Modulation of hearing related proteins in the brain and inner ear following repeated blast exposures

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(Received: March 15, 2012; Accepted after revision: May 21, 2012)

Abstract: Emerging studies show that blast exposure causes traumatic brain injury (TBI) and auditory dysfunction without rupture of tympanic membrane, suggesting central auditory processing impairment after blast exposure. There is limited information on the mechanisms of blast-induced TBI and associated peripheral and central auditory processing impairments. We utilized a repetitive blast exposure mouse model to unravel the mechanisms of blast TBI and auditory impairment. C57BL/6J mice were exposed to three repeated blasts (20.6 psi) using a shock tube, and the cerebellum was subjected to proteomic analysis. The data showed that calretinin and parvalbumin, two major calcium buffering proteins, were significantly up-regulated after repeated blast exposures, and this was confirmed by Western blotting. Since these proteins are reportedly involved in auditory dysfunction, we examined the inner ear and found both calretinin and parvalbumin were up-regulated, suggesting that modulation of these proteins plays a role in blast-induced peripheral and central auditory processing impairments. Expression of cleaved caspase-3 was also up-regulated in both regions indicating ongoing cellular apoptosis, possibly due to altered calcium homeostasis. These results provide a molecular basis for changes in central and peripheral auditory processing involving abnormal calcium homeostasis resulting in hearing impairment after blast exposure.

Keywords: blast exposure, calcium signaling, calretinin, cerebellum, inner ear, NMDA receptor, parvalbumin, traumatic brain injury

Introduction

Hearing impairment and tinnitus are the most widespread dysfunctions associated with traumatic brain injury (TBI) in the current wars [1]. Blast injury produces up to 60% hearing loss compared to non-blast related TBI [2]. It is also one of the most frequent occupational disorders in the United States which is linked to industrial and recreational high intensity noises [3]. In a minor scale, blast exposure includes natural gas explosions, industrial accidents, fireworks, mining and building constructions, and demolitions and homemade bombs [4]. The symptoms of auditory impairment present particular challenges for blast research community due to possible overlapping with post-traumatic stress disorder, mental illness and cognitive deficits, where apparent hearing loss may arise from different underlying psycho-traumatic mechanisms [1, 5]. There are limited approaches to properly assess the severity of central auditory processing impairment after blast exposure, when the tympanic membrane is intact, especially in animal models. Accurate differentiation of auditory impairments from TBI related psychiatric symptoms using specific biomarkers and development of effective treatment strategies require an understanding of the molecular basis of abnormalities in auditory signal processing and perception of sound by the brain after blast exposure.

The incidence of central auditory processing damage in service members exposed to blast in the current war is unknown. Anecdotal evidence from United States Department of Veterans Affairs suggests that a significant number of blast victims maintain hearing sensitivity but have difficulties with hearing noise owing to central auditory processing damage [1]. Blast exposure damages the central auditory processing involved in auditory patterns essential for speech perception and sound localization (http://asha.org/policy/) [1]. Blast shockwave transmission through the skull and reflection in the brain can lead to shearing and stretching resulting in axonal and microcapillary injuries, and subsequent disruption of signal inputs to auditory processing brain stem nuclei [1]. It has been shown that blast exposure damages auditory processing regions in the brain such as inferior colliculus and medial geniculate body, sug-

gesting that blast causes significant effects on the auditory system through the auditory pathway in addition to the possible direct impact on the brain parenchyma through the skull [6]. Detailed investigations on central auditory processing impairments after blast exposure are required to locate the damages in the brain and develop novel tools for clinical diagnosis, prevention, and effective treatments for rehabilitation.

There are limited studies on the pathophysiology of auditory dysfunction following repeated blast exposures. It has been reported that repeated blast exposure causes more severe auditory impairment than a single high level exposure [1]. Repeated blast exposure also caused more severe brain injury especially in the cerebellum [7]. Repeated blast exposure studies using sub-lethal blast levels showed decreased threshold for auditory dysfunction [8].

We had postulated the involvement of shockwave/impulse noise transmission through the auditory/vestibular system in the etiology of blast-induced TBI and associated central auditory/vestibular injuries. In this paper, we utilized the mouse model of repetitive blast exposure with shock tube and studied differential expression of proteins after blast exposure in the cerebellum and inner ear. We demonstrate that blast exposure leads to significant changes in the levels of brain and inner ear proteins involved in auditory function and apoptosis and propose a potential mechanism of blast-induced central and peripheral auditory processing defects.

Materials and Methods

Animals and blast injury model

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. It adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, published by the National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended. Male C57BL/6J mice (8–10 weeks old, 21–26 g) obtained from Jackson Laboratory (Bar Harbor, ME) were used in this study, since the blast TBI model was developed using the same species and the possibility of utilizing genetic knockout/transgenic technologies in the future. Moreover, the same species has been widely used for studying auditory impairment. The animals were housed on a 12-h/12-h light-dark cycle and were provided standard mice chow and water *ad libitum*. All the mice were used in accordance with an experimental protocol that was approved by the Institute Animal Care and Use Committee, Walter Reed Army Institute of Research, and all the experiments were conducted in Association for Assessment and Accreditation of Laboratory Animal Care approved laboratories.

Blast exposure

A well-characterized blast overpressure exposure using a compressed air-driven shock tube described earlier was used for the study [7, 9]. Animals were subjected to repeated blast exposures as described earlier [7]. Briefly, after anesthetizing with 4% isoflurane gas (O_2 flow rate 2 L/min) for 8 min, mice were placed 2.5 feet inside the shock tube in a prone position perpendicular to the direction of shockwaves. The animals were exposed to blast overpressure (20.6 psi) twice with 1 min interval between each blast followed by a third blast exposure at 30 min after the second blast [7]. The blast overpressure of 20.6 psi was selected from earlier studies that showed significant injury with low mortality [7]. Repeated blasts were used to mimic multiple blast exposures in the battlefield. Sham controls received anesthesia but were not exposed to blast. The animals were sacrificed at 6 and 24 h after the third blast exposure. Brain tissues were removed and cerebellum was initially separated for analysis. The inner ear/cochlea was dissected using a dissection microscope and frozen immediately.

Extraction of proteins

Proteins were isolated from the cerebellum of sham control and repeated blast exposed mice (three animals/group) using the ToPI-DIGE™ total protein isolation kit (ITSI-Biosciences, Johnstown, PA). Briefly, the tissue was rapidly homogenized in 50 µL of ToPI Buffer-2 (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% NP-40, 5 mM magnesium acetate, 30 mM Tris-HCl, pH 8.5) using clean disposable plastic pestles supplied with the kit. After homogenization, samples were incubated on ice for 30 min, with four intermittent vortex mixings, and centrifuged at 16,000 *g* for 10 min. Supernatant was collected and the total protein concentration was determined using the ToPA™ protein assay kit (ITSI-Biosciences, PA) according to the manufacturer's instructions.

Two-dimensional differential in-gel electrophoresis (2D-DIGE)

For 2D-DIGE, 50 µg each of total protein was labeled with 200 pmol of Cy3 or Cy5, and Cy2 labeling was used as internal standard using the 'minimal labeling' protocol [10]. The Cy2, and Cy3/Cy5 labeled samples were mixed and co-separated by isoelectric focusing (IEF) with pH 3–10 linear Immobiline Drystrips (GE Healthcare) in the first dimension. IEF was for a total of 65,500 V h in an IPGphor electrophoresis unit (GE Healthcare). The focused strips were equilibrated for 15

min in sodium dodecyl sulfate (SDS) equilibration buffer containing 1% dithiothreitol followed by a second 15-min equilibration in SDS equilibration buffer containing 2.5% iodoacetamide. The strips were then placed on 24×20 cm, 12.5% SDS-polyacrylamide gels and electrophoresed in an *Ettan* DALT6 (GE Healthcare) at 15 W per gel for about 4.5 h.

Image analysis

After the second dimension electrophoresis, all the gels were scanned on a DIGE-enabled Typhoon Trio Variable Mode Digital Imager (GE Healthcare, Piscataway, NJ) using the following excitation/emission wavelengths: Cy2, 488/520 nm; Cy3, 532/580 nm; and Cy5, 633/670 nm. All the images generated (three per gel) were imported into the Biological Variation Analysis module of DeCyder™ software (Version 6.5, GE Healthcare, Piscataway, NJ) for matching, normalization, and identification of differentially abundant spots, with the False Discovery algorithm enabled. The images obtained from sham control samples were compared to the images of the corresponding repeated blast exposed samples to identify the protein spots that showed ≥twofold difference in abundance.

Identification of differentially expressed proteins by liquid chromatography/tandem mass spectrometry (LC/MS/MS)

The candidate spots were picked with the Ettan Spot Picker (GE Healthcare, Piscataway, NJ) and in-gel digested overnight with trypsin using the Ettan Spot Digester (GE Healthcare, Piscataway, NJ). The in-gel digested samples were extracted in 50 µL of 50% acetonitrile/0.1% formic acid for 20 min, dried down completely at 45 °C, and sequenced by LC/MS/MS using a nanobore electrospray column constructed from 360 mm outside diameter and 75 mm inside diameter fused silica capillary with the column tip tapered to a 15-mm opening. The column was packed with 200-A, 5-µm C_{18} beads (Michrom BioResources, Auburn, CA) to a length of 10 cm. The mobile phase used for gradient elution consisted of (a) 0.3% acetic acid, 99.7% water, and (b) 0.3% acetic acid, 99.7% acetonitrile at a flow rate of 350 nL/min. All tandem mass spectra were acquired in a Thermo LTQ ion trap mass spectrometer (Thermo Corp., San Jose, CA) with the needle voltage set at 3 kV. The MS/MS spectra were searched against the NCBI non-redundant protein sequence database using the SEQUEST computer algorithm to establish the protein identity.

Western blot analysis

Polyclonal rabbit antibodies against calretinin and parvalbumin were obtained from Sigma-Aldrich (St. Louis, MO), and rabbit polyclonal antibodies against cleaved caspase-3 was obtained from Chemicon Internationals (Billerica, MA). Secondary antibody labeled with horseradish peroxidase (HRP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to β-actin conjugated with HRP (Sigma-Aldrich) was used as gel loading control. Western blot analysis was performed using tissue homogenates of inner ear and cerebellum using tissue protein extraction reagent (Pierce Chemical Co, Rockford, IL). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 30 µg total protein with precast 10% Tris-glycine gels (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ) using Novex transfer apparatus (Invitrogen, Carlsbad, CA). The membrane was blocked with blocking buffer [4% powdered milk made in phosphate-buffered saline containing 0.001% Tween-20 (PBST)] for 1 h, washed once with PBST buffer, and kept overnight at 4 °C in primary antibody made in blocking buffer. Calretinin, cleaved caspase-3, and parvalbumin antibodies were used at a dilution of 1:1000, and β-actin at a dilution of 1:50,000. The membranes were washed five times with PBST and incubated with secondary antibody made in blocking buffer for 1 h. Secondary antibody was not used in the case of β-actin. The membranes were washed again with PBST, the protein bands were detected using ECL-Plus Western blot detecting reagent (GE Healthcare, Piscataway, NJ), and the chemiluminescence was measured in an AlphaImage reader (Cell Biosciences, Santa Clara, CA).

Results

Effect of blast exposure on the expression of calretinin and parvalbumin

Figure 1 shows representative 2D-DIGE images depicting differential expression of calretinin and parvalbumin at 6 h in the cerebellum after repeated blast exposures. Protein identification by LC/MS/MS showed six matching peptides of calretinin from the protein spot labeled as calretinin. In the case of protein spot labeled as parvalbumin, only one peptide sequence corresponding to parvalbumin was obtained possibly due to the low abundance of the protein in the cerebellum. Both proteins were consistently up-regulated after repeated blast exposures with calretinin showing the highest increase compared to parvalbumin *(Fig. 1)*.

Confirmation of differential expression of calretinin and parvalbumin by Western blotting

Western blotting of cerebellar proteins of sham control and repeated blast exposed mice using calretinin and parvalbumin specific antibodies is shown in *Fig. 2.* Western blotting confirmed the proteomic analysis data showing the up-regulation of both calretinin and parvalbumin after repeated blast exposures. Both proteins showed increased expression at 24 h compared to 6 h post-blast exposures.

Expression of calretinin and parvalbumin in the inner ear

Figure 3 shows the expression of calretinin and parvalbumin in the inner ear of mice at different intervals after repeated blast exposures. The level of both calcium buffering proteins increased in the inner ear after blast exposure. The up-regulation of calcium buffering proteins after blast exposure was significantly higher in the inner ear compared to cerebellum *(Figs 2 and 3)*. Calretinin showed higher expression at 24 h whereas parvalbumin showed higher expression at 6 h post-blast exposures.

Expression of cleaved caspase-3

Expression of caspase-3 in the cerebellum and inner ear showed significant increase at 6 and 24 h post blast *(Figs 2 and 3)*. The increased expression was similar at both the time points after blast exposure.

Discussion

Our studies by proteomic and Western blotting analyses indicate that repeated blast exposures in mice result in alterations in multiple proteins in the brain which are reported to be associated with hearing impairment. Calretinin and parvalbumin, the calcium binding proteins which are found to be up-regulated in the mouse brain cerebellum after repeated blast exposures, are the major calcium buffering proteins present in the auditory system including auditory neurons [11–13]. Evidence indicates that calcium binding proteins play major roles in central auditory processing [14, 15], and our data suggest that blast exposure results in central auditory processing signaling abnormalities. In the cochlear nucleus, they function more specifically in hearing function rather than mere buffering of intracellular calcium fluctuations during signaling. Calretinin and parvalbumin modulation in the brain including the cochlear nucleus has been reported to be associated with hearing impairment [16, 17]. Based on the biological functions

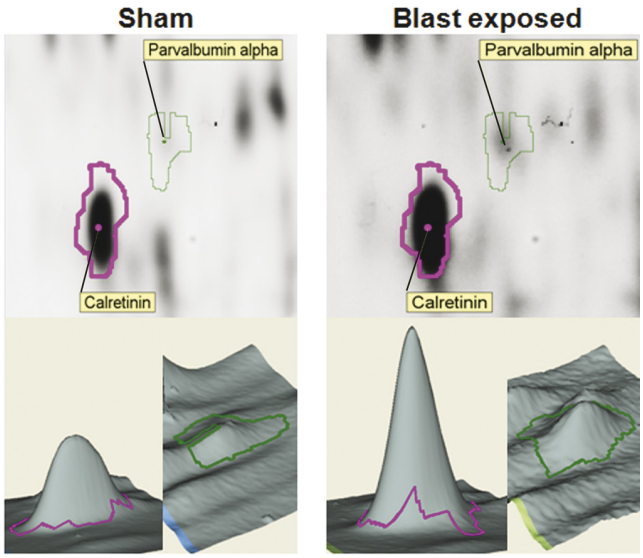

Fig. 1. Proteomic analysis of sham and repeated blast exposed mouse cerebellum samples using 2D-DIGE followed by densitometry and mass spectroscopy. Representative picture (from three different animals) showing the differential expression of calretinin and parvalbumin in the mouse brain cerebellum after repeated blast exposures

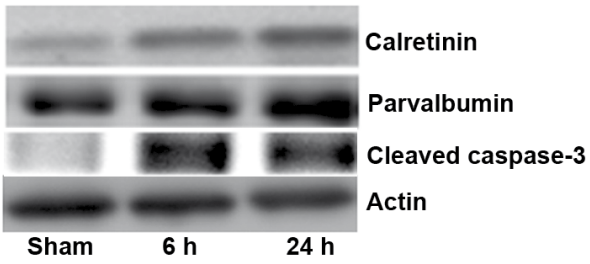

Fig. 2. Western blotting analysis of sham and repeated blast exposed mouse cerebellum samples. Representative picture from three animals in each group. Cerebellum extracts were subjected to Western blotting using specific antibodies to confirm the modulation and identity of calretinin and parvalbumin after repeated blast exposures. Modulation of cleaved caspase-3 was used as a marker of ongoing apoptosis. Western blotting with β-actin is used as loading control

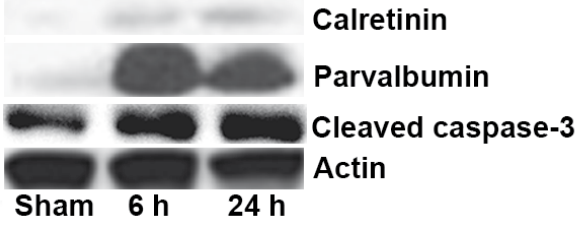

Fig. 3. Western blotting analysis of sham and repeated blast exposed mouse inner ear samples. Representative picture from three animals in each group. Inner ear extracts were subjected to Western blotting using specific antibodies to determine the expression of calretinin and parvalbumin and cleaved caspase-3 after repeated blast exposures. Western blotting with β-actin is used as loading control

of the proteins, we further studied the expression of the proteins at the peripheral auditory region and observed their differential expression after blast exposure.

The cerebellum is considered as one of the key regions in the brain involved in auditory signal processing and sound perception [18]. The role of cerebellum in auditory signal processing was first observed when studies showed that the cerebellum of cats was found to receive auditory signal senses and transmit them to the cortical auditory pathways [19]. Later, multiple studies confirmed the role of cerebellum in auditory signal processing and determined different cerebellar auditory processing areas and their connections to the central and peripheral auditory system [20–23]. Thus, our results involving the cerebellar modulation of proteins involved in auditory function indicate the possible involvement of auditory neurons of cerebellum in the pathogenesis/protection of hearing impairment and tinnitus after blast exposure. In this study, cerebellum was investigated first mainly due to the pronounced effects of blast exposures in the cerebellum compared to other brain regions [7, 24–28]. It is quite likely that similar changes can be observed in other regions of the brain involved in auditory signal processing after repeated blast exposures which needs to be investigated further.

Noise exposure in mice resulted in the up-regulation of calretinin and parvalbumin in the cochlear nucleus in a noise intensity dependent manner, suggesting a possible protective role of the calcium binding proteins in the brain stem after noise exposure [16]. In addition to cochlear nucleus, noise stimulation also leads to up-regulation of these proteins in other regions of the brain including dorsal cortex, inferior colliculus, and commissural nucleus [29]. No studies so far reported the increased expression of calretinin and parvalbumin in the inner ear and/or brain after blast exposure. Our results indicate for the first time a possible role of these proteins in the development/prevention of hearing impairment and tinnitus commonly seen in service members returning from the battlefield.

It has been demonstrated that 24 h after cochlear ablation, a significant increase in calretinin immunoreactivity was observed in the superior and inferior colliculus of adult ferrets, indicating that cochlear-driven activity appears to affect calcium binding protein levels not only in auditory nuclei but also in other neural structures whose response properties may be influenced by auditory-related activities [17, 30]. The expression of calretinin and parvalbumin was found to be up-regulated in the cochlear nucleus with aging and/or associated hearing

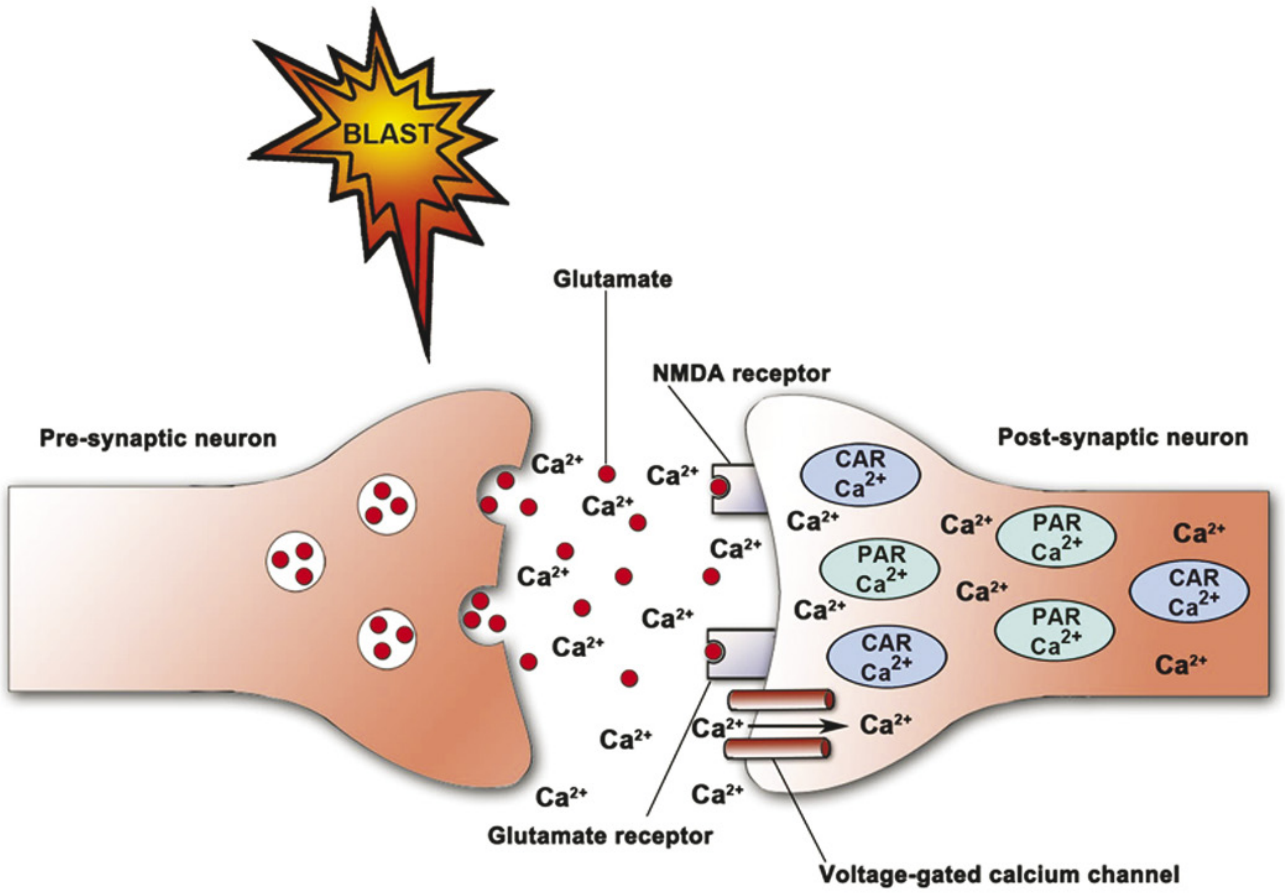

Fig. 4. Schematic representation of the potential mechanism of auditory injury and the role of up-regulated calretinin (CAR) and parvalbumin (PAR) in the brain after blast exposure

impairment, suggesting the role of these proteins in age-related auditory dysfunction [31]. A recent study showed that the expression of calcium buffering proteins including calretinin and parvalbumin decreased significantly in the hippocampus of circling mouse, a murine model of deafness, and suggested that the decreased expression could be related to the loss of auditory information modulating processes in various hippocampal areas [32].

As shown in *Fig. 4*, blast and impulse noise exposure through the auditory system can cause long-lasting depolarization of auditory neurons and release of glutamate leading to glutamate excitotoxicity resulting in calcium influx via voltage-gated calcium channels. Such a potential mechanism for auditory dysfunction has been reported in the case of chemical and noise-induced hearing impairment [33–35]. Thus, therapeutics which can counteract glutamate excitotoxicity such as N-methyl-*D*-aspartic acid (NMDA) receptor antagonists could be used as potential treatments against blast-induced hearing impairment. NMDA receptor antagonists have been found to be effective for protection against noise-induced hearing impairment [36–38]. Voltage-gated calcium channels are reported to be involved in the pathogenesis of acoustic injury in the cochlea [36, 39]. Thus, voltage-gated calcium channel blockers can reduce calcium influx and subsequent damage to the auditory neurons after blast exposure. The up-regulation of calretinin and parvalbumin in the inner ear and cerebellum after repeated blast exposures could be due to the very high demand of calcium buffering in the auditory system. It is quite likely that, in the auditory neurons, the up-regulated calretinin and parvalbumin will bind to the free calcium ions entering through the calcium channels after blast exposure and protect against calcium-induced cell death *(Fig. 4)*. Such a protective role for these calcium binding proteins in auditory neurons has been proposed earlier [29, 40].

In addition to different brain regions involved in central auditory processing system, the inner ear/cochlea involved in peripheral auditory system also significantly expresses the calcium binding proteins suggesting their roles in peripheral auditory processing [13, 41]. Our present data on the up-regulation of calretinin and parvalbumin in the inner ear after repeated blast exposures suggest similar roles for these calcium buffering proteins in the peripheral auditory processing. The up-regulation of calretinin and parvalbumin was highest in the inner ear compared to cerebellum suggesting significant alteration in calcium homeostasis in the peripheral auditory system compared to the central auditory processing regions after blast exposure.

Since altered calcium homeostasis is associated with cellular apoptosis, we determined the expression of cleaved caspase-3 in the cerebellum and inner ear, and our results indicate ongoing cellular apoptosis in both regions. Previous studies have shown increased cellular apoptosis in the brain at different intervals after blast exposure [7, 42]. Cellular apoptosis in the cochlea has been reported as a mechanism involved in noise-induced hearing loss [43]. Thus, increased signalling abnormalities and cellular apoptosis in the central and peripheral auditory processing regions could be potential molecular mechanisms of hearing impairment associated with blast exposure.

Acknowledgements

Supports from COL Paul Bliese, Director, Center for Military Psychiatry and Neurosciences, and Ms. Patricia Stroy, Biometrics Division, Walter Reed Army Institute of Research, are greatly acknowledged.

Disclaimer

The contents, opinions, and assertions contained herein are private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense. The authors report no conflict of interest.

Abbreviations

DIGE: differential in-gel electrophoresis; NMDA: N-methyl-*D*-aspartic acid; SDS: sodium dodecyl sulfate; TBI: traumatic brain injury

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