Full Length Research Paper

Pneumococcal meningitis induced neuronal injury in motor cortex and cerebellum in Wistar rats

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Bacterial meningitis is known to cause neurodegenerations in the hippocampus, a region of the brain concerned with learning and memory. Aim of the present study was to investigate the effect of pneumococcal meningitis on motor cortex and cerebellum in rats. 30 days old rats were divided into normal control (NC) and meningitis (M) groups. Rats in the meningitis group were infected with Streptococcus pneumonia intracisternally on postnatal day 31. The concentration of the bacterial suspension in phosphate-buffered saline (PBS) was 1 × 106 cfu/ml (colony forming units/ml). The rats were kept on observation for 18 h for clinical symptoms of meningitis to develop. After 18 to 24 h of incubation, 10 to 50 µl of the cerebrospinal fluid (CSF) sample was collected. Grams staining of the CSF smear was done and observed under oil immersion objective (100×) for Gram positive, lanceolate diplococci. The rats were perfused transcardially with saline followed by 10% formalin. Brain was removed, processed for paraffin sectioning, and stained with cresyl violet stain. The motor cortex and cerebellum showed neurodegeneration. Significant fractions of neurons in the motor cortex were darkly stained and a number of healthy neurons per unit area were decreased significantly. The purkinje cells were shrunken and their number was decreased significantly. Meningitis not only affected the hippocampus, but also other regions of the brain. Neurodegenerative changes were comparable to that observed in the hippocampus.

Key words: Meningitis, motor cortex, cerebellum, purkinje cells, neurodegeneration.

INTRODUCTION

Meningitis is a disease of the protective coverings of the brain caused by specific bacteria. Bacterial meningitis due to *Streptococcus pneumoniae* is associated with a significant mortality rate and persisting neurologic sequelae including sensory-motor deficits, seizures, and impairments of learning and memory. The histomorphological analysis correlating with these sequelae is a pattern of brain damage characterized by necrotic tissue damage in the cerebral cortex and apoptosis of neurons in the hippocampal dentate gyrus.

There is severe neuronal loss in bacterial meningitis due to the activation of apoptotic and necrotic pathways. Programmed cell death of the neurons in the granular layer of the hippocampal dentate gyrus and necrosis of the pyramidal neurons in the hippocampus are reported. The above pathways lead to severe neurological sequelae or even to death (Koedel et al., 2002; Leib, 1999; Quagliarello and Scheld, 1992; Leib, 2000; Nau and Bruck, 2002; Tuomanen, 1998; Townsend and Scheld, 1993; Pfister et al., 1994).

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Magnetic resonance studies of survivors of bacterial meningitis have been shown to cause hippocampal atrophy (Free et al., 1996). Long term sequelae due to these pathways that result in neuronal death leads to deafness, intellectual and cognitive impairments ranging from severe intellectual disability to educational deficits and behavioral problems, and less commonly to epilepsy, spasticity or focal neurologic deficits (Baraff et al., 1993; Berg et al., 2002; Bohr et al., 1984; De Beek et al., 2002; Grimwood et al., 2000; Schuchat et al., 1995).

Mild to severe axonal injury in the white matter in the cortex, cerebellum, basal ganglia has been reported in the victims of meningitis in humans (Gerber et al., 2009). Inflammation of the subarachnoid and ventricular space contributes to the development of brain damage that is, cortical necrosis and hippocampal apoptosis in pneumococcal meningitis. Neuronal necroses/apoptosis in the neocortex, striatum and hippocampal formation has been reported in the mouse model of *S. pneumoniae* meningitis (Gerber et al., 2001; Gehre et al., 2008; Grandgirard et al., 2007; Li et al., 2007). Magnetic resonance imaging revealed lesions in both cerebellar hemispheres, suggesting cerebellitis in patients suffering from pneumococcal meningitis (Drost et al., 2000).

Around 5 to 40% of survivors depending on patient age and pathogen show permanent neurologic sequelae (Arditi et al., 1998; Erickson and Wals, 1998; Grimwood et al., 2000; Pfister et al., 1992). Two-thirds of meningitis related deaths are due to dismal outcomes primarily to central nervous system damage and the rest are due to systemic complication related to septic shock (Pfister et al., 1992). The pathophysiological studies of bacterial meningitis has shown that the inflammatory host responses still exist after the killing of bacteria by antibiotic therapy and are responsible for tissue damage in the central nervous system (Lorenzo et al., 1995; Mertsola et al., 1991; Mustafa et al., 1989; Schneider et al., 1999). Though brain lesions were reported in experimental studies, there were no anatomical differences in the brain or hippocampus related to bacterial meningitis, nor to the academic and/or behavioral limitations seen after bacterial meningitis in children (de Jonge et al., 2008).

Though there are ample amounts of literature on meningitis induced neuronal injury in the hippocampal region, there are only a few on meningitis induced neuronal necrosis in the cerebral cortex and cerebellum. Accordingly, aim of the present study was to investigate neuronal injury in the motor cortex and cerebellum quantitatively, after experimentally inducing meningitis in Wistar rats.

MATERIALS AND METHODS

Animals

Twelve 1-month-old Wistar rats were housed in the Central Animal Research Facility of Manipal University. The rats were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. The animals were maintained under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and a 12 h light-dark cycle. All animals were allowed free access to water 4 and were fed on a commercial diet. All the studies conducted were approved by the Institutional Animal Ethical Committee, Melaka Manipal medical college, Manipal, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Experimental protocol

a. Isolation of Streptococcus pneumoniae

Oxacillin sensitive *S. pneumoniae* ATCC 33400 obtained from Christian Medical College, Vellore, India was used for the study. The pneumococci were plated and isolated on chocolate agar. The colonies having typical colony morphology of draughtsman appearance were subjected to Gram's staining for microscopical analysis. Gram positive, lanceolate-shaped diplococci were seen on examination at 100× magnification. The colonies were emulsified in Hiss's serum water for a further battery of biochemical tests for confirmation. The pneumococci were tested for the fermentation abilities with acid only, for sugars along with Insulin. The pneumococcal colonies were lyophilized and cryopreserved at -70°C for further use.

b. Experimental groups

The young rats (30 days old) were divided into Normal control (NC) and Meningitis (M) groups (n=6 in each group). The normal control group of rats remained undisturbed in their home cages. Rats in the meningitis group were infected with *S. pneumoniae* intracisternally on postnatal day 31.

c. Induction of acute pneumococcal meningitis in rats

The rats were anaesthetized with 3%vol/vol halothane and were fixed in the stereotaxic frame (Pfister et al., 1993). 10 µl of the pneumococcal suspension was inoculated into the cisterna magna under stereotaxic guidance. Skull was exposed through a skin incision and location of cisterna magna was identified. A burr hole was drilled and bacterial suspension was injected using 10 µl Hamilton syringe. The concentration of the bacterial suspension in phosphate-buffered saline (PBS) was 1×10^6 cfu/ml (colony forming units/ml). The rats were kept under observation for every 6 h for clinical symptoms of meningitis. After 18 to 24 h of incubation, 10 to 50 µl of the CSF sample was collected through lumbar puncture, for further tests. Grams staining of the CSF smear was done and observed under oil immersion objective (100X) for Gram positive, lanceolate diplococci.

Culture was also done to assess the bacterial colonies. CSF samples suspended in 20 µl were fixed with methanol (20 µl) for 30 min, prior to Geimsa staining. The polymorphs were counted using a hemocytometer and assigned total cell count as cells/ml as defined for infection (Pfister et al., 1993).

d. Tissue processing for histological study

The rats were perfused transcardially with 100 ml of saline, followed by 200 ml of 10% formalin. Brain was removed and post fixed for 48 h in the same fixative. The tissue was processed for paraffin sectioning. Motor cortex and cerebellar tissue were selected for study.

Tissue pieces were dehydrated in the ascending grades of ethyl alcohol and then cleared with Xylene. The tissue was further
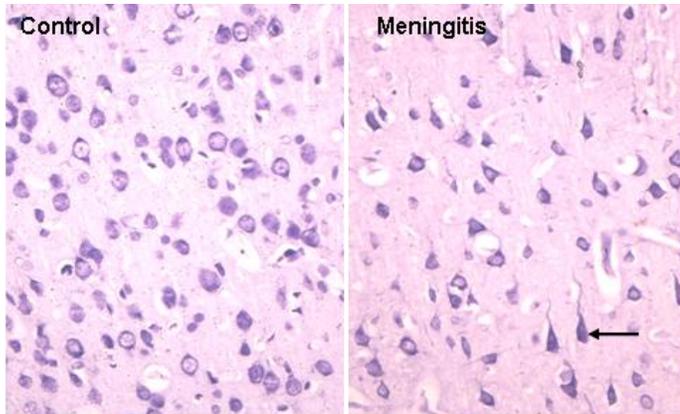
Figure 1. Photomicrographs of motor cortex of normal control (A) and Meningitis (B) rats brain. Note the degenerative changes in cortical neurons (arrows in B) in meningitis group and decreased number of neurons in B. Cresyl violet stain, Scale bar = 25 µm.

embedded in paraffin wax. Coronal sections of motor cortex and sagittal sections of cerebellum were taken at 5 µm thickness in a rotory microtome. The sections were stained with 0.1% cresyl violet stain in distilled water. Briefly, the sections were deparaffinized in xylene, and hydrated in the descending grades of ethyl alcohol. The sections were then stained with cresyl violet stain at 60°C for 20 min. Sections were differentiated in 70% alcohol and then dehydrated with 90% alcohol and absolute alcohol. The sections were mounted with DPX mounting media.

e. Neuronal quantification

1. Motor cortex:

(i) Neuronal counts: Number of neurons in 1 mm^2 of motor cortex was counted at 400× magnification. The neurons with clear cell boundary and nucleus were only counted and cells with irregular shapes and that which stained darkly were excluded from quantification. A total of 15 sections spaced 20 microns apart were selected for quantification. Numbers of neurons were expressed as no./mm^2 area.

(ii) Cross sectional area and diameter of neurons: The cross sectional area of the neurons and diameter were done using the Scion image analysis software. The digital images were used for this purpose. From each animal, five randomly selected fields with a minimum of 6 neurons were selected (From each animal a minimum of 30 neurons were analyzed).

2. Purkinje cells in cerebellum

(i) Purkinje neuronal counts: Number of purkinje neurons in 200 µm length of cerebellar cortex was counted at 400× magnification. The neurons with clear cell boundary and nucleus were only counted and cells with irregular shape and that which stained darkly were excluded from quantification. A total of 15 sections spaced 20 µm apart were selected for quantification. Number of neurons was expressed as Number/ 200 µm length.

(ii) Cross sectional area and diameter of purkinje neurons: The cross sectional area of purkinje neurons and diameter were done

using the Scion image analysis software. The digital images were used for this purpose. From each animal, five randomly selected fields of cerebellar purkinje cell layer with a minimum of 6 neurons were selected (From each animal a minimum of 30 neurons were analyzed, n=6).

f. Statistical analysis

Data was expressed as mean ± SEM. Data was analysed by two tailed Student's t-test. Probability (P) value less than 0.05 was considered as significant.

RESULTS

Behavioral observations

The rats infected with pneumococci were found to have symptoms of meningitis. They were sluggish, and showed decreased ambulatory movements. Their CSF showed Gram positive lanceolate diplococci. The CSF culture also confirmed the same on Gram's staining.

Effect of meningitis on motor cortex

The cerebral cortexes of meningitic rats were significantly different from the normal cortex. There was significant gliosis in the meningitis cortex. The cortical thickness marginally decreased, and all the layers decreased in their thickness in the meningitis brain compared to normal control brain (Figure 1). The majority of the cortical neurons showed degenerative features. The neurons are irregular in shape and are darkly stained.

i. Neuronal density

Number of neurons in mm^2 area decreased significantly (62%) in the meningitis group compared to normal control group [783.5 ± 19.5 (Mean ± SEM) in control group (n=6), 381.00 ± 19.06 in meningitis group (n=6), P< 0.0001, two tailed, Student's t-test] (Figure 2).

ii. Diameter of V layer motor neurons

The diameter of V layer motor neurons decreased significantly (27%) in the meningitis group compared to normal control group (29.98.5±0.64 in control group (n=6), 21.8±0.39 in meningitis group (n=6), P< 0.001, two tailed, Student's t-test) (Figure 3A).

iii. Cross sectional area of V layer motor neurons

The cross sectional area of V layer motor neurons
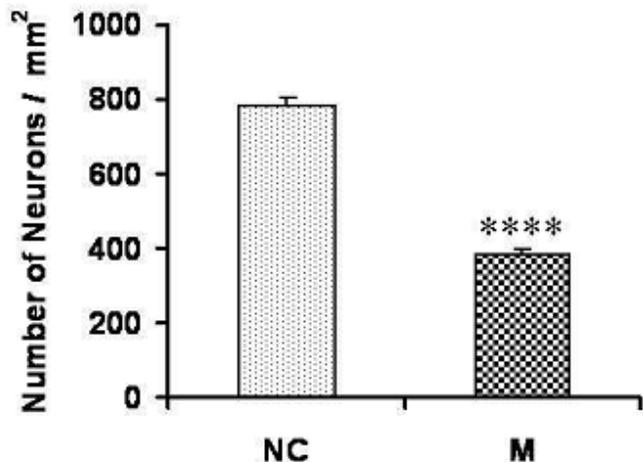
Figure 2. Number of neurons in the motor cortex of normal control (NC) and meningitis (M) group. Each bar represents mean ± SEM. Note there is a significant decrease in the number of neurons in meningitis group. NC versus M, *** * P<0.0001; Two tailed student t-test.
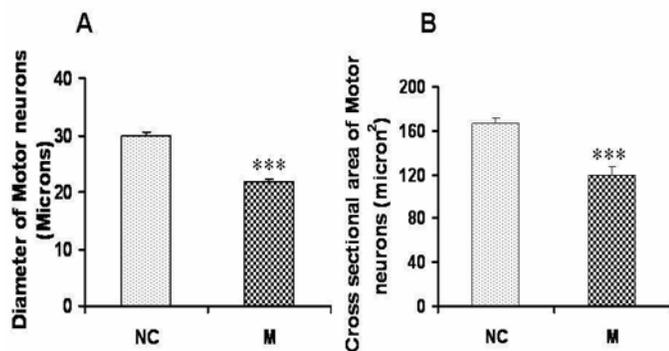
Figure 3. Diameter (A) and cross sectional area of V layer motor neuron (B) in normal control(NC) and meningitis(M) group. Each bar represents mean ± SEM. Note there is a significant decrease in the diameter of the neurons and cross sectional area of the neurons in meningitis group. NC versus M, *** P<0.001; Two tailed student t-test.

decreased significantly (28%) in the meningitis group compared to normal control group (166.1±4.73 in control group (n=6) 119.66±8.27 in meningitis group (n=6), P< 0.001, two tailed, Student's t-test) (Figure 3B).

Effect of meningitis on cerebellum

The cerebellar folium of meningitis infected rat was significantly shrunk compared to normal cerebellar folium. There was significant gliosis in the meningitis cerebellum. The thickness of molecular and granule cell layer significantly decreased (Figure 4). The Purkinje cells showed degenerative features. The neurons are smaller in size and are darkly stained. The granule cell
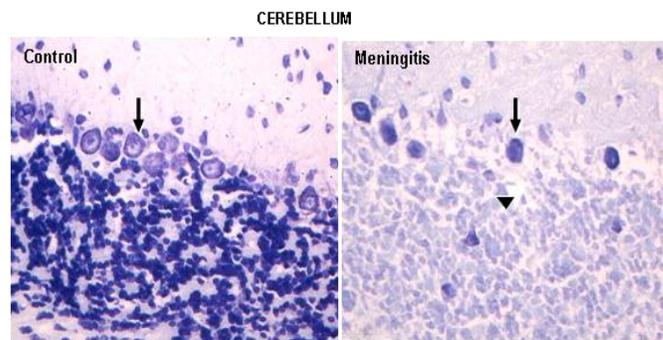
Figure 4. Photomicrographs of cerebellar cortex of normal control (A) and Meningitis (B) rats brain. Note the degenerative changes in purkinje neurons (arrows in B) in meningitis group. Cresyl violet stain, Scale bar = 25 μm.
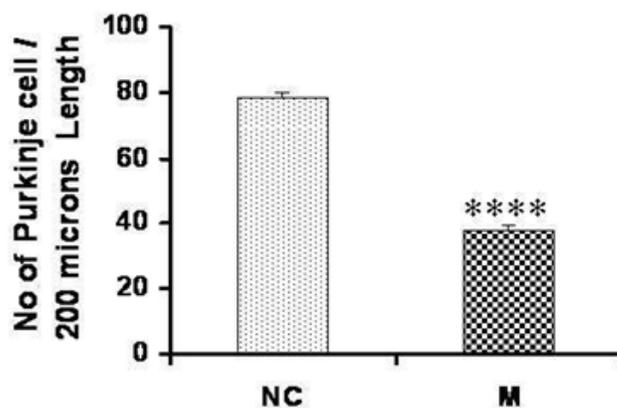
Figure 5. Number of neurons in the purkinje cell layer of cerebellar cortex of normal control (NC) and meningitis (M) group. Each bar represents mean ± SEM. Note there is a significant decrease in the number of purkinje neurons in meningitis group. NC versus M, *** * P<0.0001; Two tailed student t-test.

layer was lightly stained.

i. *Purkinje neuronal count*

Number of neurons in 200 μm length of purkinje cell layer decreased significantly (51%) in the meningitis group compared to normal control group (78.00 ± 2.02 in control group (n=6) 38.00±1.86 in meningitis group (n=6), P< 0.0001, two tailed, Student's t-test) (Figure 5).

ii. Diameter of Purkinje neurons

The diameter of purkinje neurons decreased significantly (28%) in the meningitis group compared to normal control group (27.44.5 ± 0.78 in control group (n=6) Vs 19.59 ± 0.10 in meningitis group (n=6), P< 0.001, two tailed, Student's t-test) (Figure 6A).

A

B
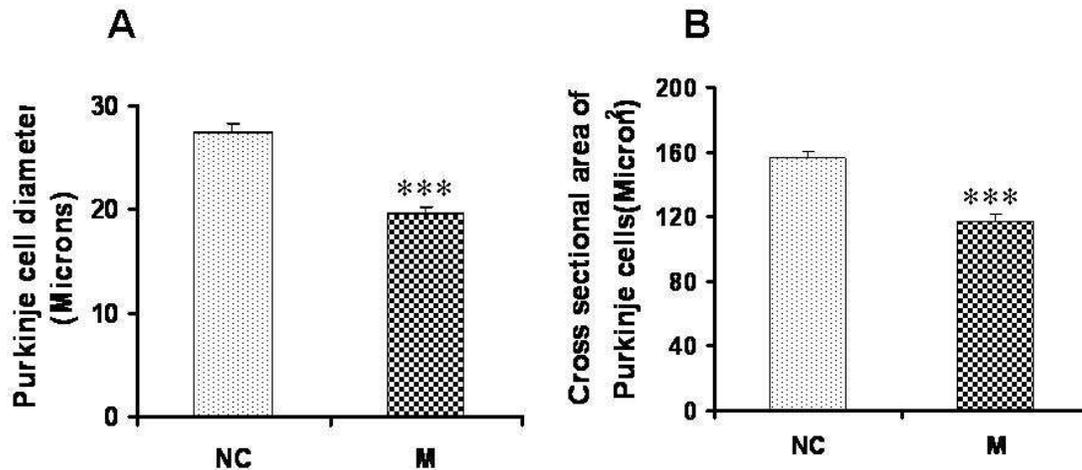
Figure 6. Graph showing the diameter (A) and cross sectional area of purkinje neuron (B) in normal control(NC) and meningitis(M) group. Each bar represents mean ± SEM. Note there is a significant decrease in the diameter of the purkinje neurons and cross sectional area of the purkinje neurons in meningitis group. NC Vs M, *** P<0.001; Two tailed student t-test. 254 ×190 mm (96 × 96 DPI).

iii. Cross sectional area of purkinje neurons

The cross sectional area of V layer motor neurons decreased significantly (29%) in the meningitis group compared to normal control group (156.1 ± 4.73 in control group (n=6) 111.49 ± 4.24 in meningitis group (n=6), P< 0.001, two tailed, Student's t-test) (Figure 6B).

DISCUSSION

The results of the present experiments revealed that pneumococcal meningitis will lead to extensive lesion in the motor cortex part of the brain concerned with motor function and cerebellar cortex concerned with motor function, equilibrium and balance. Neuronal damage was very severe in both regions. In the case of cerebral cortex, there was 60 to 70% neuronal loss, was documented. Further the surviving neurons in the meningitis brain decreased in their size as well. In the case of the cerebellum, the number of granule cells decreased significantly and the size of the surviving neurons decreased.

The mechanism and consequences of observed neuronal damage is of clinical importance. The animals that suffered from meningitis were sick, lethargic and had the symptoms of meningitis.

Disruption of the blood brain barrier is one of the most important phases in bacterial meningitis.

Normal lining of the blood vessels, the endothelium is a high resistance barrier to circulating macromolecules (Van der Flier et al., 2003). The physiological disturbance of the endothelial barrier results in vasogenic cerebral edema seen in 30% of cases and cerebral herniation in 6 to 8% of clinical cases (Pfister et al., 1993). Cerebral edema

causes increased intracranial pressure that results in impaired tissue perfusion associated with severe complicating neurological sequelae or death. This allows bacterial toxins, cytotoxins in blood and CSF that engage Toll-like receptors of the endothelium that trigger a downstream cascade. The endothelial cells activate the release of Tumor necrosis factor-alpha (TNF-α), nitric oxide and matrix etalloproteinase-2 (MMP-2). This activation leads to increased permeability (Koedel et al., 2002; Leib et al., 1999; Quagliarello and Scheld, 1992).

The endothelium also expresses several receptors for leukocyte adhesion thereby enabling the presence of chemotactic factors like Interleukin-8 (IL-8) activated by inflammatory mediators. This further promotes neutrophil adherence to their target receptors thereby enabling transendothelial migration through selectins and integrins. The activation of tissue factor on the endothelium is one of the causes for the culmination into procoagulant state resulting in thrombosis and hemorrhage that are quite frequently found in autopsy cases. Neuronal injury in bacterial meningitis is caused due to hypoxia, neurotoxic bacterial products and inflammatory mediators of the host response. The final tissue damage oxidants are the free radicles. These include reactive oxygen species (ROS), reactive nitrogen species (NOS) and they directly affect the neurons (Kastenbauer et al., 2002).

Endothelial activation and vascular inflammation have a deleterious effect on cerebral blood flow. Due to vasculitis, there occurs subintimal infiltration of cerebral blood vessels by neutrophils causing constricture of the vascular lumen leading to vasospasm (Pfister, 1992). The intrinsic pathways that are involved in neuronal death involve the necrotic pathway, caspase-independent apoptotic pathway and caspase-depenedent apoptotic pathway. The process of necrosis and apoptosis are intertwined and rely

basically on the presence/absence of bacterial toxins that increase/decrease endogenous factors like Calcium, poly (ADP ribose) polymerase, PARP and Oxidants. The Oxidants cause loss of membrane integrity thereby leading to depolarisation via lipid peroxidation, finally causing neuronal necrosis. Oxidants also cause DNA damage activating PARP which leads to energy depletion resulting in cell death. High concentrations of bacterial pneumolysin, neurotoxin, excitatory amino acids (EAA) and oxidants trigger massive Calcium release along with increase in cytosolic apoptosis inducing factor (AIF) that results in Caspase-independent apoptotic pathway with the activation of PARP that damages the mitochondrial membrane to release cytochrome-c. During inflammation, there is release of inflammatory mediators like TNF-ɑ that inactivates PARP that will inhibit necrosis and push the cell-death dynamics to apoptosis through the release of caspases.

The observed neuronal injury may be the consequence of axonal injury as reported in the human studies (Gerber et al., 2009). Cerebral infarction in chronic meningitis may be another factor causing the neuronal injury (Floret et al., 1989; Lan et al., 2001). Studies implicate an important role of monocytic inflammatory cells in bacterial meningitis by the release of glutamate, which may contribute to neuronal cell death (Spranger et al., 1996). Galectin-3 was localized to polymorphonuclear neutrophils, microglia, monocytes and macrophages, suggesting an involvement of galectin-3 in the neuroinflammatory processes leading to brain damage in pneumococcal meningitis (Bellac et al., 2007). In the late phase of acute PM, a significant transcriptional upregulation of kynurenine-3-hydroxylase and an accumulation of the neurotoxic metabolites 3-hydroxykynurenine (3-HKYN) and 3-hydroxyanthranilic acid in cortex and hippocampus has been reported. The positive correlation between the concentration of 3-HKYN and the extent of hippocampal apoptosis adds support to the concept that 3-HKYN contributes to brain injury in PM (Bellac et al., 2006).

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