Acute Streptococcus pneumoniae meningitis causes Neurodegeneration in the Subregions of the Hippocampus in Wistar Rats

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***Abstract*- Background:** Bacterial meningitis is known to cause neurodegenerations in the hippocampus, motor cortex and cerebellum. Aim of the present study was to investigate quantitatively the effect of pneumococcal meningitis on hippocampal sub regions in Wistar rats.

Materials and Methods: Thirty days old rats were divided into normal control (NC) and meningitis (M) groups. Rats in the meningitis group were infected with *Streptococcus pneumoniae* intracisternally on postnatal day 31. 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 18 hrs for clinical symptoms of meningitis to develop. After 18-24 hrs of incubation, 10-50µl of the CSF sample was collected. Gram's staining of the CSF smear was done and observed under oil immersion objective (100X) for Gram positive, lanceolate diplococci. The rats were perfused transcardially with saline followed by 10% formalin. Brains were removed, processed for paraffin sectioning and stained with cresyl violet stain. Neurodegeneration in the hippocampal CA1, CA3 and dentate hilus were quantified.

Results: The hippocampal sub-regions showed neurodegeneration. Significant fractions of neurons in the above regions were darkly stained and were irregular in shape. There was 56-81% neuronal loss in these regions. The surviving neurons showed 34-45% decrease in cell diameter and 28-29% decrease in the cross-sectional area in the hippocampal sub regions.

Conclusion: Meningitis affects the hippocampal subregions equally and may be the neural basis of cognitive deficit. The mechanism of such neurodegeneration could have been due to necrosis or apoptosis.

***Index Terms*-** Neurodegeneration, pneumococcal meningitis, hippocampus, CA1, CA3, Dentate hilus

I. Introduction

Pneumococcal meningitis (PM) is associated with high mortality and morbidity. Great majority of survivors are affected by neurological sequelae due to a wide spectrum of brain injury mainly affecting the cortex and hippocampus. Bacterial meningitis due to *Streptococcus pneumoniae* is associated with neurologic sequelae including sensory-motor deficits, seizures, and impairments of learning and memory. The histomorphological observations of these sequelae suggest a pattern of brain damage characterized by necrotic tissue damage in the cerebral cortex and apoptosis of neurons in the hippocampal dentate gyrus [2,3,4,5]. The only mode of treatment for bacterial meningitis has been antibiotics [1]. Two-thirds of meningitis related deaths are due to miserable outcomes, primarily from central nervous system damage. Rest of the outcomes are due to systemic complications related to septic shock [6].Spatial memory and learning deficits were reported in mice after experimental pneumococcal meningitis. It has been shown that inflammatory host responses still continue even after killing the bacteria with antibiotics. This inflammatory response of the host is said to be responsible for tissue damage in the central nervous system [7,8,9,10].

In bacterial meningitis neuronal injury or damage is a common feature, which occurs in the hippocampus due to apoptotic cell death pathways. Neuronal injury in bacterial meningitis is caused by hypoxia, neurotoxic bacterial products and inflammatory mediators of the host response. The final tissue damaging oxidants are the free radicals. These include reactive oxygen species, reactive nitrogen species and they directly affect the neurons [11]. Programmed cell death of the neurons in the granular layer of the hippocampal dentate gyrus and necrosis of the pyramidal neurons is seen in the hippocampus. The above pathways lead to severe neurological sequelae or even to death [12,13,14,15,16,17,18,19].

Magnetic resonance studies of survivors of bacterial meningitis have shown hippocampal atrophy [20]. Long term bacterial meningitis leads to neuronal death, deafness, intellectual and cognitive impairments and behavioral problems and less commonly to epilepsy, spasticity or focal neurologic deficits [3,21,22,23,24,25].

Though there is ample literature on meningitis induced neuronal injury in the hippocampal region, none of them seem to address the neurodegeneration quantitatively in different subregions of the hippocampus. Hence the present study was performed to investigate neuronal injury quantitatively in the sub regions of the hippocampus, after experimentally inducing meningitis in Wistar rats.

II. Materials and Methods

Animal ethical approval

Twelve one 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 and were fed on a commercial diet. All the studies conducted were approved by the Institutional Animal Ethical Clearance 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 *Streptococcus 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 alpha haemolytic colonies were subjected to Gram's staining for microscopical analysis. Gram positive, lanceolate-shaped diplococci were seen on examination at 100X 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 Inulin. The pneumococcal colonies were lyophilized and cryopreserved at-70^{o}C for further use.

b.Experimentalgroups:

30 days old Wistar rats 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 *Streptococcus 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 (Robert et al., 2003). 10µl of the pneumococcal suspension was inoculated into the cisterna magna under steriotactic 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 10µl 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 hrs for clinical symptoms of meningitis. After 18-24 hrs of incubation, 10-50µl of the CSF (cerebrospinal fluid) sample was collected through lumbar puncture, for further tests. Gram's 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 20µl were fixed with methanol (20µl) for 30 minutes, prior to Geimsa staining. The polymorphs were counted using a hemocytometer and assigned total cell count as cells/ml as defined for infection (Robert et al., 2003).

d. Tissue processing for histological study

After 18hrs of incubation, the rats in the meningitic group and the rats of the control group were euthanized and sacrificed on post natal day 31. 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 hrs in the same fixative. The tissue was processed for paraffin sectioning. Hippocampal tissue was selected for study. Tissue was dehydrated in the ascending grades of ethyl alcohol and then cleared with xylene. The tissue was further embedded in paraffin wax. Coronal sections of hippocampus were taken at 5µm 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^{o}C for 20 minutes. 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

i. Neuronal cell counts:

Number of neurons in 250µm length of CA1, CA3 and dentate hilus region were counted at 400X 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. Number of neurons was expressed as Number/ 250µm length of the given region.

ii.Cross sectional area and diameter of neurons: The cross sectional area of the neurons and diameter were measured 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).

f. Statistical analysis

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

III. RESULTS

Behavioral observations and general histological changes

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. Gram's staining of the CSF culture also confirmed the same. The hippocampus of meningitis rats showed significant neurodegeneration. There was significant gliosis in all the sub regions of the hippocampus in the meningitis rat. The hippocampus was decreased significantly in its size and all the layers were decreased in their thickness in the meningitis induced rat brain compared to the normal control brain. The majority of the neurons showed degenerative features. The degenerating neurons were irregular in shape and were darkly stained

Effect of meningitis on hippocampal CA1 region

There were severe neurodegenerative changes in the hippocampal CA1 region (Fig 1.)

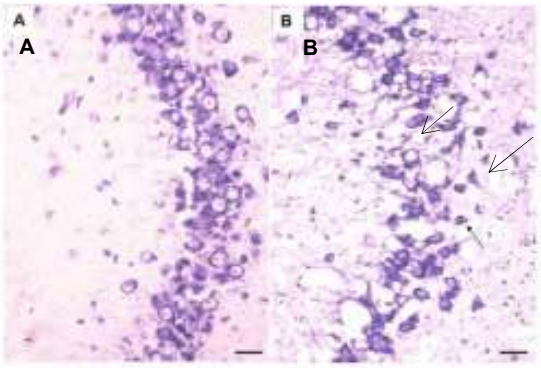

***Fig 1**: Photomicrographs of hippocampus: CA1 region of normal control (A) and Meningitis (B) rat brain. Note the degenerative changes (arrows in B) and decreased number of neurons in B. Cresyl violet stain, Scale bar = 25µm.*

i. Neuronal cell density in CA1 region (Fig 2)

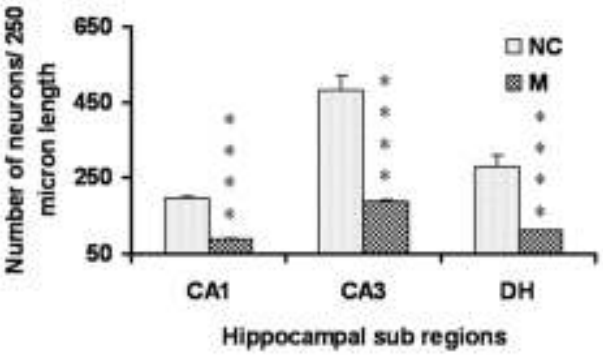

***Fig 2:** Graph showing the number of neurons in 250µm length of CA1, CA3 and dentate hilus of normal control (NC) and meningitis (M) groups. Each bar represents mean±SEM(n=6). Note there is a significant decrease in the number of neurons in meningitis group. NC Vs M, * P<0.0001; Two tailed student t-test.*

Number of neurons in CA1 region was decreased significantly (56%) in the meningitis group compared to normal control group,[198.16 ± 3.42 cells in 250µm length of CA1 region (Mean ± SEM) in control group,(n=6) Vs 86.83 ± 3.58 in meningitis group, (n=6), P< 0.0001, two tailed, Student's t-test].

ii. Diameter of CA1 neurons (Fig 3)

The diameter of CA1 neurons was decreased significantly (36%) in the meningitis group compared to normal control group (25.64 ± 0.96 in control group, (n=6) Vs 16.31 ± 0.67

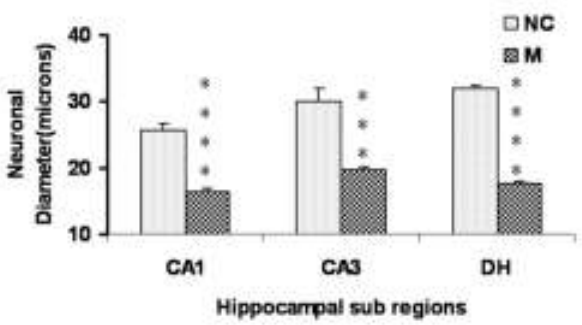

***Fig 3:** Graph showing the diameter hippocampal CA1, CA3 and dentate hilar neurons in normal control (NC) and meningitis (M) groups. Each bar represents mean ± SEM(n=6). Note there is a significant decrease in the diameter of the neurons in meningitis group. NC Vs M, *** P<0.001; Two tailed student t-test.*

in meningitis group, (n=6), P< 0.0001, two tailed, Student's t-test).

iii. Cross sectional area of CA1 neurons (Fig 4)

The cross sectional area of CA3 neurons was decreased significantly (28%) in the meningitis group compared to normal control group (181.10 ± 4.33 in control group, (n=6) Vs 130.10 ± 8.29 in meningitis group, (n=6), P< 0.001, two tailed, Student's t-test).

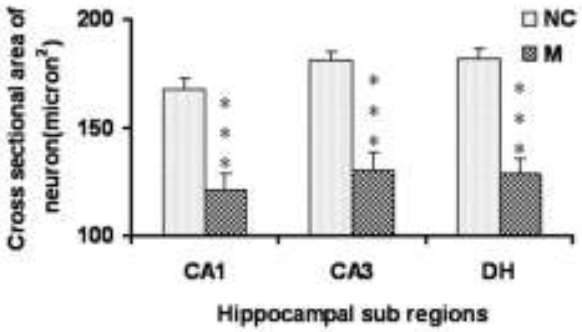

***Fig 4:** Graph showing the cross sectional area of hippocampal CA1, CA3 and dentate hilar neurons in normal control (NC) and meningitis (M) groups. Each bar represents mean ± SEM (n=6). Note there is a significant decrease in the cross sectional area of the neurons in meningitis group. NC Vs M, *** P<0.001; Two tailed student t-test.*

Effect of meningitis on hippocampal CA3 region

There were severe neurodegenerative changes in the hippocampal CA3 region (Fig 5)

i. Neuronal cell density in CA3 region (Figure 2)

Number of neurons in CA3 region was decreased significantly (81%) in the meningitis group compared to normal control group (481.5 ± 37.46 cell in 250µm length of CA3 region in control group,(n=6) Vs 188.00 ± 3.96 in meningitis group,(n=6),

P<0.0001, two tailed, Student's t-test)

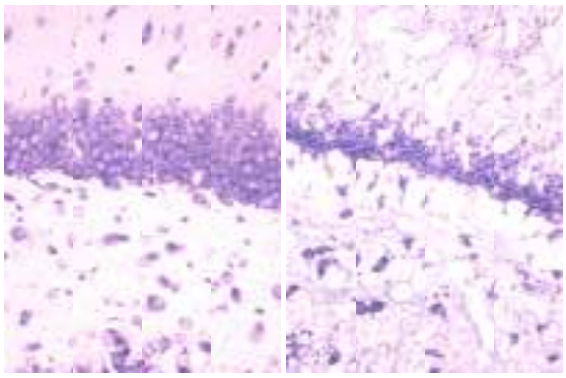

Fig 5: *Photomicrographs of hippocampal CA3 region in normal control (A) and Meningitis (B) rat brains. Note the degenerative changes in hippocampal CA3 neurons (arrows in B) and decreased number of neurons in meningitis group. Cresyl violet stain, Scale bar = 25µm.*

ii. Diameter of CA3 neurons (Figure 3)

The diameter of CA3 neurons was decreased significantly (34%) in the meningitis group compared to normal control group (29.96±2.02 in control group, (n=6) Vs 19.64±0.50 in meningitis group, (n=6), P< 0.001, two tailed, Student's t-test).

iii. Cross sectional area of CA3 neurons (Fig 4)

The cross sectional area of CA3 neurons was decreased significantly (28%) in the meningitis group compared to normal control group (181.10 ± 4.33 in control group, (n=6) Vs 130.10 ± 8.29 in meningitis group, (n=6), P< 0.001, two tailed, Student's t-test).

Effect of meningitis on dentate hilus region

There were severe neurodegenerative changes in the hippocampal CA1 region (Figure 6.)

i. Neuronal cell density in dentate hilus region (Fig 2)

Number of neurons in dentate hilus was decreased significantly (60%) in the meningitis group compared to normal control group (281.5 ± 27.75 cell in 250µm length of dentate hilus region in control group, (n=6) Vs 113.00 ± 2.01 in meningitis group, (n=6), P< 0.0001, two tailed, Student's t-test).

ii. Diameter of dentate hilar neurons (Fig 3)

The diameter of dentate hilar neuron was decreased significantly (45%) in the meningitis group compared to normal control group (31.96 ± 0.48 in control group(n=6) Vs 17.62 ± 0.41 in meningitis group(n=6), P< 0.0001, two tailed, Student's t-test).

iii. Cross sectional area dentate hilar neurons (Figure 4)

The cross sectional area of dentate hilar neurons was decreased significantly (29%) in the meningitis group

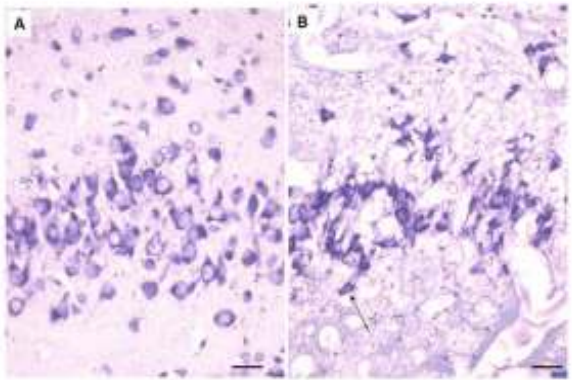

Fig 6: *Photomicrographs of dentate hilus region of the hippocampus in normal control (A) and Meningitis (B) rat brains. Note the degenerative changes (arrows in B) and decreased number of neurons in B. Cresyl violet stain, Scale bar=25µm*

compared to normal control group (181.1 ± 5.20 in control group, (n=6) Vs 128.76 ± 6.55 in meningitis group, (n=6), P< 0.001, two tailed, Student's t-test)

IV. Discussion

The results of the present experiment revealed that pneumoccocal meningitis will lead to extensive lesion in the sub regions of the hippocampus, the part of the brain concerned with cognitive functions. Hippocampus on the whole has been attributed to spatial memory and long term potentiation (LTP) and LTP is widely believed to be one of the main neural mechanisms by which memory is stored in the brain. Some neuroscientists no longer believe that the concept of a unified "limbic system" is valid, though[35]. However, the hippocampus with the areas of the CA1 to CA4 along with the dentate gyrus, is anatomically connected to parts of the brain that are involved with emotional behavior-the septum, the hypothalamic mammillary body, and the anterior nuclear complex in the thalamus so it's role as a limbic structure cannot be completely dismissed. The hippocampus is especially vulnerable to damage caused by metabolic dysregulation and in the present study the dysregulation being brought about by septic meningitis mediated by the gram positive pneumococci. However distinct sub-regions within the hippocampus differ by their relative susceptibility to such damage. Region CA1 pyramidal neurons are most sensitive to metabolic perturbations while region CA3 pyramidal neurons show more resistance, and these unique profiles of susceptibility are but one example that differentiates CA1/CA3 neurons[36]. However, despite the importance of hippocampus in learning, memory and cognitive deficits, little information is available about the integrity of hippocampal neurons during acute pneumococcal meningitis. Thus, we have investigated the effects

of meningitis on the hippocampal morphology in a rat model of intracisternal inoculation inducing pneumococcal

meningitis and our results revealed neuronal damage which was very severe in all sub regions of hippocampus as revealed by our quantitative data. In the case of CA1, CA3 and dentate hilus regions, 56-81% neuronal loss was documented. Further more there was 34-45% decrease in the diameter and 28-29% decrease in the cross sectional area was observed in the surviving neurons in the hippocampal sub regions. The neurons also showed shrunken and darkly stained cytoplasm with clear nuclei that showed no sign of death signifying that there was no DNA damage. This is in line with the works done by Kafa et al. during sepsis where they also show that a significantly higher neuronal density together with widespread dark, shrunken neurons with dense cytoplasm and irregular membrane boundaries in the CA1 and CA3 areas of the hippocampus in faecal peritonitis group as compared to the sham and unoperated groups [37]. One possible explanation for the decreased neuronal cell volumes is that it could be due to a more condensed alignment of neurons within these areas that could significantly lower nuclear diameter of healthy looking pyramidal neurons of CA1 area. The dark, shrunken neurons frequently observed in these regions in the present study may represent an early stage of a neuronal degeneration. The presence of such neurons have also been reported previously in different brain areas including hippocampus after studies of ischemia, trauma and other brain insults as brought out by Gallays et al. and Messaris et al.

Although, the pathophysiological basis of these changes (shrunken size with slightly condensed chromatin and darkly stained neurons) is not entirely described they may be regarded as an early sign of programmed cell death (Ito *et al.*, 2007). There is compelling evidence to support this assumption. Increased apoptosis is reported in neurons of CA1 regions of the hippocampus, in choroid plexus and in purkinje cells of the cerebellum in models of sepsis.

Hippocampal neuronal death reported here could be the result of apoptosis or as an inflammatory response [26]. Alternatively it could also have been an excitotoxic injury. Indeed studies implicate an important role of monocytic inflammatory cells in bacterial meningitis by the release of glutamate, which may contribute to neuronal cell death. Animals treated with kynurenic acid showed significantly less neuronal injury ($P < .03$) in the cortex and the hippocampus than did untreated controls. These results suggest an important contribution of glutamate to neurotoxicity in the animal model of neonatal meningitis [27].

The observed neuronal injury may also be the consequence of axonal injury as reported in the human studies [28]. Infarction in chronic meningitis may be another factor causing the neuronal injury [29,30]. The neuronal degeneration could take the necrotic pathway or the apoptotic pathway. It has been demonstrated that genetic inactivation of inducible nitric oxide synthase (iNOS) results in a marked reduction of caspase-3-mediated neuronal damage in experimental murine pneumococcal meningitis [26]. It has been demonstrated that the broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone(z-VAD-fmk) prevented hippocampal neuronal cell death and white blood cell influx into the cerebrospinal fluid compartment in experimental pneumococcal meningitis [31]. Further more gene expression studies in cortex and hippocampus during acute pneumococcal meningitis have shown the upregulation of the cell death/survival genes in hippocampus [32].

Experimental analysis during the acute phase of pneumococcal meningitis showed the localisation of Galectin-3 to polymorphonuclear neutrophils, microglia, monocytes and macrophages, suggesting an involvement of galectin-3 in the neuroinflammatory processes leading to brain damage in pneumococcal meningitis [33]. In the late phase of acute PM, a significant transcriptional upregulation of kynurenine-3-hydroxylaseandan 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 [33].

Another mechanism of neuronal injury in the hippocampus could be due to the down regulation of neurotrophic factor secretion during meningitis insult. The experimental BDNF infusion has shown to protect a large number of neurons in cerebral cortex and hippocampus from inflammatory brain injury in bacterial meningitis [34].

Furthermore the morphological changes associated with cell shrinkage and density decrease could also precipitate dysfunction in synaptic integrity of the regions of the hippocampus that are connected with each other through pathways. Many neurons in the rat and mouse hippocampus respond as place cells: that is, they fire bursts of action potentials when the animal passes through a specific part of its environment. Hippocampal place cells interact extensively with head direction cells, whose activity acts as an inertial compass, and with grid cells in the neighboring entorhinal cortex. In particular, the extensive excitatory recurrent connections of region CA3 have been proposed to play a role in encoding and retrieval of associations, including autoassociative completion of a single pattern, or associative retrieval of the next pattern in a sequence. Lee and Kessner et al in their work on lesions to selective regions of the hippocampus have brought out significant functions attributed to the CA1, CA3 and the dentate gyrus regions of the hippocampus. They show that selective lesions of the dentate gyrus or region CA3 impair the detection of novel locations of familiar items, beyond the effect of CA1 lesions. Control rats showed a normal increase in exploration when a familiar object was moved to an unfamiliar location, whereas rats with CA3 lesions did not show this enhanced exploration. The authors interpret this finding as indicating a role of region CA3 in detecting the mismatch between the memory for the spatial context of each item and the current sensory input about the spatial position of the item. They contrast this with a more modest change in mismatch detection found in rats with lesions of region CA1, which has traditionally been proposed as the region that computes mismatch. They also find a reduction of exploration with dentate gyrus lesions, which

they interpret as impairment in the capacity to create the distinct representations of spatial context necessary for detection of the mismatch[38]. The results for the present study highlight the complexity of the changes that could be brought about by acute pneumococcal meningitis causing the dysfunction of synaptic integrity which could be effected as an early sign for neuronal degeneration and this could very well explain the pattern of neurodegeneration associated with metabolic dysfunction that could lead to impairment of spatial memory, acquisition and short-term/LTP associated with the hippocampal regions of CA1, CA3 and the dentate hilus as a whole. Hence this study could well be helpful in curtailing the effects of neurodegeneration via conjunctive therapy targeted against neuroinflammation, free radical damage of the neuronal mass due to apoptosis and necrosis and several signal transduction pathways involving Brain derived neurotrophic factor (BDNF) that can directly or indirectly control neurogenesis to replace the damage done to the various regions in the brain

V. Conclusion

The consequences of the observed neurodegeneration in the different sub regions of the hippocampus from our study lines up with the facts published by Wellmer et al [28], that state that the result of untreated pneumococcal meningitis may result in cognitive deficit . These findings may well be the neuronal basis for the reported cognitive deficit reported in the human survivors of meningitis, especially in children [20, 3]. This study could pave avenues for research into therapeutic modalities that could thwart the process of neurodegeneration through pathways that could trigger salvage of the destroyed neurons; as the study indicates only early signs of neuronal damage but not DNA damage.

VI. Acknowledgments

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