Kindling & mossy fibre sprouting in the rat hippocampus following hot water induced hyperthermic seizures


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Background & objectives: Hot water epilepsy (HWE) is well recognized reflex epilepsy with possible genetic susceptibility. Rat model and human experimentation had proven that HWE is a type of hyperthermic seizure with possible kindling on repeated stimulation in animals. The present study was undertaken to investigate kindling associated with hyperthermic seizures induced by repeated hot water stimulation in the rat model and to prove hyperthermic kindling.

Methods: Epileptic seizures were induced in 36 male Wistar albino rats by means of hot water sprays at 48 h time intervals. Progression of seizure activity was investigated by studying the behaviour, severity and duration of the seizure. Threshold of rectal temperatures and timed latency for seizure induction were studied. Seizure discharges (EEG) were recorded from ventral hippocampus in six of these rats. Timm’s staining was used to study the neuronal sprouting as a consequence of kindling. Studying the seizure threshold, latency, duration of seizure discharge and behavioural seizure following a stimulus-free interval of 30 days tested permanence of kindling.

Results: Following 8-12 episodes of hot water stimulations there was progressive epileptic activity manifested in the form of lowering of rectal temperature thresholds from 41.5 to 40.0°C, drop in latency for developing seizures from 185 to 118 sec, increase in duration of hippocampal seizure discharge from 15 to 140 sec, along with progressive increase in complexity of EEG after discharges, increase in behavioural seizure severity from Grade 1 to 5 in all the rats, and neuronal sprouting observed in supragranular molecular layer and in stratum lacunosum.

Interpretation & conclusion: Our study covered all aspects of kindling and provided a useful animal model for human hot water epilepsy. Hyperthermic seizures induced by hot water in the rat model kindle as demonstrated by Timm’s staining.

Key words Hot water epilepsy - hyperthermic kindling - mossy fibre sprouting - reflex epilepsy - Timm’s staining
Kindling involves a progressive increase in neuronal activity on repeated stimulation generally of the forebrain regions, which could be induced by various types of stimulations such as, electrical and chemical. Hot water bath induced epilepsy commonly referred to as hot water epilepsy (HWE), is a common reflex epileptic disorder described from southern India. However, isolated cases have also been reported from other parts of the world. In view of the subsequent development of spontaneous non-reflex seizures in 25 per cent of the cases, Satishchandra et al postulated a phenomenon of hyperthermic kindling as a mechanism for this unique type of epilepsy. Klauenberg et al reported progressive increase in seizure activity - a kindling like phenomenon in rats, following repeated immersion in hot water tub and Jiang et al provided histopathological evidence in the form of hippocampal sprouting following hyperthermia induced stimulation. To understand the underlying pathogenesis of hyperthermic seizures in HWE, we developed a rat model that mimics the human form of hot water epilepsy and reported the clinical features, the stimulus threshold, latency, electroencephalographic and pathological features. In the present study, we investigated the effect of repeated hot water induced stimulations on the nature of seizure among adult rats, with special reference to rectal temperature thresholds, seizure latency, duration, behavioural grading of seizures and electroencephalographic after-discharges to evaluate whether there is evidence of hyperthermic kindling. As kindling is associated with aberrant sprouting of mossy fiber in the hippocampal regions and there are reports of such a phenomenon occurring following repeated hyperthermic seizures, we have also examined the structural evidence of mossy fibre sprouting following kindling as a basis for the progression of human hot water epilepsy in this study.

Material & Methods

Thirty six male Wistar albino rats of 20-22 wk age weighing 120-150 g, procured from Central Animal Research Facility, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India, were the subjects for the study. The animals had free access to food and water. Of the 36 rats, 12 had electrodes implanted stereotactically into their ventral hippocampus to study the progression in epileptic seizure discharges following repeated hot water stimulation. All the rats were further subdivided into equal groups of tests and controls. The study has been approved by the institutional animal ethics committee.

Animal surgery: Bipolar stainless steel electrodes with diameter of 0.1 mm, and inter-electrode distance of 0.5 mm, insulated with epoxy resin except at the tips, were stereotactically implanted into the right ventral hippocampus of the rats using the standard co-ordinates under chloral hydrate anaesthesia (400 mg/kg) administered intraperitoneally. Standard co-ordinates as mentioned in the Sterotaxic Atlas of Rat brain (2.6 mm behind bregma; 4.6 mm laterally and 7.5 mm below corticle surface) were used to place these electrodes. The electrodes were anchored with eyeglass screws and dental cement.

Hot water stimulation: The rats were freely ambulant in 40 × 30 × 15 cm plastic chamber, having a fine mesh at the bottom for continuous drainage of water, thus preventing immersion of the body. Hot water (55°C) stimulation was carried out by directing jets of water over the head with 5 ml syringe at a rate of 30 times/min, as described...
earlier\textsuperscript{24,25}. Duration of each stimulation was to a maximum of 10 min with seizure commencement as the end point for stimulation. The water was poured on the head followed by body, each time to mimic the practice of hot water bathing by human subjects. The rats were subjected to 15 stimulations at intervals of 48 h. The 16\textsuperscript{th} stimulation was given after a stimulus free period of 30 days. Two minutes after each hot water stimulation protocol, the rats were cooled using water at room temperature (25°C). The control animals were placed in the stimulation chamber for the same duration of time and exposed to water jets of 25°C (ambient room temperature).

Parameters of seizures studied were rectal temperature thresholds, latency, seizure severity, and duration. Further, reassessment of the epileptic activity was done in all the animals, to evaluate relative stability (permanence) of the kindling phenomenon after a 30 day stimulus free interval. Electroencephalographic recordings of seizure discharges were made using a single channel Polyrite\textsuperscript{®} (Inco-Ambala-India) EEG machine. In the test rats, EEG recording was commenced at the onset of the seizure and continued for 30 min during the post-ictal period. Rectal temperatures were recorded using a digital thermometer (Taiwan Electronics, Taiwan KD) as described earlier\textsuperscript{24} before the stimulation, at the onset of seizure (threshold), 2 min after the seizure and following the post-stimulation cooling protocol. Latency defined as the time interval between the commencement of stimulation to the onset of seizure, the duration of clinical seizure and rectal temperature thresholds to initiate the seizure were recorded with each stimulation.Behavioural components of the seizures were recorded on a video. Clinical grading of seizures was done using the criteria of Klauenberg and Sparber\textsuperscript{21}; Grade 1: Head and body twitching, Grade 2: Forelimb clonus, Grade 3: Kangaroo posturing - the animals sitting on the hind limb drawing the fore limb close to the body, and head erect, Grade 4: Animal falling on its back drawing limbs close to the body in a flexed posture, Grade 5: Explosive “Pop-Corn” posturing followed by running - where the animal is in prone position, has bursts of clonic seizures of the limbs resulting in jumping posture followed by attempts at aimless running. The control rats were handled in an identical manner except that they were exposed to head and body stimulation with water at room temperature (25°C) for 5 min. None of the control rats developed any seizure and rectal temperature recorded did not show any change.

\textit{Timm’s staining protocol:} All perfusions were performed 24 h following the last stimulation. The rats were injected 0.2 ml heparin into the heart and intracardially perfused with 0.37 per cent sodium sulphide solution, pH 7.2 (150-200 ml) for 20 min, followed by 100 ml of 4 per cent buffered paraformaldehyde for 10 min. The brains were stored in 4 per cent paraformaldehyde at room temperature for 3-5 days. They were sliced in coronal plane, processed for paraffin embedding in an automated tissue processor and 15 µm thick slices were collected on poly-L-lysine coated glass slides. Sections from rats those experienced single and multiple seizures were processed along with those from the matched controls. Deparaffinised sections were rehydrated through graded alcohol and brought to glass distilled water. Batches of sections from the control and various test groups were placed in a glass jar containing a freshly prepared developer, maintained at 24-28°C in the dark. The developer contained a mixture of 240 ml gum Arabic solution (1 kg Arabic gum powder in 2 l distilled water (dw), 40 ml citrate solution (9.4 g sodium citrate, 10.2 g citric acid in 40 ml dw), 120 ml of 5.6 g per cent hydroquinone
blind coded and the features were recorded. The staining procedure was repeated 3-4 times and slides showing consistent results were analyzed for evidence of neuronal sprouting.  

**Results**

Resting rectal temperature of the control rats was 37°C. On the first stimulation in the experimental rats, rats developed seizures at a mean threshold temperature of 41.5°C. During subsequent stimulation, the threshold gradually dropped to 40.5°C by the 4th stimulation, and reached 40.2°C by the 11th trial and remained relatively stable thereafter till the 15th stimulation (Fig. 1). Following a stimulus free period of 30 days, when the animals were stimulated once again (16th stimulation), the seizure threshold was 40°C.

Seizure latency - the time lag between the point of stimulation to the onset of seizure, dropped from a mean value of 185 sec. on the first trial to 118 sec. on the 7th stimulation (Fig. 2). Beyond this, the latency remained relatively stable till the 15th stimulation. When stimulated once again, after the
of 25 ± 6 sec on first stimulation. The seizure discharge gradually increased to 65 ± 10 sec and 150 ± 15 sec on the 6th and 8th stimulation respectively. Thereafter till the 15th stimulation, there was no significant difference in the EEG pattern or its duration. When subsequently tested with hot water stimulation following the 30 day stimulus free interval, the average seizure discharge lasted 145 ± 10 sec and did not show much of a qualitative change from the 15th stimulus. No spontaneous clinical seizures or EEG discharges were observed any time during the study.

Fig. 4 (A). Rat dorsal hippocampus - Timm’s staining control animal. H, hilus; GL, granular layer of dentate gyrus; IM, internal molecular layer of dentate gyrus; OM, outer molecular layer of dentate gyrus; SL, stratum lacunosum; SR, stratum radiatum; P, pyramidal layer. Bar 100 µ.

Fig. 4 (B). Rat dorsal hippocampus - animal experienced repeated seizures and showed features of kindling. Note dark band of Timm’s silver stain along the outer molecular layer of dentate gyrus. (arrow heads) H, hilum; GL, granular layer; IM, internal molecular layer. Bar 100 µ.
The histology of the control animals revealed occasional anoxic neurons randomly distributed in the hippocampus, cerebral cortex, diencephalic areas and cerebellum. The brainstem neurons were normal. In these control animals, the Timm's staining in the hippocampus was found along the mossy fibre axons of dentate granule cells, which heavily innervated the hilus and extended into the stratum lacunosum of CA_3 zone, ending at C_3-CA_2 border (Fig. 4A). Some reaction product was observed occasionally outlining a few dendrites of granule cell layer. No reaction products as distinct tracts or clusters of granules were seen in the internal (IML) and outer (OML) molecular layers. An occasional section from the control animal revealed small amount of granular silver reaction product at the tips of dentate gyrus and on the granule cell-IML junction.

In a 'single seizure group' of rats (sacrificed one day after the seizure activity) the routine histological features were not distinguishable from the control animals in the anatomical distribution of the anoxic change in the neurons. The white matter and the glial component are normal, similar to control animals.
oligodendroglia or astrocytes or spongy neuropil were evident. There was no evidence of microglial reaction. The only distinguishing feature in the restimulated animals after multiple seizures and a period of rest from those initially stimulated to have multiple seizures was more of argentophilic apical dendrites and granular deposits in neuropil in cerebral cortex, especially hippocampus, but not cerebellum.

On Timm’s stain, fine granules at the tip of dentate gyrus as thin, sparse branching and linear streaks traversing the internal molecular layer on both sides of the brain was observed (Fig. 5A, 6A). Argentophilic fine deposits were found in the neocortex and Ammon’s horn of hippocampus, but not in cerebellum.

The rats which had multiple seizures (7-15 stimulations), those which remained stimulation free for 30 days (after 15 stimulations) and those again resubjected to hot water stimulation after a quiescent period showed variable neuronal shrinkage, cork-screwing and shrinkage of apical dendrites of many of CA1 pyramidal neurons while the rest of the hippocampal zones were minimally affected. In addition to degenerating neurons in hippocampus, similar but variable degree of pathology was noted in cingulate gyrus, temporal lobe, the neurons becoming shrunken, eosinophilic, with loss of nuclei. The Purkinje cells in cerebellum, the large neurons of reticular formation and cranial nerve nuclei revealed shrinkage, loss of Nissl substance and eosinophilic condensation of the cell bodies. In the white matter no discernible reactive cytological change in oligodendroglia or astrocytes or spongy neuropil were evident. There was no evidence of microglial reaction. The only distinguishing feature in the restimulated animals after multiple seizures and a period of rest from those initially stimulated to have multiple seizures was more of argentophilic apical dendrites and granular deposits in neuropil in cerebral cortex, especially hippocampus, but not cerebellum.

On Timm’s staining, in the multiple seizure rats, the staining in the internal molecular layer at the tips of the blades of dentate gyrus and the external...
Fig. 7 (A). CA3 area of Ammon’s horn - Dorsal hippocampus in control animal - Suprapyramidal in control animal - Supra pyramidal area has no sprouts. Bar 50 µ.

Fig. 7 (B). Identical area (CA3) in animal which experienced single seizure reveals suprapyramidal mossy fiber sprouts arborising with basal dendrites of pyramidal neurons (arrowheads). Bar 50 µ.

Fig. 7 (C). Identical suprapyramidal zone of CA3 in animal which experienced multiple seizures shows exuberant clumps of sprouts (arrows) labeled by Timm’s silver stain. Bar 50 µ.
molecular layer was denser than in animals subjected to single seizure (Fig. 5B, 6B). In contrast to control animal and the ones experienced single seizure, in multiple seizure group, the hilar zone of dentate gyrus showed diffuse loss of silver staining and dark labeling of remaining CA4 neurons. The argentophilic labeling of the molecular layer, the zone of mossy fibre sprouts has extended as a continuous band merging the internal and external molecular layer, till the angles of dentate gyrus. At the tips of the dentate gyrus, linear tracts and dense clusters of granules were noted. The internal molecular layer revealed irregular thin silver positive tracts traversing discontinuously at different places, while the border of external molecular layer had a thick band.

The labeling at the angles of the dentate gyrus blades was variable from animal to animal in multiple seizure group but denser than in the single seizure group. Stratum lacunosum, above the molecular layer also had similar thin Timm’s positive tracts, in all the multiple seizure groups of animals. The cap of the CA3 curvature, in multiple stimulation group had many dense and coarse aggregates of silver grains (Fig. 7C), more than the ones noted following single stimulation. The stain density along the cap of CA3 zone appeared to correlate with increase in number of seizures and the temporal distance from the first seizure. A time point correlation of the neurophysiological events with the morphological features was not clear, though a definite evidence of long term sequelae like greater amount of new mossy fibre sprouting, reflecting kindling and formation of new neural circuits was evident.

Discussion

In this study, we established hyperthermic kindling in rats, following repeated hot water exposures on the head. There has been a clear drop in the rectal temperature thresholds for seizures, reduction in latency for seizure response after commencement of the stimulation protocol, and prolongation of duration of seizure response. Further, following repeated stimulations, there was a progression in severity grade of seizures as well. The kindled responses remained relatively stable over a 30 day stimulus free interval. However, we have not observed any spontaneous seizures or epileptic EEG discharges during the random observations in the inter-stimulus interval during the initial 14 stimulations or in the 30 day rest period between 15th and 16th stimulation. Seizure activity could be established in adult rats with hot water stimulation, unlike previous studies in the young rats30,31, suggesting kindling phenomenon can occur even in mature adult brains by repeated seizures. Differential kindling response of young immature brain to repeated seizure activity in contrast to adult mature brain needs to be probed further. Aberrant sprouting of hippocampal neurons is a commonly observed phenomenon accompanying electrical1 and chemical kindling2. Using the progressive increase in the behavioural component of the seizures with repeated hyperthermic stimulations as a criterion, Klauenberg and Sparber21 have demonstrated a kindling phenomenon in rats. Later, Jiang et al22 have demonstrated neurodegenerative changes and mossy fiber sprouting with hyperthermic seizures in rats pups, as a model for sequel of prolonged febrile seizure. Thus, although in the past, there have been occasional reports of kindling following hyperthermia, our study is different in that it covers all aspects of kindling such as the seizure thresholds, latencies, seizure severity, electrical afterdischarges, as well as the issues of permanence of kindling and neuronal sprouting.
in adult animals. It also forms a useful animal model for the human hot water epilepsy, which is frequently reported from south India, with probable genetic basis\textsuperscript{32,34}. Placement of electrodes in the hippocampus was based on an \textit{in vitro} slice study\textsuperscript{31}, which demonstrated that hippocampus is sensitive to hyperthermia and is capable of producing seizure like discharges with a slight increase in temperature of the surrounding medium. Hot water stimulations were administered at 48 h intervals, based on the observation from our earlier study in which we found that kindling failed to occur when the inter-stimulus interval was 24 h. We also had similar experience in our pilot studies with longer stimulation free intervals beyond 48 h. This feature is similar to the phenomenon reported with electrical kindling\textsuperscript{1}. Degree of mossy fiber sprouting has shown positive correlation to the number of stimulations with hot water, similar to the feature observed in the hippocampus from patients with temporal lobe epilepsy with Ammon’s horn sclerosis\textsuperscript{35} with some variation. The degree of sprouting in the adult rats in our study with hot water multiple stimulation of short duration was akin to, that reported by Jiang \textit{et al}\textsuperscript{16}. None of the rats in our study, ever entered a phase of status epilepticus. An interesting observation was the evidence of sprouting a day after single stimulation indicating the acute phase of neuronal sprouting. The extent of sprouting increased with increase in the number of hot water stimulations. We need to study the Timm’s staining pattern in animals, who had stimulation free period of 30 days followed by 15\textsuperscript{th} and 16\textsuperscript{th} events of stimulation. Unexpectedly Jiang \textit{et al}\textsuperscript{16} recorded a decrease in sprouting following 32 seizures, in contrast to groups subjected to less number of seizures. Till date no study has been carried out for mossy fiber sprouting on temporal lobectomy specimens from cases of human hot water epilepsy. The pattern of hippocampal injury in our rat model was bilateral, but asymmetrical, roughly corresponding to the feature noted in humans. The rats did not have afterdischarges or developed spontaneous seizure during the study period. In human cases of hot water epilepsy after long interval, some of the cases are known to progress to generalized seizures. It is not clear, how the mossy fiber sprouting and kindling phenomenon contributes to this progression, though it is tempting to ascribe a cause and effect relationship. In an attempt to elucidate some of the molecular mechanisms, studies with glutamate (glu) channel antagonists for various subtypes and gamma amino butyric acid (GABA) receptor antagonist (g-vinyl- GABA) in our laboratory suggested a role for glu and GABA in kindling (unpublished observations).

In conclusion, the rat model we described replicates the events in human hot water epilepsy and also provides insight into hyperthermic seizures. This also proves the phenomenon of hyperthermic kindling in our rat model for HWE.

References


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