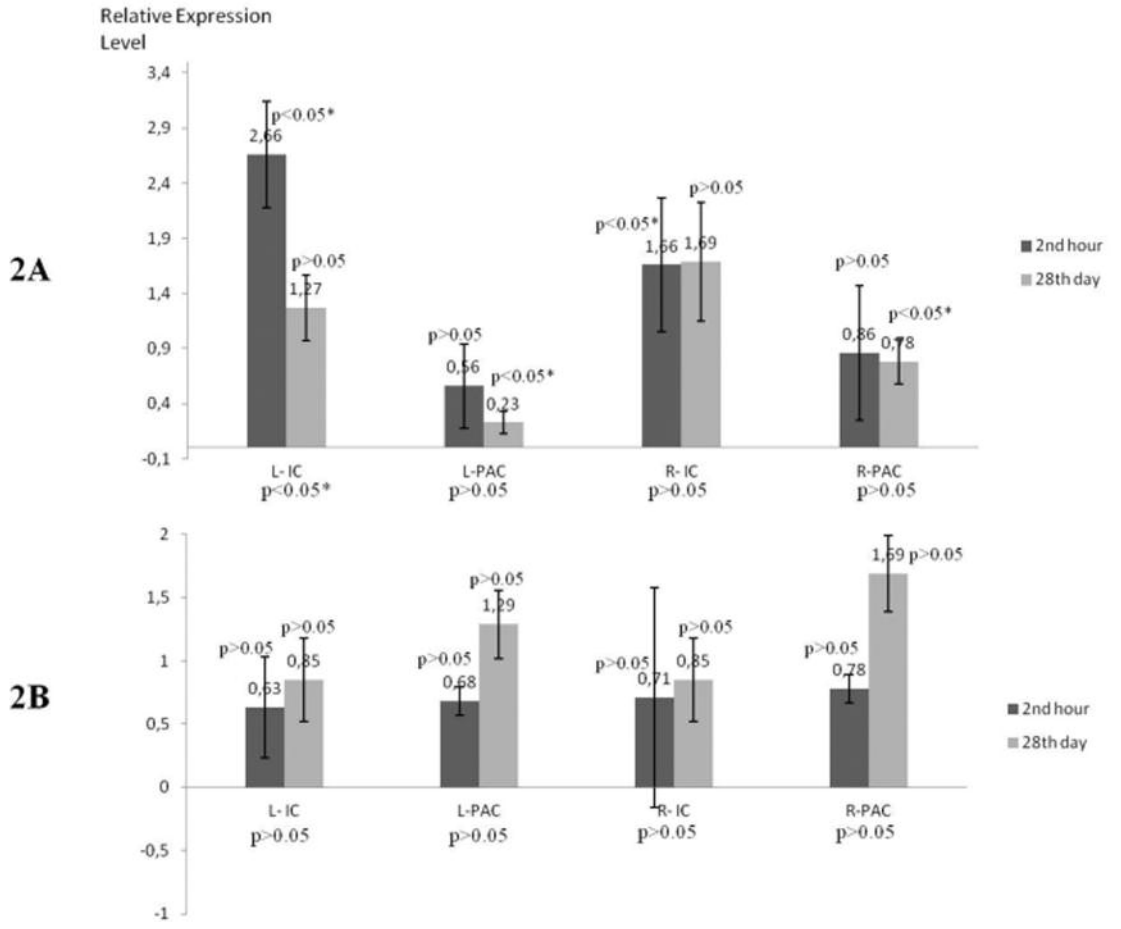

Figure 2. A. Relative quantitation of GAD_{65} expression (median±75th percentile – 25th percentile) in primary auditory cortex (PAC) and inferior colliculus (IC) in ablation group. **B.** Relative quantitation of BDNF exon IV expression (75th percentile – 25th percentile) in primary auditory cortex (PAC) and inferior colliculus (IC) in ablation group. 1 in vertical scale represents normalized value of control group. P values above the columns represent comparison between control and group of interest. P values under x axis represent comparison between 2nd hour and 28th day values.

Table 1. Summary of changes at the time of interest with reference to control value of 1

	Noise Group		Ablation Group	
	GAD_{65}	BDNF	GAD_{65}	BDNF
Right IC at 2nd hour	Ø	↑	↑	Ø
Left IC at 2nd hour	↑	Ø	↑	Ø
Right IC on 28th day	Ø	Ø	Ø	Ø
Left IC on 28th day	Ø	Ø	Ø	Ø
Right PAC at 2th hour	↓	Ø	Ø	Ø
Left PAC at 2th hour	↓	Ø	Ø	Ø
Right PAC on 28th day	Ø	↑	↓	Ø
Left PAC on 28th day	Ø	↑	↓	Ø

Ø: no statistically significant change with reference to control value ($p>0.05$). ↑ : statistically significant upregulation with reference to control value ($p<0.05$). ↓ : statistically significant downregulation with reference to control value ($p<0.05$).

Table 2. Summary of changes on 28th day values with reference to 2nd hour values

	Noise Group		Ablation Group	
	GAD_{65}	BDNF	GAD_{65}	BDNF
Right IC	Ø	Ø	Ø	Ø
Left IC	Ø	Ø	↑	Ø
Right IC	↑	Ø	Ø	Ø
Left IC	↑	Ø	Ø	Ø

Ø: no statistically significant change on 28th day ($p>0.05$). ↑ : statistically significant upregulation on 28th day ($p<0.05$)

became normal in noise group on the 28th day, GAD_{65} expression returned to control levels. In ablation group as being a model of permanent hearing loss, GAD_{65} expression on 28th day showed a gradual downregulation bilaterally with more obvious contralateral downregulationIt would be reasonable to deduce that GAD_{65} expression in PAC is parallel to hearing level. One can further propose that contralateral PAC was affected more obviously which was in comply with crossing pattern of afferent auditory pathway.

For ablation groups, there was a strong elevation in GAD65 expression of bilateral IC's at 2nd hour. In ablation group GAD65 expression of contralateral IC appeared to be more upregulated. In noise group only contralateral expression was elevated significantly. Even though median value of ipsilateral expression seemed to be elevated, there was no significance which might have resulted from small number of samples and wide range of results. Down regulated GAD_{65} expression in IC may be explained with hearing recovery in noise group. However, slightly up regulated ipsilateral and contralateral expressions were noted in ablation group at 2nd hour which might imply a linkage of GAD_{65} expression in IC with or excessive stimulus and permanency of hearing loss.

It was an unexpected finding for us to witness to higher GAD_{65} expression in contralateral IC when there was noise-induced hearing loss at 2nd hour which was opposed to PAC response. Higher GAD_{65} expression might have been a protective effect of IC for PAC. Currently we still don't know as to whether these different responses of IC and PAC arose from counterbalancing effect between the two.

A gradual up regulation of BDNF exon IV expression in bilateral PAC's was noted in response to the noise at both time periods even though hearing was normal on the 28th day. We did not expect BDNF response any kind in ablation group as no nerve damage was created. However, ipsilateral and contralateral expressions on the 28th day was unexpectedly high (but not significantly) which might have been explained with unintentional-intracochlear neural damage during the ablation. No significant change in BDNF exon IV expression was observed in IC's except for ipsilateral expression at 2nd hour in noise group. This limited

upregulation could be accounted for intracochlear neural damage caused by noise application. In general, response of PAC was greater than response of IC in noise group. There was almost no BDNF response in ipsilateral and contralateral IC's as expected.

Abbott et al. (1999) measured GAD_{65} and GAD_{67} levels in rats that were exposed to 10 kHz tone at 100 dB SPL for 9 hours. While GAD_{65} showed slight elevation in IC immediately after the exposure and 2 days post-exposure, it was below unexposed controls on the 30th day. In contrast, GAD_{67} displayed a more than two-fold increase immediately post-exposure. It was well below controls on the 2nd and 30th days. [12] Milbrandt et al. (2000) investigated GAD_{65} immunoreactivity in rats exposed to 12 kHz tone at 106 dB SPL for 10 hours. In contrast to Abbott's and our findings, they found a decline in IC at 0 and 42 hours post-exposure. Then it returned to baseline level.[13] Using Western blotting analysis Mossop et al. (2000) found a decreased ratio of contralateral GAD to ipsilateral GAD in samples from IC at 24 hour and 7 days post-ablation. There was no right and left difference at 4 hour and 1 year post-ablation. [14] Pouyatos et al. (2004) had the rats expose to 97 dB SPL at 8 kHz tone for 6 hours a day, 5 days/week for 4 weeks. Using ELISA they measured a 37% decline in GAD_{67} in IC at 6 weeks post-exposure.[15] Holt et al. (2005) witnessed to an increase in GABA-A receptor subunits β2, β3 and γ2 in IC at 3, 21 and 90 days following bilateral cochlear ablation.[16] Dong et al. (2010) had guinea pigs expose to 10 kHz tone at 124 dB SPL for 1 hour and measured GAD1 along with some other proteins right after, 2 and 4 weeks after the acoustic trauma. Whereas there was no significant difference in ipsilateral IC between the recovery time groups, significant difference was only noted in contralateral IC between the right after group and week 2 group.[17]

Knowledge on GAD or GABA changes in PAC following acoustic trauma or ablation is limited. Sarro et al. (2008) noted reduced GABAA β2/3 subunits in layers 2/3 of auditory cortex in gerbils whose cochleas were ablated at postnatal very early period. Then, they assumed that GABA synthesis might have been upregulated by a retrograde signal of reduced GABAAR.[18] Using Western blotting Xu et al. (2010) investigated effects of noise at early postnatal period on GABAA α1, GABAA α3 and GAD_{65} of PAC, and found a decline in GABAA α1 subunit and GAD_{65} expression and an increase in GABAA α3 relative to controls 24 hours after the noise.[19]

There is only limited data available regarding BDNF expression from PAC and IC following noise exposure and cochlear ablation. Tan et al. (2007) found a decline and an increase in BDNF expression in AC and IC on 6-day post-acoustic trauma respectively.[20] Oh et al. (2007) found decreased BDNF and increased BDNF expressions in PAC following 2 and 4 weeks respectively after cochlear ablation.[21] Meltser and Canlon (2010) studied effects of temporary and permanent hearing loss induced by acoustic trauma on BDNF expression in IC. There was effect of temporary hearing loss on BDNF expression at 30th minute and 24th hour. Permanent hearing loss resulted in upregulation in BDNF at 30th minute and returned to baseline at 72nd hour.[22]

Conclusion

To conclude, the results revealed clear differences in GAD_{65} expression of PAC and IC in response to permanent and temporary hearing loss. GAD_{65} expression in PAC is almost exactly opposite to GAD_{65} expression in IC. Whereas GAD_{65} expression in PAC is parallel to presence of hearing loss, its expression in IC might be linked to existence of excessive stimuli and permanency of hearing loss. This is also likely to be related to protective action of IC. The results showed slow reaction of BDNF exon IV in response to noise exposure which accounts for late increase in response to noise exposure. Clearly BDNF response of PAC was greater than response of IC. Ipsilateral IC had slight BDNF exon IV expression in case of noise exposure. Ablation did not cause BDNF exon IV expression. Late BDNF response of PAC may become noticeable in response to ablation. Further studies are needed since this preliminary report has included limited number of rats only.

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Conflict of Interest: None.

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