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A Possible Molecular Mechanism of Hearing loss during cerebral ischemia in mice

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Abstract

Ischemic brain stroke is a leading cause of disability, including the hearing loss. Clinical reports also suggest hearing loss in stroke patients but the mechanism is still unclear. Therefore, we hypothesize that hearing loss after cerebral ischemia may be associated with alteration in synapse, gap junctions, and sodium channel (NaC) proteins. Ischemia reperfusion injury was performed in wild type mice. Lesion volume was determined by TTC staining of the brain sections. BBB disruption was confirmed by Evans blue and BSA-FITC leakage. We found that brain edema, infarct size and permeability were increased in ischemic mice as compared to the sham. Caspase-3, -9 and TUNEL positive cells were increased in I/R mice indicates neuronal apoptosis. Moreover, increased expressions of matrix metalloprotease's (MMP-2, -3, -9, -13), IL-6, and decreased expressions of tight junction proteins (TJP) in I/R group, as compared to the sham group signifies inflammation and BBB disruption. We also observed decreased levels of PSD95, SAP97, Connexin-43, NaC- α , NaC- β , and increased expression of Connexin-45, whereas no substantial change was observed in Connexin-26 expression in I/R group. Interestingly, auditory response was reduced in I/R mice indicates hearing loss. This data suggests that hearing loss in ischemic mice was primarily due to the alteration in connexin, synapse, and NaC channels.

Keywords

Cerebral ischemia; Blood brain barrier; Apoptosis; Hearing loss; Neurotransmission

Introduction

Hearing impairment, the most common sensory disability worldwide, has a profound effect on one's ability to function at a personal, social, and occupational level. Many different conditions cause hearing impairment such as inflammation, trauma, aging, genetic disorders, and stroke (Caplan 2000). Some clinical reports show hearing impairment in stroke patients (Edwards et al. 2006; Bamiou et al. 2012). However, the interruption of hearing attributes to stroke pathology within the auditory pathways is a largely unexplored. It is known that auditory processing is to facilitate sound perception, recognition, attention, memory and

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Conflict of interest

The authors declare that they have no conflicts of interest.

learning- which are all integral components of auditory cognition (Griffiths et al. 2010; Paludetti et al. 2012). Although acute loss of hearing is an unusual characteristic in stroke pathology, elderly stroke patients seem to suffer more commonly from it (Gopinath et al. 2009). Furthermore, strokes may also cause auditory processing disorders (Maineri et al. 2007; Rey et al. 2007). Similarly, patients with cerebrovascular auditory lesions report auditory perceptual problems with sound localization (Blaettner et al. 1989; Edwards et al. 2006). All of these reports above suggest the involvement of cerebro-vascular pathology in hearing loss during cerebral stroke.

Cerebral ischemia induces neuronal death primarily by necrosis in the ischemic core, while loss of neurons is severely affected in the infarct core by apoptosis (Broughton et al. 2009; Tyagi et al. 2012; Belayev et al. 2004). The apoptosis is characterized by activation of caspase-9, and subsequently of caspase-3. It has been suggested that caspase-3 activation leads to the activation of apoptotic cascades (Lee et al. 2005; Kamat et al. 2011; Chen et al. 2013) during ischemic stroke which is mediated by the early activation of matrix metalloproteinases (MMPs). Activation of MMPs (MMP-2 and MMP-9) exert neurotoxic effects (Rosenberg and Yang 2007; McColl et al. 2008). Activation of MMPs also promotes degradation of the extracellular matrix and further leads to the opening of the blood brain barrier (BBB), resulting in infiltration of inflammatory cells which often lead to neuronal damage at the infarct site (Candelario-Jalil et al. 2009; Jin et al. 2010). BBB is a highly selective barrier that separates the blood from the brain's extracellular fluid in the central nervous system (CNS). Because of this, the BBB has a serious role in cerebrovascular pathophysiology and disease progression of the central nervous system (Summerfield et al. 2007). Tight junctions (TJ) are complexes of the multi-protein that mediate cell to cell adhesion and control the transportation through the extra cellular matrix in brain and could potentially influence the ability of the TJ complexes to limit BBB function (Hawkins and Davis 2005; Kalani et al. 2014).

Neurons are usually connected to the micro-vessel via astrocytes to assist in their nutritional uptake, oxygenation, and normal function. A disruption in the micro-vessel leads to an interruption in nutritional exchange and physiological disturbance in neurons. Such a condition is also known as neurovascular impairment. Neurovascular component which contains neurons and vessels get disturbs during pathology and impair the neuronal communication. Neuronal communication is usually occurs through the neurochemical synapse. Neurochemical synapses are specialized junctions which serve as functional connections between neurons through which neuronal, as well as non-neuronal cells, communicate to each other (Chandler et al. 2006; Squire et al. 2009). Neurochemical synapses are primary involved in the information-processing functions of the central nervous system which allow neurons to form neuronal circuits. Axons are connected with dendrites which forms the synapse. Therefore, any impairment in Na⁺ channels also delays neurochemical transmission. Intracellular connection as wells as intercellular communication of synapse takes place via gap junction proteins (connexins). Ischemia/ reperfusion injury may cause gap junction proteins impairment that's led to disruption of intercellular communication by hindering protein synthesis or inducing channels aberrant function.

In spite of the fact that hearing loss is observed in cerebral stroke patients, the mechanism is still unclear. Therefore, in present study, we question the possible mechanism by which cerebral ischemia causes and whether the auditory cortex damage affect auditory function by altering GAP junctions, sodium channels, and synaptic neurotransmissions which may later summate to affect central auditory function.

Material and methods

Chemicals and Antibodies

Antibodies MMP-9, MMP-2, MMP-3, MMP-13 Caspase-3, Caspase-9, PSD95, SAP97, GFAP, IL-6, Cx-26, Cx-43, claudin-5, Occludin, Nac- α , Nac- β , and GAPDH antibodies were purchased from Abcam (Cambridge, MA). HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bradford protein assay reagents, PVDF membrane were purchased from BIO-RAD (Hercules, CA). Other analytical grade chemicals (BSA, FITC, Evans blue) were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Procedures

Animals—Male C57BL/6J (Wild Type) mice, (10–12 week-old; n = 5 animals/group for each study like Western blotting, Immunohistochemistry; microvascular permeability; cerebral edema, Infarct volume and Evans Blue assay) were used for experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee accordance with of the University of Louisville. Animal were kept at suitable and standard housing conditions in the animal facility center at the University of Louisville.

Grouping of Animal

1. WT Sham
2. WT+ Ischemia Reperfusion (I/R) injury

Focal Cerebral Ischemia—Animals weighing 27–30 g were used for experiment and were allowed free access to food and water till one hour of surgery. Mice were anesthetized with sodium pentobarbital (50 mg/kg body wt.) before surgery and during the surgery body temperature was maintained at $37\pm0.5^{\circ}\text{C}$. Ischemia reperfusion (I/R) injury was induced by occlusion of the middle cerebral artery (MCAO). The monofilament was inserted for a duration of 60 min. MCAO was created by a silicone resin-coated 5–0 nylon monofilament (Ethicon, Titusville, N.J) which was introduced into a small incision of the left common carotid artery and advanced distal to the carotid bifurcation for temporary occlusion of the middle cerebral artery. Blood flow was restored by withdrawal of the monofilament after 60 min of middle cerebral artery occlusion. Same anesthetic and surgical procedure were subjected to the sham operated mice except the insertion of monofilament (Longa et al. 1989; Tyagi et al. 2012).

Neurobehavioral Assessment in mice—The neurobehavioral assessment in mice of both the group was performed after I/R injury as described in the method of Longa and coworkers (1989). Briefly, the neurobehavioral observations in sham and I/R mice were

scored on a five-point scale. Evaluation of scoring and grading were done in mice; in which a score of 0 designates no neurologic deficit, a score of 1 means failure to extend opposite forepaw fully (a mild focal neurologic deficit), a score of 2 was assigned when sham circling was observed (a moderate focal neurological deficit), and a score of 3 was given when a mice was not able to hold the grip of wire mesh when hanged on it and fell to the side opposite to the brain damage (a severe focal deficit). Furthermore, when the mice did not walk spontaneously and had a depressed level of consciousness, a score of 4 was assigned. The scores of neurobehavioral response obtained after analysis on each scale were summed to get a neurological deficit score of each group of animals.

Auditory brainstem response (ABR)—The hearing assessments of the C57BL/6J (Wild Type) sham and ischemic male mice were conducted. Mice were placed in 2 groups as mentioned above. Twenty-four hours after the ischemia, the auditory threshold of animals was conducted again alongside the sham operated animal. Animals were anesthetized intraperitoneally (i.p) injection of 2, 2, 2 tribromo-ethanol (TBE). Body temperature of mice was maintained constant at 37°C using a controlled heating pad. For stimulus generation, presentation and data acquisition, we used Tucker-Davis-Technologies (TDT) III systems (Tucker-Davis-technologies, TDT; Ft Lauderdale, FL, USA) operating with BioSig32 software (TDT). Auditory responses were recorded by registration of the potential difference between sub dermal electrodes positioned at the vertex, and the mastoid process. A ground electrode was inserted over the body blow neck. In general, ABR waveforms were recorded for 10 mili sec. ABR waveforms were recorded in decreasing 90 to 05-dB sound pressure level (SPL) intervals from the maximum amplitude until straight waveforms visualized.

Determination of Infarct Volume (IV)

Animals were sacrificed after 24 h to measure the infarct volume in I/R injury and sham operated mice. Mouse brains were quickly removed, washed with PBS and kept at –20°C for 15 min to make a proper section. Coronal sections of 2 mm were cut into slices using a mouse brain matrix cutter (Harvard Apparatus, Holliston, MA, USA). The slices were stained with mitochondrial viability dye of 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich, Taufkirchen, Germany) for 30 min at 37°C and subsequently image were captured. After the staining the brain slices were fixed with 4% paraformaldehyde to fix the stained area by TTC (Tyagi et al. 2012; Braeuninger et al. 2010). The stained brain sections were digitally photographed, and the infarct area (pale white) of each brain section was measured using Biovis Image Plus software version 1.5, which was then further used to derive infarct volume. Infarct volume of ischemic brain was determined by percentage (%) of (infarct area of ipsilateral side/contralateral side) × 100.

Determination of BBB disruption

Evans blue extravasation—BBB integrity in mice was investigated by measuring the extravasation of Evans blue (Belayev et al. 1996; Uyama et al. 1988). BBB disruption of the mice was assessed after 24 h of I/R injury using Evans Blue dye (n=7 for each group). Evan's blue dye (2%, 4 mL/kg) was injected slowly into the left femoral vein and allowed to circulate for 60 min. The mice were anesthetized with TBE and perfused intra-cardiac with PBS. The brain was removed after decapitation, and coronal sections were divided into the

right and left hemispheres. The dissected brain were weighed and extracted in 1.0 ml of 50% trichloroacetic acid solution and centrifuged at 8000g. The amount of extravagated Evans Blue dye in the brain was determined by spectrophotometry at 620 nm and quantified as micrograms per gram of brain tissue (Spectra Max 3000, CA, USA).

Micro vascular permeability in brain—Microvascular permeability in mice brain was measured in anesthetized mice fitted in stereotaxic apparatus to prevent mechanical damage during drilling. A cranial window was created in the skull of mice by using micro driller (FST). The exposed area was regularly flushed with artificial cerebrospinal fluid. A Syringe pump was fitted with two injections, one containing heparin saline to prevent clotting, and the other with FITC solution. Fluorescein isothiocyanate (300mg/mL) bound to BSA (FITC-BSA) was infused slowly through carotid artery (0.2 mL/100 g of body wt.) by a syringe pump (Harvard Apparatus) and allowed to circulate for about 5 minutes (Tyagi et al. 2010). The pial circulation was observed to ensure that there was no spontaneous leakage in the spotted area that would indicate decreased vascular integrity. Venules were identified by observing the topology of the blood flow and vascular diameters increasing in the direction of blood flow. The exposed area of brain was observed using in vivo imaging fluorescent intra vital microscopy (Olympus, Japan).

Cerebral edema—Brain edema was measured 24 hours after stroke as described previously by (Liu et al. 2009; Tyagi et al. 2012). Briefly, the brain was quickly removed after the animal was sacrificed. Then the brain was blotted to remove residual absorbent moisture, and dissected through the inter-hemispheric fissure into right and left hemispheres (n = 5 animals/group). Mainly the cortex was used for this purpose and the rest of parts were discarded. Wet weight was determined before drying the tissue. Dry weight of the whole ischemic and sham hemispheres was determined after heating the tissue for 3 days at 100 °C in a drying oven. Absolute water content was calculated as % H₂O = (1 – dry weight/wet weight) × 100%. It was measured in ipsilateral side/contralateral side.

Cryosectioning of brain tissue—At the end of the experiment mice were euthanized, decapitated, and the brain was harvested. Further, brain tissues were washed properly in phosphate buffered saline and preserved in Peel-AWay disposable plastic tissue embedding molds (Polysciences inc., Warrington, PA., USA) having tissue freezing media (Triangle Biomedical Sciences, Durham, N.C., USA), and kept at –80° C. Sections (25 µm) were made using cryotome (Leica CM 1850) and placed on poly-L-lysine coated slides and stored at –80°C till use.

TUNEL assay for Apoptosis—TUNEL staining is used for assessment of apoptosis in mice brain. Briefly, the assay was conducted on frozen brain section from both the groups using a commercially available kit (Dead End Fluorometric TUNEL System; Promega). Staining was done according to manufacturer's instructions keeping positive control. Confocal microscopy was performed for image capture for apoptotic cells in mice brain tissue.

Immunohistochemistry—The frozen brain tissues were blocked with blocking solution (5% BSA in TBS-T) and incubated with a primary antibody (Abcam, USA) at 1:100

dilutions overnight at 4 °C. The unbound antibody was washed with TBS and then the sections were incubated with goat anti-mouse alexa flour 488 and goat anti-rabbit taxas red for 60 min at RT. The slides were further stained with DAPI (1:10,000) for 10 min, washed, and mounted with anti-fade mounting media. The images were acquired using a laser scanning confocal microscope (60 × objectives, FluoView 1000, Olympus, PA, USA). Total fluorescence intensity in five random fields (for each experiment) was measured with image analysis software (Image-Pro Plus, Media Cybernetics, Rockville, MD, USA).

Western blot analysis—Western blot assay is performed for protein expression. Briefly, protein was extracted using 1x RIPA buffer. Equal amount of proteins from brain were separated by SDS-PAGE. Further, protein from the gel was transferred onto PVDF membrane (BioRad, Hercules, CA) by wet transfer method. Non-specific sites of protein on membrane were blocked with 5% non-fat dry milk in TBS-T (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween- 20, pH 7.4) for 1 h at room temperature. Membranes were washed with washing buffer (pH 7.6, TBS, 0.1% Tween 20) for 3 times, 10 min each. The blot was then incubated for overnight at 4°C with appropriate primary antibody in blocking solution according to the supplier's specific instructions. The blots were washed with TBS-T (3 times, 10 min each) and incubated with appropriate HRP- conjugated secondary antibody for 2 h at room temperature. After washing with TBST-T, ECL Plus substrate (Thermo scientific, inc.) was as applied to the blot for protein expression and images were capture in gel documentation system. Relative optical density of protein bands was analyzed by using software image lab 3.0. The same membranes were used for estimation of GAPDH protein which was stripped with stripping buffer and re-probed with GAPDH. The GAPDH was used as a loading control for all the proteins of interest.

Statistical analysis

The results are expressed as mean ± S.E.M. Statistical analysis of the biochemical and molecular data were analyzed by student T test. A *p* value <0.05 is considered to be significant.

Results

Cerebral Blood Flow

There was no significant change observed in cerebral blood flow as compared to sham operated mice after 60 minute of MCAO. Observed data indicates the blood flow was restored after removal of monofilament (bar graph not shown).

Neurological deficit (ND)

The neurological deficit (ND) was scored in all the experimental mice after the 24hr of ischemia/reperfusion (I/R) injury (Fig. 1A). The mice exhibited varying degree of flexion, circling, hemiparesis, and non-spontaneity behavior, showing impaired sensorimotor ability as compared to sham operated mice group.

Cerebral Infarct Volume

The effect of I/R on brain infarct volume was measured by TTC staining. As shown in Fig. B, a clear difference between viable tissue (a deep red) and damaged tissue (pale colored white mass). Infarct volume was significantly increased in the ipsi lateral side as compared to sham (Fig. 1B–C). There was no infarct area observed in the sham operated group and contralateral side of ischemic brain (Fig. 1).

Cerebral edema

Cerebral edema was confirmed by measuring water contents in cortical areas. We found a significant increase in water content of the cortex of ischemic brain as compared to sham operated group (Fig. 1D). These observations indicate ischemia/reperfusion induced brain injury (Fig. 1).

GFAP and IL6 expression

There was a significant increase observed in GFAP and IL6 protein expression in ischemic cortex as compared to sham cortex (Fig. 2A–B). The results suggest the increased inflammation with activated glial cells.

Protein expression of MMP-2, MMP-3, MMP-9 and MMP-13

There was a significant increase in MMP-2, MMP-3, MMP-9, and MMP13 protein expression in ischemic cortex as compared to sham cortex (Fig. 3A–D). The results signify activation of MMPs during I/R injury (Fig. 3).

Tight Junction (TJ) protein expression

Claudin-5 and Occludin, a marker of BBB dysfunction was investigated in the cortex of ischemic and sham operated mice. Claudin-5 and Occludin expression was significantly less in ischemic cortex as compared to sham cortex (Fig. 4A–B). The results signify loss of BBB in the ischemic hemisphere (Fig. 4).

Apoptosis

To test whether cerebral ischemia caused apoptosis. We measured protein expression caspase-3 and -9. Protein levels of caspase-3 and -9 were significantly up-regulated in ischemic cortex as compared to sham operated cortex (Fig. 5A–5B). Further, TUNEL assay also confirmed apoptosis in ischemic hemisphere as compared to contra lateral hemisphere of ischemic mice and sham mice (Fig. 5C–5D). I/R injury demonstrated a number of morphological changes characteristic of cell death; such as the blubbing of the nuclei and DNA fragmentation, characteristic of apoptotic bodies (Fig. 5C–D). The co-labeling of TUNEL with DAPI (nuclei) suggested DNA fragmentation in the nucleus of ischemic cortex as compared to sham operated cortex (Fig. 5C–D). The results suggested that I/R induce apoptosis in part by generating DNA fragmentation (Fig. 5).

Synapse protein (SAP-97 and PSD-95) expression

Our data noticeably showed significant decrease in SAP-97 and PSD-95 protein expression which clearly indicates synaptic damage (Fig. 6A–6B). We re-confirm these results with

immunofluorescence assay and again found a significant decreased level of SAP-97 and PSD-95 expression in ischemic mouse brain as compared to sham, which indicates disruption of synapse (Fig. 6C–6D). This data indirectly supports the impairment of neuronal communication through synapse (Fig. 6).

Sodium channel protein

Experimental data of sodium channel proteins showed decrease levels of sodium protein (NaC- α & NaC- β) in ischemic mice as compared to sham (Fig. 7A–7B). Further, confocal analysis of NaC- α and NaC- β also showed decreased levels of expression which delineates the loss of sodium channel protein (Fig. 7C–7D). These observations clearly inferred the disturbed electrical transmission during ischemia/reperfusion induced brain injury (Fig. 7).

Cx-26, Cx-43 and Cx-45 expression

We measured proteins expression of gap junction (connexins). Stimulation of ischemia significantly decreases the expression of Cx-43 whereas Cx-45 protein expression was increased in ischemic cortex as compared to sham operated cortex. Interestingly, there was no change observed in Cx-26 expression in ischemic mice as compared to sham (Fig. 8A–8B). We re-confirm these results with immunofluorescence assay and found the same pattern of expression (Fig. 8C–8D). This observation openly provides evidence of altered levels of gap junction proteins during ischemia/reperfusion induced brain injury in ischemic brain (Fig. 8).

Micro vascular leakage by BSA-FITC

Increased microvascular permeability and blood brain barrier (BBB) disruption could be an initiating factors for the induction of brain injury and critical factors for the post-ischemic neuronal damage severity. Blood brain barrier integrity was measured by Evans blue leakage in cortical area. The levels of Evans blue were significantly increased in ischemic side as compare to non-ischemic side (Fig. 9C). However, the Evans blue leakage was not observed in sham-operated group (Fig. 9A). To confirm these results we also check micro vascular leakage by BSA-FITC. We measured interstitial diffusion of BSA-FITC in brains of all groups of mice by intra-vital microscopy. We observed that BBB permeability was significantly higher in ischemia group as compared to sham operated mice. These results suggested an increased vascular permeability in part by edema formation in cerebral ischemic mice which is a major complication in stroke.

Auditory brain stem response

To determine the Ischemia injury associated physiological consequences on hearing functions. ABRs were measured in both the groups. In wild type sham mice, auditory response thresholds using tone stimuli at 16 khz are between 25–30 dB sound pressure level (SPL). In contrast, auditory response thresholds was significantly increased between 70–90 dB SPL at 16 KHz in Ischemic brain mice that further indicate severe hearing impairment in ischemic brain as compare to sham group (Fig. 10A–C). The auditory brain response in mice was measured at the ischemic created side.

Discussion

According to survey, it has been considered that adult-onset hearing impairment is the third leading cause of disability in world (WHO, 2008). Several clinical studies have documented that hearing loss is associated with cerebral stroke patients (Kim et al. 2014). However, the direct impact of cerebral ischemia on the hearing loss and its physiological consequences remains to be un-explained.

In the present study, we demonstrated that cerebral ischemia causes damage of cerebral cortex and activates MMPs in-parallel disruption of tight junction protein which results a subsequent increased in inflammation and neurodegeneration, in part by increased micro-vascular permeability that could lead to damage of auditory cortex. Furthermore, auditory cortex damage might affect central auditory function by altering GAP junctions, sodium channels, and synaptic neurotransmissions as shown in Fig. 11. We measured auditory brain response in sham and ischemic mice. Ischemic mice showed lower auditory brain response (ABR) and lowered sound pressure level (SPL) as compared to sham operated mice. Our present data clearly indicates that hearing impairment occurs in cerebral stroke mice (Fig. 10A–C). It is speculated that the most critical change associated with hearing impairment in early development is the damage of auditory cortex. Previous studies in deaf white cats suggested that specific association areas of the auditory cortex becoming abnormally responsive in hearing impairment (Lomber et al. 2010; Fine et al. 2005). The auditory cortex (AC), a sub cortical part of cerebral cortex, is the source of a large set of down regulated pathways that can affect neural processing at every level of the auditory system (Suga et al. 2000). To take this into account, we performed an entire molecular and immunohistochemistry assay in the cerebral cortex only. Since, we performed an ischemia reperfusion (I/R) injury in mice; we found infarct area in ipsilateral side through TTC staining (Fig. 1).

During inflammation, the brain releases a range of pro-inflammatory cytokines, including interleukin (IL-6) and leukocyte recruiting protein (ICAM), which indicates increased BBB permeability (Savarin et al. 2011). Furthermore, we also found increased brain water contents which signify inflammation (Fig. 1). In support with the above information, we also found increased expression of GFAP and IL-6; an astrocyte marker indicates glial activation as well as neuroinflammation (Fig. 2). In the patho-physiology of the central nervous system, MMPs aggravate cerebral ischemia and edema (Yang and Rosenberg 2007; Ethell and Ethell 2007).

Altered expression of MMPs has been shown to play a destructive role in brain during cerebral ischemia (Tyagi et al. 2012; Ruhul Amin et al. 2003). Among all the MMPs, MMP-2 and MMP-9 is a key enzyme which has a major role in brain injury after cerebral ischemia (Lee et al. 2004; Romanic et al. 1998). In agreement with above studies, we also found elevated expression of MMPs (MMP-2, MMP-9, MMP-13, and MMP-3) in the ischemic brain which indicates that MMPs are actively engaged in the pathology of ischemia (Fig. 3). MMPs cause reversible degradation of tight junction proteins early after the onset of ischemia, and a delayed secondary opening during a neuroinflammatory response (Yang and Rosenberg 2011a, 2011b). In a later stage MMPs breakdown the extracellular matrix

(ECM) that disrupts the blood-brain barrier (BBB) during reperfusion. An earlier report by Zehendner et al. (2011) suggests that apoptosis leads to TJ protein alterations by causing degradation of occludin and Claudin-5 (Cl-5). In brain tissue, claudin-1 and claudin-5 together with occludin, a TJ protein, have been described to be present in endothelial cells which are required for the formation of the BBB (Liebner et al. 2000; Yang et al. 2007; Ballabh et al. 2004). The decreased expression of occludin in extra cellular junctions also results in the formation of gaps between the cells with a marked increase in permeability (Patibandla et al. 2009). Concurrently, we also found decreased expression of claudin-5 and occludin which confirm disruption of BBB during ischemic insult (Fig. 4). Therefore, decreased expression of tight junction proteins (Claudin-5 and Occludin) confirms the loss of microvascular integrity (Fig. 4). We also confirmed it by BSA-FITC assay where prominent leakage observed in ischemic mice brain (Fig. 9) indicates BBB disruption. These events are considered crucial events to initiate the apoptosis, and cause post-ischemic brain infiltration of inflammatory cytokines into the brain (Shichita et al. 2009; Bojarski et al. 2004). Concomitantly, we also found increased level of caspase-3 and caspase-9 protein (Fig. 5A&B) along with increased TUNEL positive cells which is quite suggestive of neuronal apoptosis (Fig. 5).

All the above observed data clearly confirm the pathological condition of cerebral ischemia. Since, we establish that cerebral ischemia causes hearing loss; we also checked the protein which is associated with neuronal communication and signal processing between neurons. So, we can connect the dot between cerebral ischemia and hearing loss. Neurons communicate through synapse via pre-synapse and post synapse; where an exchange of information occurs. The flow of information around the brain is achieved by electrical activity and chemical processes. In this study, we checked the pre-synapse marker (SAP-97) and post synapse marker (PSD-95). We found there was a significant decreased in protein expression of both the markers which indicates aberrant synapse function (Fig 6A&B). Immunofluorescence assay also reveals the loss of SAP-97 and PSD-95; which confirm the synapse disruption in ischemic mice (Fig. 6C&D). We predicted that abnormal synapse function may not allow proper communication between two neurons.

Sodium channels (NaC) consist of a large α subunit that associates with other proteins, such as β subunits. This protein is expressed by a cell and forms channels that conducts Na^+ . The highly selective NaC belongs to a new class of proteins called the NaC/degenerin (DEG) superfamily and are involved in trans-epithelial Na^+ transport, mechano-transduction, and neurotransmission (Mano and Driscoll 1999; Kellenberger and Schild 2002). Action potential and resting potential which occurs in the axonal membrane is maintained by Na^+ channels. The activation or deactivation of channel control the flow of positively-charged Na^+ ions into the neuron through the channels, and balancing the voltage across the neuronal membrane. Our data showed decreased expression of NaC- α and NaC- β protein (Fig. 7A&B). Furthermore, Immunofluorescence assay also confirmed the loss of NaC- α and NaC- β protein which clearly indicates the disruption or deactivation of electrical current mediated through Na^+ ions (Fig. 7C&D). This could be one of the possible mechanisms of hearing impairment in ischemic mice.

Gap junction channels provide the basis for a unique direct cell-to-cell communication, whereas Connexin (Cx) hemi-channels allow the exchange of ions and signaling molecules between the cytoplasm and the extracellular medium (Giaume et al. 2013). In the present study, we found significant decrease in Cx-43 protein expression and an increase in Cx-45 (Fig. 8A&B). Moreover, immunofluorescence assay also showed decreased expression of Cx-43 and increased expression of Cx-45 in ischemic mice (Fig. 8C&D). However, no significant change was observed in expression of Cx-26 which signifies altered cell to cell communication (Fig. 8).

Conclusion

Taken together, our observations determine the novel mechanism for hearing loss during stroke along with BBB dysfunction in ischemic mice. In conclusion, our present data clearly indicates that hearing impairment occurs in cerebral stroke mice. During I/R injury, infarct area in ipsilateral side cause an increase in water contents along with increased expression of IL-6, GFAP expression and activates MMPs which lead to open the BBB by degrading TJPs that can contribute to cell death. There was significant decreased in pre-synapse marker (SAP-97) and post synapse marker (PSD-95) protein expression, which indicates aberrant synapse function. Furthermore significant decrease in Cx-43 and an increase in Cx-45, however, no significant change was observed in expression of Cx-26 which signifies altered cell to cell communication in part by decreased expression of NaC- α and NaC- β protein, which clearly indicates the disruption or deactivation of electrical current mediated through Na⁺ ions. Impairment of the carotid artery blood flow may also have resulted in diminished blood supply and ischemia in the cochlea. Thus, our study unequivocally underscores that hearing impairment might have occurred due to the altered expression of synapse, sodium channel and connexin proteins which are actively engaged in signal transduction and neuronal communication. This information may be helpful in developing a novel therapeutic drug for hearing loss associated with stroke.

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Abbreviations

MMP	Matrix metalloproteases
BBB	Blood brain barrier
NaC	Sodium channel
SPL	Sound pressure level
TJP	Tight junction protein
Cx	Connexin
MCAO	Middle cerebral artery occlusion
I/R	Ischemia reperfusion injury

ABR Auditory brainstem response

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Figure 1. Effect of Ischemia reperfusion injury (I/R) on (A) Neurological deficit (ND), (B) Cerebral Infarct core (C) cerebral infarct Volume and (D) Cerebral edema. A *P<0.05 value represents the significance of difference between sham and ischemia.

Figure 2.

Fig A–B, Represents the expression level of GFAP and IL6 expression in sham and ischemic mice brain. A * $P < 0.05$ value represents the significance of difference between Sham and Ischemia.

Figure 3. Western blots analysis of the matrix metalloproteases (MMP) protein expression
Figure and western blot image (A–D) shows the expression level of MMP-2 and MMP-9, MMP-3 and MMP-13 in sham and ischemic group of mice. A significance of difference between sham and ischemia were represented by * $P < 0.05$ value.

Figure 4. Western blot analysis of tight junction proteins

Western blot and bar graph represents expression of Claudin-5 and Occludin in sham and ischemic group of animals (4A–B). A * $P < 0.05$ value represents the significance of difference between sham and ischemia group.

Figure 5. Effect of I/R on apoptotic cell death

Western blot analysis shows the protein expression of caspase-3 and caspase-9 (5A–B).

TUNEL assay represents the apoptotic cells in sham and ischemic group and representative graph showing the expression of caspase-3 and caspase-9 and extent of apoptosis in sham and ischemic group (5C–D). A significance of difference between group represented by * $P < 0.05$ value.

Figure 6. Effect of I/R on synapse protein (SAP-97 and PSD-95) expression

Western blot analysis of pre and post synapse markers and expression level of SAP-97 and PSD-95 (6A–B). Immunofluorescence analysis demonstrate the protein expression of SAP-97 and PSD-95 expression and bar graph represents the expression level of synapse marker in different group (6C–D). A significance of difference between group represented by * $P < 0.05$ value.

Figure 7. Effect of I/R on Sodium channel protein

Figure (7A) represents the protein expression of NaC- α and NaC- β ; and (7B) represents the expression level of NaC- α and NaC- β in sham and ischemic group. Immunofluorescence analysis displays the protein expression of NaC- α and NaC- β expression. Bar graph denotes the expression level of sodium channel protein in different group (7C–D). A significance of difference between group represented by * $P < 0.05$ value.

Figure 8. Effect of I/R on Cx-26, Cx-43 and Cx-45 expression

Figure (8A) represents the protein expression of Cx-26, Cx-43 and Cx-45 and (8B) represents the expression level of Cx-26, Cx-43 and Cx-45 in sham and ischemic group. Immunofluorescence assay illustrate the protein expression of Cx-43 and Cx-45 expression (8C–D). Bar graph embodies the expression level of Cx-43 and Cx-45 in sham and ischemic group. A * $P < 0.05$ represent significance of difference between sham and ischemia group.

Figure 9. Analysis of BBB disruption in ischemic mice brain

(A) Micro vascular leakage by BSA FITC method and (B) Vascular leakage shown in fluorescence unit and (C) represents BBB integrity (Evans blue leakage). A significance of difference between group represented by * $P < 0.05$ value.

Figure 10. Effect of I/R on Brain Auditory response

Auditory brainstem response recording showing ischemic mice increases hearing thresholds.

(A–B) Representative traces from sham and ischemic mice in response stimuli showing the intensity of sound pressure levels. (C) Average hearing thresholds level for click-evoked auditory brain response between sham and ischemic group. A * $P < 0.05$ value represent significance of difference between group.

Figure 11.

Representative Image depicting the alteration of sodium channels and gap junctions in neuron which causes delayed synaptic and electrical transmission of message from brain to cochlea. These abnormalities in ischemic case may not allow cortex to process the auditory function in brain and further experience the hearing impairment.