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Memory and learning seems to be related to cholinergic dysfunction in the JE rat model

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HIGHLIGHTS

- Intracerebral inoculation of JEV (3×10^6 pfu/ml) on 12 day Wistar rats.
- Spatial memory and learning was impaired on 10 and 33 dpi and recovered on 48 dpi.
- Cholinergic markers were reduced in cortex, hippocampus, striatum, and cerebellum.
- Correlation between spatial memory and brain cholinergic parameters was found.
- Transient form of spatial memory impairment was observed in JE infected rats.

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ABSTRACT

Cognitive changes have been known in encephalitis but in Japanese encephalitis (JE) such studies are limited. This study aims at evaluating the spatial memory and learning and correlate with markers of cholinergic activity in the brain.12 day old Wistar rats were inoculated with dose of 3×10^6 pfu/ml of JE virus. On 10, 33 and 48 days post-inoculation (dpi), spatial memory and learning was assessed by Y maze. Brain biopsies from frontal cortex, corpus striatum, hippocampus and cerebellum were taken. Muscarinic cholinergic receptor was assayed by Quinuclidinyl benzylate (H3-QNB) binding, CHRM2 gene expression by real time PCR and choline acetyl transferase (ChAT) by Western blot. Spatial learning and memory showed significant decline in rats inoculated with JEV on 10 and 33 dpi (47.5%, $p < 0.01$; 30.2%, $p < 0.01$). It started recovering on 48 dpi. Muscarinic cholinergic receptors showed significant decrease in frontal cortex (31%, $p = 0.001$; 26%, $p = 0.003$), hippocampus (57%, $p = 0.001$; 39.9%, $p = 0.002$) and cerebellum (31.2%, $p = 0.008$; 21.6%, $p = 0.007$) but not in corpus striatum as compared to control. The mRNA expression of CHRM2 receptor gene showed significant decrease in the expression in frontal cortex (48%, $p < 0.001$; 38%, $p < 0.01$), hippocampus (43%, $p < 0.001$; 37%, $p < 0.05$) and cerebellum (46%, $p < 0.001$; 42%, $p < 0.05$) on 10 and 33 dpi. ChAT showed significant fold decrease in the expression in frontal cortex (2.11, $p < 0.01$, 1.12, $p < 0.05$) and hippocampus (2.2, $p < 0.01$, 1.41, $p < 0.05$) on 10 and 33 dpi. Correlation between ChAT, CHRM2 and total muscarinic receptor activity with spatial memory were found at different dpi. There was transient spatial learning and memory impairment which was associated with reduction of total muscarinic receptor binding, CHRM2 gene and ChAT expression in different brain region of rat infected with JE Virus.

1. Introduction

Japanese encephalitis is one of the commonest causes of infectious encephalitis in the world and affects nearly 70,000 cases with 25–40% annual mortality. Nearly half of survivors of JE develop neurological sequelae [14,21,24,31]. Cognitive and behavioral changes constitute about 20–30% of the sequelae [47,50]. JE affects specific areas of brain as has been shown in MRI and autopsy studies. MRI study in six patients with JE revealed bilateral thalamic involvement in five, brainstem in three and basal ganglia in one [32]. Signal changes were also seen in midbrain and cerebellum in three each of seven patients and the basal ganglia in one [25] and another study on seventeen patients also revealed similar findings [20]. MR imaging in 62 patients with JE revealed temporal lobe (mostly hippocampus) involvement in 11 (17.7%) patients in addition to thalamic and brainstem involvement [16].These changes are consistent with human autopsy studies in which the lesions

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were noted in thalamus, basal ganglia, midbrain, cerebral cortex and cerebellum [44]. The histopathological findings in 34 autopsy cases form Karnataka India revealed edema, hazy meninges, focal congestion in white matter and deep gray matter along with necrosis especially in thalamus, corpus striatum, midbrain, pons and cerebellum [44]. These results were in agreement with that of 11 autopsies from Japan [53].

Cholinergic neurons are present in the basal forebrain complex, frontal cortex, striatum, hippocampus, amygdala and brainstem [4], these region are also involved in JE. Cholinergic projections from basal forebrain (nucleus basalis of Meynert) to the frontal cortex and limbic structures (hippocampus) are associated with learning and memory [30]. Cholinergic dysfunction has been linked to neurobehavioral alterations in various neurological disorders such as Alzheimer's disease (AD) [2], Huntington disease [46] schizophrenia [41] and organophosphate poisoning (OP) [6].

Choline acetyl transferase (ChAT) is a sensitive indicator of cholinergic activity in the central nervous system (CNS). In AD, ChAT activity is reduced by 90% in nBM (basal forebrain), along with other sub-cortical areas such as hypothalamus, caudate, and thalamus but is normal in striatum [1]. Loss of about 90% AChE activity has been reported in different cortical areas in AD [36]. There is also decrease of up to 30% in the density of muscarinic receptors predominantly in the hippocampus [36,37]. Out of five muscarinic receptors (M1-M5), loss of M2 subtype is the most prevalent in AD [28].

Brain cholinergic activity can be evaluated in different brain regions of experimental animal and these can be correlated with memory and learning abnormalities. Such studies may provide valuable information on the possible mechanism of CNS infection on memory and behavioral changes. There is paucity of studies evaluating the role of cholinergic functions in CNS infections.

We hypothesize that JE may result in, memory and learning impairment which will partially or completely recover on follow up as the infection subsides. The memory and learning functions may correlate with cholinergic activity in brain.

We aim to study the memory and learning in JEV infected rats at different time points after JEV infection and to correlate these with cholinergic activity in the brain. We also study these changes at different time points to study the recovery of cognitive and biochemical markers.

2. Material and methods

2.1. Virus

Indian neuro-virulent strain of JE virus, GP 78668A (GP-78) was used for the viral titration by standard plaque assay [52]. To increase the viral titer, intracerebral inoculation with 50 μl, 2% MEM (Gibco, NY, USA) of JEV was injected in 3–4 days old suckling mice.

2.2. Animals

In the present study 12 days old suckling Wistar rats were used. The rats were maintained under hygienic conditions in an air conditioned room at 25 ± 2 °C with 12 h light/dark cycle. The rats were housed with free access to food and water. The experimental protocol was approved by the institutional Animal Ethics Committee. All the experimental procedures were carried out in accordance with the institutional guidelines.

2.3. Rat model of JE

120 rats were divided into two groups viz. Group 1 and Group 2. The details of experimental design are presented in Fig. 1. JEV (3×10^6 pfu/ml) was inoculated with minimum penetration of 5 mm into the cerebral cortex, taking care to avoid injury in any specific brain region [39]. 3×10^6 pfu virus dose of GP 78668A (GP-78) was used for intracerebral inoculation because of the greater susceptibility and longer survival of younger rats compared to the older ones. The viral dose used in this study (3×10^6 pfu) was standardized on the basis of previous report [23,39,48].

Sterile phosphate buffered saline (PBS) was inoculated in the control rats following the same protocol. Both JEV inoculated and control PBS inoculated rats were monitored daily for any clinical signs and symptoms.

3. Behavioral parameters

3.1. Spatial learning and memory

3.1.1. Continuous alternation test

Spontaneous alternation in a single session was assessed in a computerized Y maze (TSE, Germany), which was used as a measure of short-term memory performance [29,43]. Each arm of Y maze was 40 cm long, 30 cm high and 15 cm wide and converged in an equilateral triangular central area with 15 cm at its longest axis. Rat was placed at the end of one arm and allowed to move freely through the maze for five minutes. The sequence of each arm entry recorded automatically by Y maze software. Measure of spatial memory was defined as the number and the sequence of the arm entries were recorded during 5 min. The alternation percentage was calculated as the number of alternations (entries into three different arms consecutively) divided by the total possible alternations (i.e. the number of arms entered minus 2).

3.1.2. Neurotransmitter receptor binding assay

Cholinergic muscarinic receptors were estimated in frontal cortex, hippocampus, corpus striatum and cerebellum, using radioligand receptor binding technique following the standard procedure [22].

3.1.3. Preparation of crude synaptic membrane

Crude synaptic membrane was prepared by homogenizing the brain regions (frontal cortex, corpus striatum, hippocampus and cerebellum) in 19 volumes of Tris–HCl buffer (5 mM, pH 7.4). The homogenate was centrifuged at 40,000 *g* for 15 min at 4 °C. The pellet was washed twice in homogenization buffer and centrifuged at 40,000 *g* for 15 min at 4 °C. The pellet was suspended in Tris–HCl buffer (40 mM, pH 7.4) and stored at −20 °C for binding assays.

3.1.4. Assay of muscarinic–cholinergic receptors in selected brain regions

Binding incubations in a final volume of 1.0 ml were carried out in triplicate. For muscarinic–cholinergic receptors, ^{3}H-QNB (42 Ci/mmol, Perkin Elmer, USA) was used and atropine sulfate (1.9×10^{-6} M) was used as a competitor (Table 1). The reaction mixture containing Tris–HCl buffer (40 mM, pH 7.4), together with membrane protein (300–400 μg) and appropriate radioligand was incubated for 15 min at 37 °C in the presence or absence of competitor to assess the non-specific and total binding respectively. At the end of incubation, contents of the binding tubes were immediately filtered on glass fiber disks (25 mm diameter, 0.3 μm pore size, Whatman GF/B) and washed twice rapidly with 5 ml chilled Tris–HCl buffer (40 mM, pH 7.4). Filters were dried and transferred into vials and scintillation mixture (2,5-diphenyl oxazole; 1,4-bis-5, phenylozazolyl-benzene; naphthalene; toluene; methanol and 1,4 dioxane) added to it. The radioactivity was counted on β scintillation counter (Packard, USA) at an efficiency of 30–40% for ^{3}H to determine membrane bound radioactivity. Specific binding was determined by subtracting the non-specific binding from the total binding and has been expressed as pmol ligand bound/g protein. The amount of membrane protein per tube was around 300–350 mg as determined by the method [27].

3.1.5. Total RNA extraction

RNA was extracted from separated brain regions (frontal cortex, corpus striatum, hippocampus and cerebellum) individually collected in 1 ml of ice-cold TRIzol (Invitrogen, Carlsbad, CA, USA) using QIAmp