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Curcumin's antiepileptic effect, and alterations in $Na_v1.1$ and $Na_v1.6$ expression in iron-induced epilepsy

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ABSTRACT

The present study was carried out to evaluate: the antiepileptic effect of dietary curcumin, and the effect of epileptic state and curcumin on the molecular expression of voltage-activated Na^+ channel subtypes $Na_v1.1$ and $Na_v1.6$ in the iron-induced experimental epilepsy in the rat. Rats were divided into four groups; Group I (control rats), Group II (epileptic rats), Group III (curcumin-fed epileptic rats), and Group IV (curcumin-fed rats). Curcumin was fed chronically to rats approximately at the dose of 100 mg/kg body wt. The animals were made epileptic by intracortical injection of $FeCl_3$. The mRNA and protein expressions of $Na_v1.1$ and $Na_v1.6$ were examined by RT-PCR analysis and immuno-histochemistry. Results showed a significant increase (upregulation) in the expression of both $Na_v1.1$ and $Na_v1.6$ with seizure activity in the cortex and hippocampus of epileptic rats. Epileptic rats fed with curcumin showed a marked decrease in epileptiform activity, and reduced mRNA and protein levels of $Na_v1.1$. It appears that the antiepileptic action of curcumin may be associated with the downregulation of $Na_v1.1$ in the cortex.

1. Introduction

Voltage-gated sodium channels are primary molecules responsible for neuronal excitability as they are involved in the generation and propagation of action potentials in excitable cells (Yin et al., 2016). In mammals, there are nine isoforms of the functional α subunit of voltage-gated sodium channels, and of them $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$ are mostly expressed in the brain (Goldin et al., 2000; Catterall et al., 2005). $Na_v1.1$ channels are predominantly abundant in cell bodies (Westenbroek et al., 1989, 1992), and $Na_v1.6$ in myelinated axons and in dendrites (Caldwell et al., 2000; Jenkins and Bennett, 2001). Alterations in the expression of voltage-gated sodium channels such as $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$ predispose neurons towards network hyperexcitability and repetitive firing, and thus may be causally associated with epileptogenesis/ seizure activity (Scharfman, 2002).

Various experimental studies have demonstrated changes in biophysical properties of voltage-gated Na^+ currents in epilepsy (Ellerkmann et al., 2003; Ketelaars et al., 2001). In addition studies have also shown altered mRNA and protein expression of some α subunits of Na^+ channels in the epileptic tissue of human and animals (Klein et al., 2004; Qiao et al., 2013; Xu et al., 2013; Zhu et al., 2016). Up-regulated mRNA and protein levels of $Na_v1.1$ and $Na_v1.6$ were observed in cortical neurons of the seizure-onset region of a rodent model of absence epilepsy (Klein et al., 2004). Increased expression of $Na_v1.6$ protein and mRNA was also observed in hippocampal CA3 neurons of kindled rats and mice (Blumenfeld et al., 2009). Some recent studies have shown abnormal changes in the expression of both $Na_v1.1$ and $Na_v1.6$ subunits in kainic acid-induced epileptic rats (Xu et al., 2013; Qiao et al., 2013).

Iron-induced epilepsy in the rat mimics human post-traumatic epilepsy (Willmore et al., 1978; Willmore and Rubin, 1981). The aim of the current study was to investigate changes in the expression of voltage-gated sodium channels: $Na_v1.1$ (SCN1A) and $Na_v1.6$ (SCN8A) in the iron-induced experimental epilepsy (Willmore et al., 1978) in the rat which models post-traumatic clinical epilepsy. This model has been widely used to investigate mechanism of epileptogenesis and pharmacology of epilepsy (Willmore et al., 1978; Willmore and Rubin, 1981; Das et al., 2017). However changes in the expression of voltage-gated sodium channels have not been studied in this model. There have, however, been studies investigating alterations in sodium channels in a variety of experimental epilepsy models; for example, kainic acid model (Qiao et al., 2013), absence epilepsy model (Klein et al., 2004).

The second aim of the present study was to investigate whether curcumin's antiepileptic action in iron-induced epileptogenesis/ epilepsy involves its effect on sodium channels. Curcumin is a major active diphenolic compound extracted from *Curcuma longa* (turmeric) root (He

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et al., 2010). It is used as a food additive as well as a herbal remedy. Curcumin has little adverse effects and easily crosses the blood-brain barrier (Mishra and Palanivelu, 2008). Some experimental studies have demonstrated its beneficial effects against epilepsy and cognition. A study has reported that dietary intake of curcumin prevents generalization of seizures and epileptogenesis-associated biochemical changes in iron-induced epilepsy in rats (Jyoti et al., 2009).

Since curcumin is an antioxidant, and oxidative stress plays a major role in iron-induced epileptogenesis it can exert an antiepileptic effect in iron-induced epilepsy (Jyoti et al., 2009). In a study, pretreatment with curcumin in a dose-dependent manner, ameliorated pentylenetetrazol-induced seizures associated oxidative stress and cognitive impairment (Mehla et al., 2010). Choudhary et al. (2013) also found that curcumin treatment significantly attenuated seizure severity and memory impairment in pentylenetetrazol-kindled mice. Curcumin was also found to have significant anticonvulsant effects in the increasing current electroshock seizure model in mice (Bharal et al., 2008). In a study by Gupta et al. (2009) also, curcumin was shown to have a protective effect against kainic acid-induced seizures.

Curcumin, however, does not seem to have an effective antiepileptic action in some models of epilepsy. Curcumin co-administration with pentylenetetrazol, could not prevent the development of pentylenetetrazol-induced seizures (Kaur et al., 2014), and it also failed to delay the onset of kainic acid-induced seizures (Sumanont et al., 2007), although it prevented epileptogenesis-associated cell death in kainic acid-induced seizures, and reduced the severity of kainic acid-induced status epilepticus (Shin et al., 2007). In a recent study, curcumin was found not to reduce seizure frequency in the rodent post-status epilepticus model of temporal lobe epilepsy (Drion et al., 2016). It seems that beneficial effects of curcumin are model dependent and may apply to some models while not to others. Therefore, it is of interest to investigate its effects in iron-induced epilepsy model that models human post-traumatic epilepsy.

2. Materials and methods

2.1. Chemicals

Ferric chloride ($FeCl_3$), curcumin, bovine serum albumin, Tri reagent, 3, 3′-diaminobenzidine (DAB), Taq polymerase and DNTPs were purchased from Sigma-Aldrich, St Louis, USA. Normal goat serum (NGS) was obtained from Abcam, Cambridge, UK. Primary polyclonal antibodies anti-Nav1.1 and anti-Nav1.6 were obtained from Alomone Labs, Jerusalem, Israel. horseradish peroxidase (HRP)-labeled secondary antibody was obtained from Sigma-Aldrich, St Louis, USA. Rest of the chemicals were obtained from Fischer Scientific, Mumbai, India. Glass redistilled water was used throughout in the present experiments.

2.2. Animals and their care

Forty male Wistar rats of one month of age were obtained from Central Laboratory Animal Resources, Jawaharlal Nehru University, New Delhi. The animals were housed in polypropylene cages (12″ × 9″ × 6″) kept in well-ventilated rooms in pathogen-free condition with 12:12 h light and dark cycle, at 22–25 °C room temperature and 50–55% relative humidity. All protocols involving the use of animals were performed by following the guidelines of Committee for the Purpose of Control and Supervision of Experiments on animals. Studies were conducted after obtaining prior approval from the Animal Ethical Committee of the Jawaharlal Nehru University, New Delhi.

2.3. Curcumin treatment

For curcumin treatment, rats were fed curcumin-supplemented standard rat food pellets. The food pellets contained curcumin 1500 ppm (Jyoti et al., 2009). Based on the amount of curcumin-mixed food consumed (diet eaten) by rats, it was estimated that rats on average ingested curcumin in the dose of approximately 100 mg/kg body weight. Curcumin treatment was administrated for 5 months before induction of epilepsy (pretreatment), and for one after induction of epilepsy (cotreatment). Pretreatment condition also mimics human situation since curcumin is consumed as a routine spice in human diet (in some parts of the world). It will be of interest to see how the dose of curcumin administered to rats in the current study would scale for humans: that is, what would be a comparable amount of curcumin in a human diet. In clinical trials on human subjects, doses ranging from 2 to 12 gm curcuminoid mixture per day have been administered. A sample of 10 gm curcuminoid mixture may contain 6–7 gm curcumin. Thus, ingestion of 10 gm curcuminoid mixture in human diet would amount to ingestion of about 7 gm curcumin per day. For a human person (60–70 Kg body weight) it would calculate to around 100 mg/Kg.

2.4. Iron-induced epilepsy induction

Surgical procedures for intracortical injection of iron and implantation of electrodes were performed according to methods described previously (Mishra et al., 2010, 2013) under 4% isoflurane-induced anaesthesia. Burr holes of 0.5 mm diameter (one for saline/$FeCl_3$ injection and four for placement of epidural electrodes) were drilled into the skull bone at stereotaxically marked sites. Saline/$FeCl_3$ (5 µl containing 100 mM $FeCl_3$ dissolved in physiological saline) was stereotaxically (at coordinates: from bregma: AP- 1, ML- 1, V- 1.5 mm) intracortically injected over 5 min (1 µl/min) using motorised injector (Stoelting, USA). For ECoG recording, stainless steel screw electrodes (Plastic One, USA) were placed (at coordinates: from bregma AP + 2 and -2, ML + 2 and -2, V -1.5 mm) over the occipital and frontal cortices. Two screws were placed in the left hemisphere and two in the right hemisphere. One screw electrode was also placed over the frontal sinus to serve as animal ground. The free ends of these electrodes were soldered to a nine-pin connector, which was fixed to the skull with dental acrylic cement to make a robust platform. After 7 days of recovery period operated rats were habituated to the recording set up for 3 days in the recording chamber.

2.5. Animals were grouped as follows

Group I (n = 10) consisted of animals that served as control for group II animals. They remained on normal diet for six months, and were implanted with electrodes for electrographic recording, and had received intracortical injection of saline in place of $FeCl_3$.

Group II (n = 10) consisted of animals that remained on normal diet for five months. Thereafter, they were made epileptic by the procedure described above, implanted with electrodes for electrographic recording, and they continued to be on normal diet for a further period of one month. These animals constituted the group of experimental epileptic animals.

Group III (n = 10) consisted of animals that received curcumin treatment for five months. Thereafter these animals were made epileptic, and implanted with electrodes for electrographic recordings, and continued to remain on curcumin-mixed diet for a further period of one month. These animals have thus been fed curcumin for five months prior to induction of epilepsy, and then for one month after induction of epilepsy. These animals constituted the group of curcumin- treated epileptic animals.

Group IV (n = 10) consisted of animals that remained on curcumin-mixed diet for six months. They had received intracortical injection of saline, and were implanted with electrodes for electrographic monitoring as in group III animals. These animals constituted the group of normal animals that received curcumin only treatment.

2.6. Recording of cortical electroencephalographic (ECoG) and multiple-unit activity (MUA)

Four rats from each group were taken for electrical activity analysis. For ECoG and MUA (Sharma et al., 2007; Mishra et al., 2013) recordings, composite extracellular signals were routed through a high impedance probe (Grass HIP 511 with FET), and amplified and filtered for ECoG and MUA recordings (1 Hz to 100 Hz and 300 Hz to 10 kHz respectively) by using Grass Polygraph (P511 AC preamplifiers). MUA count was electronically discriminated by using a window discriminator (WPI, Florida, USA). ECoG and MUA were also monitored and displayed on Grass Technologies PolyVIEW 16 Data Acquisition System. Electrophysiological recordings were performed on animals that had developed epileptiform activity after iron injection. The MUA potentials counts were used to quantify the epileptiform seizure activity.

2.7. Collection and preparation of tissue

After the treatment, rats were fasted overnight to preserve metabolic levels at basal rate, anesthetized and killed by decapitation. The whole brain was isolated, rinsed in ice-cold physiological saline. Then, cortex and hippocampus were dissected out and total RNA was isolated.

2.8. Total RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was extracted from brain tissues of three rats from each group using Tri-Reagent (Sigma-Aldrich, St Louis, USA), according to manufacturer's instructions. RNA was reverse transcribed in a total volume of 20 µl using RevertAid™ cDNA synthesis kit (Fermantas, Germany) according to manufacturer's instructions. cDNA products were subjected to semi-quantitative PCR analysis on a gradient thermal cycler (Eppendorf, Germany). PCR cycle comprised of initial denaturation at 94 °C for 2 min. The amplification was then carried out for 30 cycles consisting of 30 s each for denaturation at 94 °C and annealing, and extension at 72 °C for 1 min. Final extension was done at 72 °C for 10 min. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control. Primers were designed with Primer3 software using sequences data available on National Centre for Biotechnical Information (NCBI) database (Table 1). The PCR products were applied to 1.5% (w/v) agarose gel electrophoresis containing ethidium bromide. PCR products were visualized under UV light and photographed using gel documentation system (Alpha Innotech, CA, USA). The intensities of the bands were quantified densitometrically using Image *J* software (https://imagej.nih.gov/).

2.9. Immuno-histochemistry

Immunoreactivity studies were performed on three rats from each group. Rats were anesthetized and perfused using 4% paraformaldehyde. Intact brains were dissected out from perfused animals and were post fixed in 4% paraformaldehyde for 24 h. Brain sections (10 µm) were cut and mounted on gelatine-coated clean slides. These sections were air dried for 1 h at 37 °C, and then washed with phosphate buffer saline (PBS). The sections were then treated with 1% Triton X-100 for antigen retrieval, and were dipped in 1% H_2O_2 in PBS for 20 min in order to quench the endogenous peroxidase. For the blocking of non-specific antigen binding, sections were incubated with 3% NGS for 90 min. The slides were incubated in respective rabbit-raised polyclonal primary antibodies ($Na_v1.1$ and $Na_v1.6$, 1:1000) overnight at 4 °C, and three washings were given in PBS of 5 min each. After washing, the sections were incubated with HRP-labelled secondary antibody (1:200) at room temperature for 90 min. The sections were again washed three times with PBS and covered with DAB and 0.25% H_2O_2 solution in PBS for 10 min at room temperature followed by washing in distilled water. Finally, sections were mounted on glass slides using DPX, and photographed under light microscope (Nikon Eclipse T*i*, Tokyo, Japan). The protein levels of $Na_v1.1$ or $Na_v1.6$ were analyzed by comparing the intensity of $Na_v1.1$ or $Na_v1.6$ immuno-reactivity visualized by DAB to an unstained section to calculate relative optical intensity by using Image *J* software.

2.10. Statistical analysis

The data were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc tests using SigmaStat software (version 3.5, Systat Software Inc., San Jose, California, USA), and ORIGIN® 8.6 software (Origin lab corporation, Massachusetts, USA). Statistical significance was determined at the threshold of $p < 0.05$ and 0.015. In the current experiments multiple measurements were done, that is why statistical significance was also determined at lower threshold of 0.015 in order to apply multiple-testing correction to avoid false positives (Laken, 2016), and thus only significance at the level of 0.015 was accepted for conclusions. Data in figures are presented as univariate scatter plots (Weissgerber et al., 2015).

3. Results

3.1. Epileptiform electrographic activity

All experimental animals (Group II) given intracortical $FeCl_3$ injection developed epileptiform electrographic activity compared to controls (Fig. 1). The ECoG and corresponding multi-unit activity (MUA) recordings during, wake behaviour, showed a progressive increase in the electrical seizure activity over one month period during which recordings were made. The epileptiform activity comprised isolated spikes during 7–8 days post iron injection. Thereafter the electrographic seizure episodes consisting of polyspikes, and spike-wave complexes became spontaneous and recurrent. The increases in multi-unit activity were generally concurrent with ECoG paroxysms. Behavioural seizures (concomitant with epileptic ECoG activity) consisted of pauses in exploratory behaviour, twitching of vibrissae, jaw automatisms, mild head nodding, tonic flexion etc. Control animals (that received intracortical injection of saline instead of iron) did not exhibit any electrographic or behavioural seizure activity.

The one way ANOVA (at the 0.015 level) of MUA (at day 10, and day 30 after induction of epilepsy) indicated that there were significant differences between the four groups (treatments): ($F_{3,12} = 118.11374$, $p < 3.57149E\text{-}9$; $F_{3,12} = 156.74735$, $P < 6.88764E\text{-}10$). At the 0.05

Table 1
Primers used for RT-PCR analysis of $Na_v1.1$ and $Na_v1.6$.

Gene	Accession #	Product size	Direction	Sequence
GAPDH	XM_017593963.1	158	Forward	AACGACCCCTTCATTGAC
			Reverse	TCCACGACATACTCAGCAC
$Na_v1.1$	XM_017592058.1	186	Forward	TCCTGGAGGGTGTTTTAGATGC
			Reverse	AAAGATTTTCCCAGAAGTCCTGAG
$Na_v1.6$	NM_01266.2	355	Forward	GTTCATCGGTGTCATCATCG
			Reverse	CAAGGCAAACATTTTGAGCA

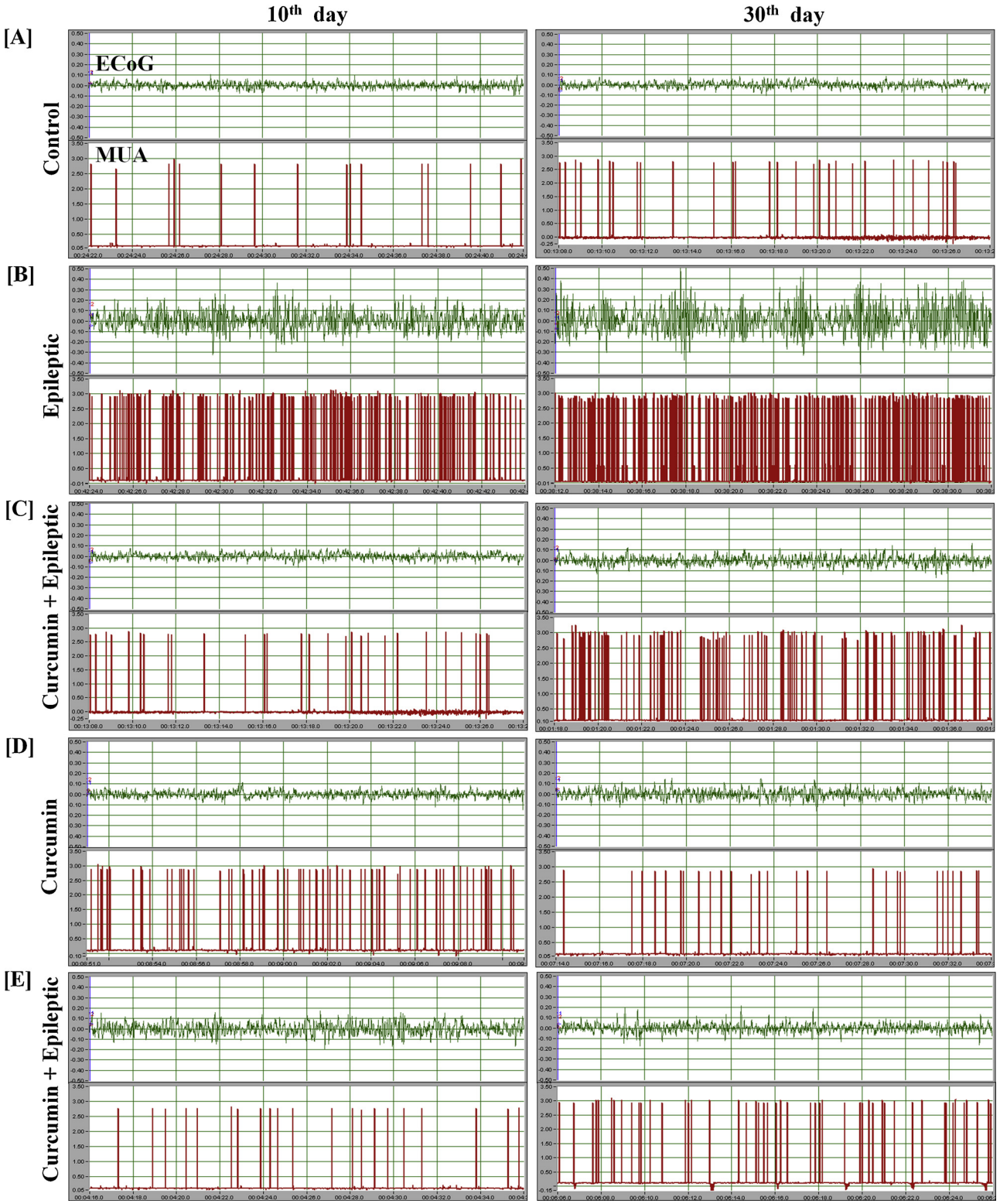

Fig. 1. Representative sample polygraph recordings showing ECoG and MUA activity on 10th and 30th day after $FeCl_3$/saline intracortical injection in control, epileptic, curcumin-fed epileptic and curcumin-fed rats. Sample records are of 20 s stretches. (Recordings-Grass Polyview Data Acquisition System).

level also, the data was significant ($F_{3,12} = 118.114$, $P < 0.001$; $F_{3,12} = 156.747$, $p < 0.001$). Statistical comparison between the MUA of controls (group I) and that of iron-induced epileptic rats (group II) showed highly significant (both at 0.015 and 0.05 levels) increases in the MUA at day 10 ($p = 0.00252$, $p = 0.009$), and at day 30 ($p = 0.00302$, $p = 0.010$) (Fig. 2) indicating significant development of iron-induced epileptiform activity and a progressive increase in the epileptiform activity over time.

3.2. Effect of curcumin on epileptiform activity

In curcumin-treated animals (group III animals that had received

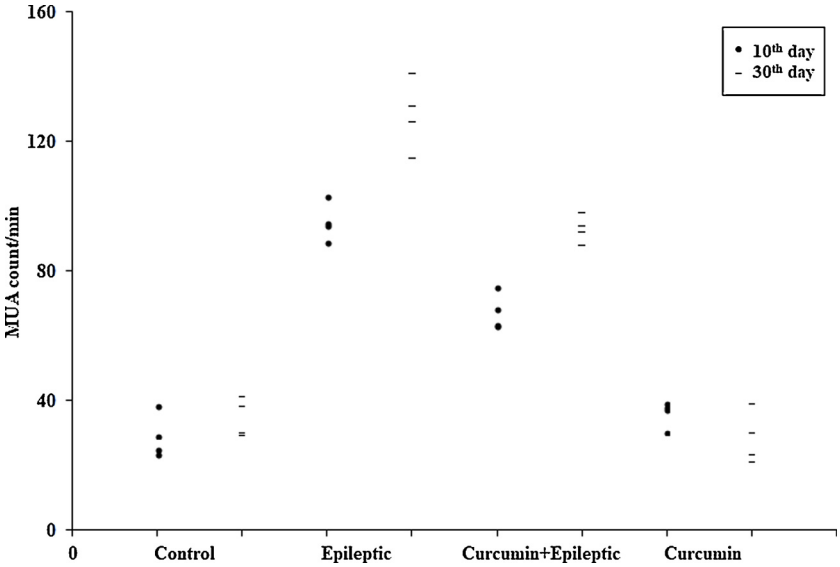

Fig. 2. Univariate scatter plots of MUA counts corresponding to ECoG of control (group I), epileptic (group II), curcumin-fed epileptic (group III) and curcumin-fed (group IV) rats. MUA increase in epileptic animals is evident compared with controls. Decline of MUA in curcumin-treated epileptic animals is evident compared with epileptic animals (curcumin's effect on MUA). No difference is evident between control and curcumin-fed rats (no effect of curcumin on basal MUA). Statistical significance is described in the text.

intracortical iron injection along with curcumin treatment), electrographic (and behavioural seizure activity) was very greatly decreased, indicating that curcumin treatment highly reduced the development and occurrence of seizures (Fig. 1). Comparisons (B vs C) showed significant decreases in MUA counts corresponding to epileptiform ECoG. As shown in Fig. 1 panel C, the ECoG of some curcumin-treated (Group III animals) appeared practically free from electrical seizure activity. However, in some animals, the ECoG did contain highly reduced/interictal type electrical seizure activity Fig. 1 panel E. Therefore, curcumin treatment did not completely suppress the electrical seizure activity.

Statistical comparisons between the MUA of (at the 0.015 level) epileptic animals (group II) and that of curcumin-treated epileptic animals (group III) showed significant declines in the MUA of the curcumin-treated animals ($p = 0.00753$ (day 10), $p = 0.00753$ (day 30)). At the 0.05 level also the decline was significant ($p = 0.025$ (day 10), $p = 0.025$ (day30). This showed that curcumin reduced the epilepsy-associated increase in the MUA (Fig. 2).

Curcumin treatment of normal rats did not alter their multi-unit activity (Fig. 2). Statistical comparisons of MUA between the control (group I) and that of curcumin-treated normal rats (group IV) were insignificant at both the levels (0.015 and 0.05): $p = 0.015$ NS, $p = 0.050$ NS). Curcumin treatment in naïve animals (group IV) did not affect the basal electrocorticographic activity or MUA indicating that curcumin treatment does not change the brain's basal electrical activity.

3.3. mRNA expression of $Na_v1.1$ and $Na_v1.6$ in epileptic animals

The data for the expression of voltage-gated sodium channels $Na_v1.1$ and $Na_v1.6$ measured at day 30 after induction of epilepsy are presented in Figs. 3 and 4. The results showed that the mRNA expression of the channels is significantly elevated both in the cortex and the hippocampus.

The one way ANOVA (at the 0.015 level) of the expression of $Na_v1.1$ in the cortex indicated that there were significant differences between four groups: $F_{3,8} = 10.17591$, $p < 0.00418$. Similarly there were significant differences in the expression of $Na_v1.1$ in the hippocampus: $F_{3,8} = 13.43372$, $p < 0.00172$. The ANOVA data was significant at the 0.05 level also: cortex-$Na_v1.1$, $F_{3,8} = 10.176$, $p < 0.004$; hippocampus, $Na_v1.1$ $F_{3,8} = 13.434$, $p < 0.002$.

Statistical comparisons (at the 0.015 level) between the expression of Nav1.1 in controls (group I) and that of epileptic rats (group II) showed highly significant elevation in the expression of $Na_v1.1$: in the cortex 50% ($p = 0.00252$), in the hippocampus 36% ($p = 0.00302$). At the 0.05 level also the comparisons showed significance in the expression of $Na_v1.1$ cortex: $p = 0.009$, and hippocampus $p = 0.010$.

The one way ANOVA (at the 0.015 level) of the expression of $Na_v1.6$ in the cortex indicated that there were significant difference between the four groups: $F_{3,8} = 12.58431$, $p < 0.00213$. Similarly there were significant difference in the expression of $Na_v1.6$ in the hippocampus: $F_{3,8} = 12.15765$, $p < 0.00238$. The ANOVA data was significant at the 0.05 level also: cortex-$Na_v1.6$ $F_{3,8} = 12.584$, $p < 0.002$; hippocampus $F_{3,8} = 12.158$, $p < 0.002$.

Statistical comparisons (at 0.015 level) between the expression of $Na_v1.6$ in controls (group I) and that of epileptic rats (group II) showed highly significant elevation in the expression : in the cortex, 89% $p = 0.00252$; in the hippocampus 91%, $p = 0.00252$. At 0.05 level also, the comparison showed significance: $p = 0.009$.

The results thus indicated that iron-induced epileptic activity was associated with increased expression of $Na_v1.1$ and $Na_v1.6$ in the cortex as well as in the hippocampus.

3.4. Effect of curcumin on the mRNA expression of $Na_v1.1$ and $Na_v1.6$ in epileptic rats

The data for the effect of curcumin are depicted in Figs. 3 and 4. The overall one way ANOVA (at the level of 0.015) of the expression of $Na_v1.1$ and $Na_v1.6$ indicated that there were significant differences between the four groups: cortex $Na_v1.1$ $F_{3,8} = 10.17591$, $p < 0.00418$; hippocampus, $Na_v1.1$. $F_{3,8} = 13.43372$, $P < 0.00172$; cortex $Na_v1.6$ $F_{3,8} = 12.15765$, $P < 0.00238$; hippocampus $Na_v1.6$ $F_{3,8} = 12.58431$, $p < 0.00213$. The ANOVA indicated significance at the 0.05 level also: cortex $Na_v1.1$ $F_{3,8} = 10.176$, $P < 0.004$; $Na_v1.6$ $F_{3,8} = 12.584$, $P < 0.004$; hippocampus $Na_v1.1$ $F_{3,8} = 13.434$, $P = 0.002$, $Na_v1.6$ $F_{3,8} = 12.158$, $P = 0.002$.

In the cortex of curcumin-treated epileptic rats (group III vs group II), the mRNA levels of both $Na_v1.1$ and $Na_v1.6$ were decreased (at the level 0.015) by about 40% ($p = 0.00377$), and 55% ($p = 0.00377$ NS) respectively with respect to epileptic control. In the hippocampus, the mRNA levels of both $Na_v1.1$ and $Na_v1.6$ were decreased by about 20% ($p = 0.00377$ NS) and 58% ($p = 0.00377$ NS)

At the 0.05 level, however, the significance was as follows: Cortex, $Na_v1.1$ ($p = 0.013$), $Na_v1.6$ ($p = 0.013$); hippocampus, $Na_v1.1$ ($p = 0.017$ NS), $Na_v1.6$ ($p = 0.013$).

In conclusion, accepting the significance at the 0.015 level it appeared that curucmin did not significantly affect the mRNA expression of both $Na_v1.1$ and $Na_v1.6$ in the hippocampus of epileptic animals. In the cortex curcumin significantly reduced the expression of $Na_v1.1$, but

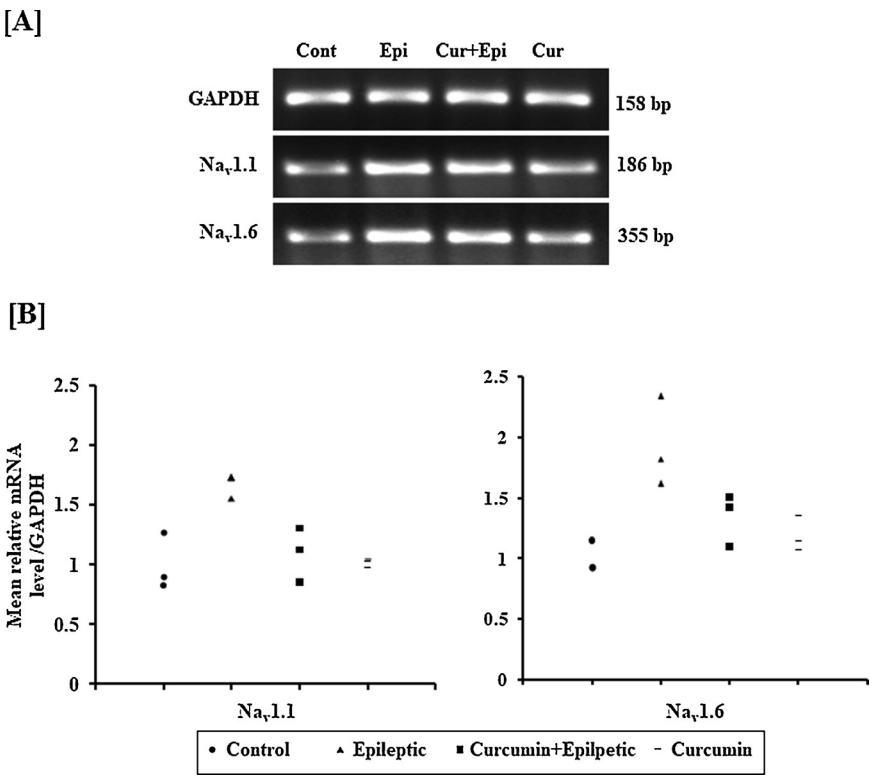

Fig. 3. mRNA expression of $Na_v1.1$ and $Na_v1.6$ in the cortex of control, epileptic, curcumin-fed epileptic and curcumin-fed rats. mRNA expression (electrophoresis bands of RT-PCR products) in rat brain cortex **(A),** univariate scatter plot of mRNA levels as analyzed by densitometry **(B)**. Increased expression of both channels in epileptic animals is evident compared with controls. Some decline in expression level of curcumin-treated epileptic animals is evident compared with epileptic animals (effect of curcummin on expression levels). No difference is evident between control (group I) and curcumin fed (group IV) animals (no effect of curcumin on basal expression of channels), statistical significance described in the text.

did not affect $Na_v1.6$. Thus, in epileptic animals, curcumin influenced (reduced) only the mRNA expression of $Na_v1.1$ in the cortex.

3.5. Immuno-histochemical expression of $Na_v1.1$ and $Na_v1.6$ in epileptic rats

In Figs. 5 and 6 images of $Na_v1.1$ and $Na_v1.6$ are presented. Immuno-reactivity for both $Na_v1.1$ and $Na_v1.6$ was increased in epileptic rats.

The one way ANOVA (at the 0.015 level) of the immuno-histochemical expression of $Na_v1.1$ and $Na_v1.6$ in epileptic rats indicated that there were significant differences between the four groups: cortex, $Na_v1.1$ and $Na_v1.6$: $F_{3,8} = 89.36046$, $p < 1.71482E\text{-}6$ and $F_{3,8} = 69.80176$, $p < 4.44518E\text{-}6$ respectively; hippocampus $Na_v1.1$ and $Na_v1.6$: $F_{3,8} = 16.6057$, $p = 8.50603E\text{-}4$ and $F_{3,8} = 152.08966$, $p < 2.1546E\text{-}7$ respectively. At the 0.05 level also there was

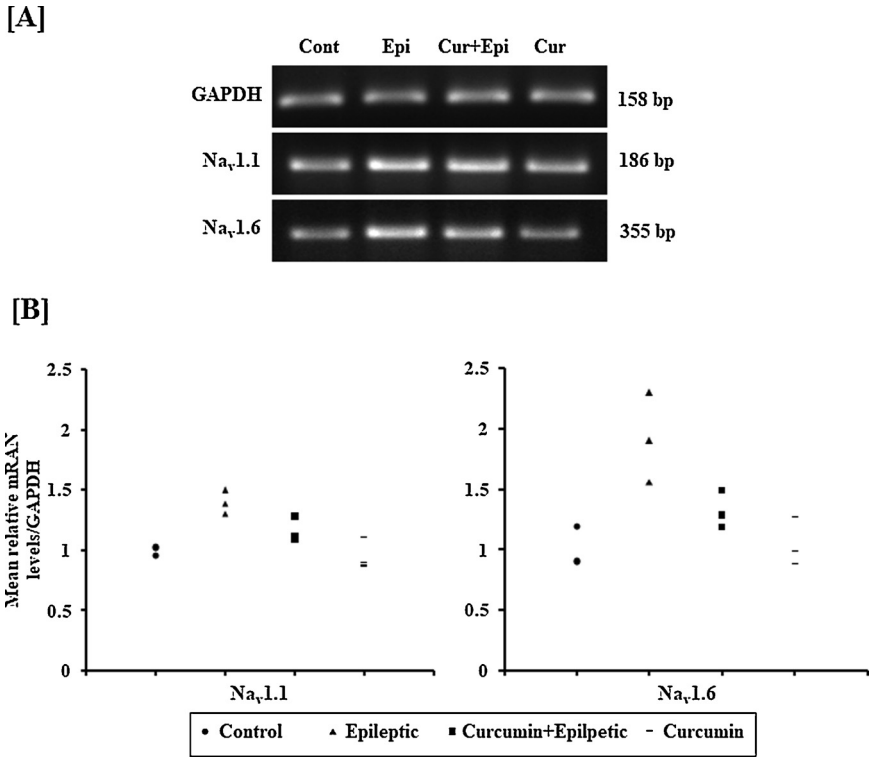

Fig. 4. mRNA expression of $Na_v1.1$ and $Na_v1.6$ in the hippocampus of control, epileptic, curcumin-fed epileptic and curcumin-fed rats. mRNA expression (electrophoresis bands of RT-PCR products) in rat hippocampus (A) and univariate scatter plots of mRNA levels as analyzed by densitometry (B). Increased expression in epileptic animals is evident compared with controls. Some decline in expression levels in curcumin-treated epileptic animals is evident compared with epileptic levels. No difference is evident between controls (group I) and curcumin-fed (group IV) animals (No effect of curcumin on basal expression levels).

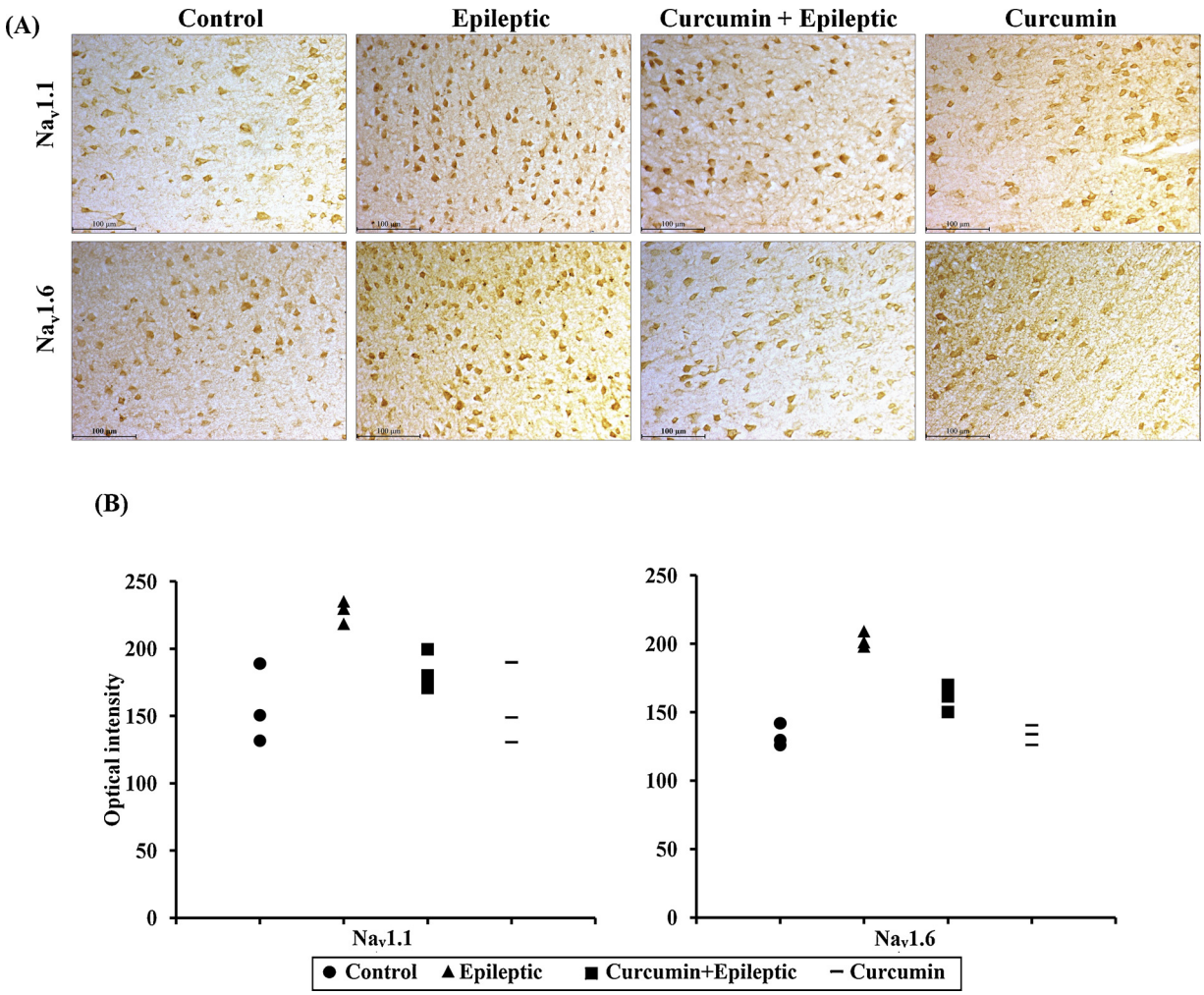

Fig. 5. Protein expression of $Na_v1.1$ and $Na_v1.6$ in the cortex of control, epileptic, curcumin-fed epileptic and curcumin-fed rats. Coronal sections of cortex showing immuno-reactivity of $Na_v1.1$ and $Na_v1.6$ in the cortex (A) univariate scatter plots of optical intensity (B). Explanation and statistical significance in the text.

significance: cortex $Na_v1.1$, $F_{3,8} = 86.360$, $p < 0.001$, $Na_v1.6$, $F_{3,8} = 69.802$, $p < 0.001$; hippocampus $Na_v1.1$, $F_{3,8} = 16.606$, $p < 0.001$, $Na_v1.6$, $F_{3,8} = 152.090$, $p < 0.001$. Statistical comparison (at the 0.015 level) between the expression of $Na_v1.1$ and $Na_v1.6$ in controls (group I) and those of epileptic rats (group II) showed highly significant upregulation of the channels in epileptic animals: cortex, $Na_v1.1$ 29%, $p = 0.0032$; $Na_v1.6$ 42%, $p = 0.00252$; hippocampus $Na_v1.1$ 33%, $p = 0.00302$, $Na_v1.6$ 49%, $p = 0.00302$. At the 0.05 level also the comparisons were significant: cortex, $Na_v1.1$, $p = 0.010$; $Na_v1.6$, $p = 0.009$; hippocampus, $Na_v1.1$ $p = 0.010$, $Na_v1.6$, $p = 0.010$. Results thus showed upregulation of $Na_v1.1$ and $Na_v1.6$ both in the cortex and hippocampus of epileptic animals. These results are entirely consistent with those of mRNA expression.

3.6. Effect of curcumin on the immuno-histochemical expression of $Na_v1.1$ and $Na_v1.6$ in epileptic animals

Immuno-histochemically, curcumin seemed to reduce the epilepsy-associated increase in the expression of $Na_v1.1$ and $Na_v1.6$ (Figs. 5, 6). The overall one way ANOVA (at the 0.015 level) of the immuno-histochemical expression of $Na_v1.1$ and $Na_v1.6$ indicated that there were significant differences between the groups: cortex, $Na_v1.1$ $F_{3,8} = 89.36046$, $p < 1.71482E\text{-}6$, $Na_v1.6$, $F_{3,8} = 69.80176$, $p < 4.44518E\text{-}6$, hippocampus $Na_v1.1$ $F_{3,8} = 16.6057$, $p < 8.50603E\text{-}4$, $Na_v1.6$ $F_{3,8} = 152.08966$, $p < 2.1546E\text{-}7$. At the 0.05 level also there was significance: cortex $Na_v1.1$ $F_{3,8} = 89.360$, $p < 0.001$, $Na_v1.6$ $F_{3,8} = 69.802$, $p < 0.001$; hippocampus, $Na_v1.1$, $F_{3,8} = 16.606$, $p < 0.001$, $Na_v1.6$, $F_{3,8} = 152.090$, $p < 0.001$.

Statistical comparisons between the immuno-histochemical expression of channels in control (epileptic animals, group II) and curcumin-treated (epileptic animals- group III) showed significance as follows: at the 0.015 level of significance: cortex, $Na_v1.1$ ($p = 0.00503$), $Na_v1.6$ ($p = 0.00377$), hippocampus; $Na_v1.1$ ($p = 0.00753$) NS, $Na_v1.6$ ($p = 0.00377$). At the 0.05 level of significance: cortex, $Na_v1.1$ ($p = 0.017$), $Na_v1.6$ ($p = 0.013$), hippocampus: $Na_v1.1$ ($p = 0.025$), $Na_v1.6$ ($p = 0.013$).

In conclusion, accepting the significance at the level of 0.015, curcumin significantly reduced the immuno-histochemical expression of both $Na_v1.1$ and $Na_v1.6$ in the cortex of epileptic animals. In the hippocampus, however, it influenced $Na_v1.6$ but not $Na_v1.1$. However, all immuno-histochemical expression (protein) data do not correlate with the mRNA expression data. Both expressions (in response to curcumin) were in accordance in respect of $Na_v1.1$ in the cortex showing that curcumin down-regulated the epilepsy-associated increase in the channel in the cortex.

The $Na_v1.6$ data in the cortex as well as in the hippocampus did not correlate as immuno-histochemically Nav1.6 was down-regulated, but the mRNA levels were not affected. The $Na_v1.1$ data on the effect of curcumin in the hippocampus did correlate but both expression were not affected by curcumin indicating that curcumin did not affect $Na_v1.6$ in epileptic animals. In view of the discrepancy between their immuno-histochemical and mRNA expression, the data concerning the effect of

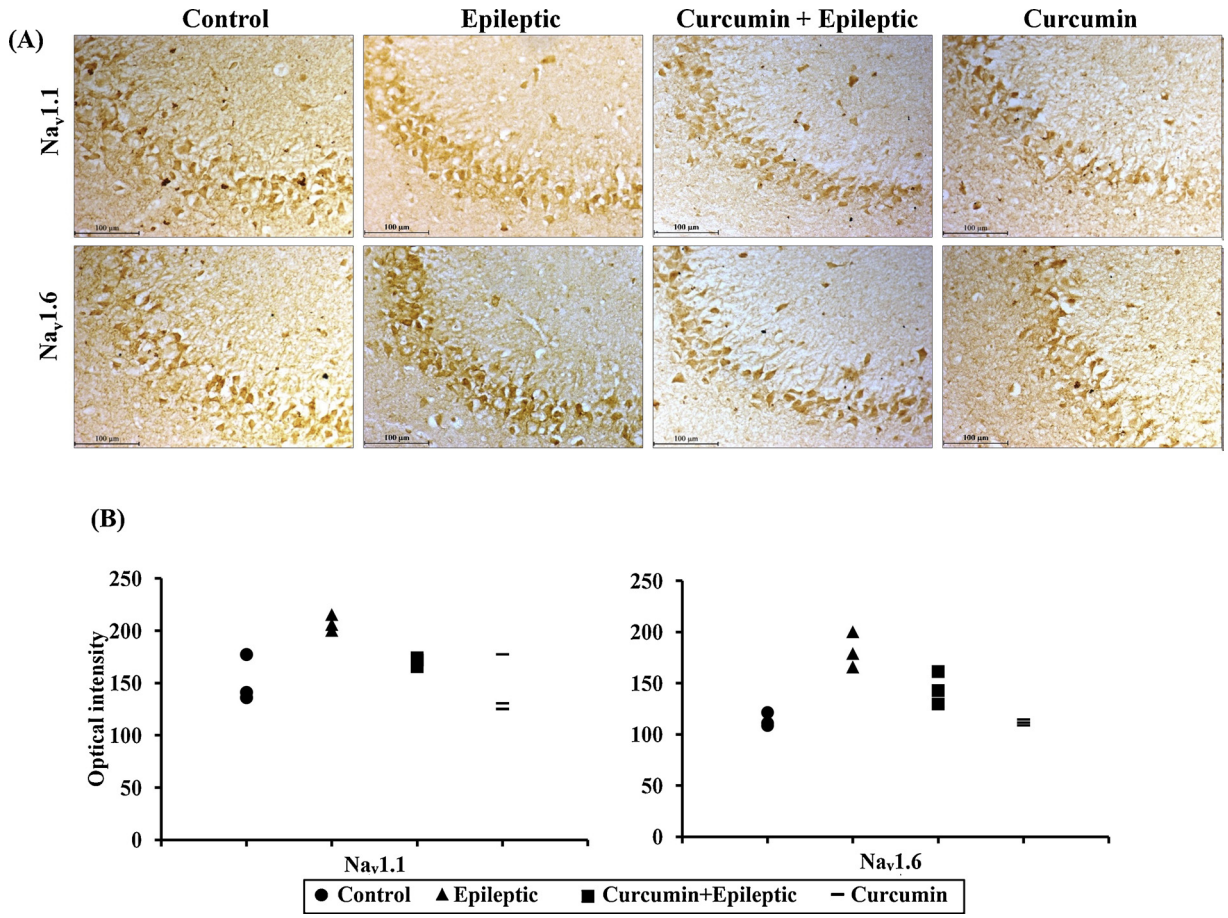

Fig. 6. Protein expression of $Na_v1.1$ and $Na_v1.6$ in the hippocampus of control, epileptic, curcumin-fed epileptic and curcumin-fed rats. Coronal sections of hippocampus showing immuno-reactivity of $Na_v1.1$ and $Na_v1.6$ in hippocampal CA3 region (A) and univariate scatter plots of optical intensity (B). Explanation and statistical significance in the text.

curcumin on $Na_v1.6$ remain inconclusive.

Curcumin treatment of normal rats did not alter the expression of $Na_v1.1$ and $Na_v1.6$. Statistical comparison between the expression of $Na_v1.1/Na_v1.6$ of group IV rats and that of $Na_v1.1/Na_v1.6$ of group I rats showed the difference to be non significant in both the cortex and hippocampus.

Therefore, in conclusion the current results indicate that in epileptic animals curcumin down-regulated only $Na_v1.1$ in the cortex.

4. Discussion

The present study demonstrates that the expression of voltage-gated sodium channel subtypes $Na_v1.1$ and $Na_v1.6$ is significantly upregulated in the iron-induced epilepsy in rats. Both $Na_v1.1$ located in the soma of neurons and $Na_v1.6$ located in the nodes of Ranvier and axon initial segments are considered important in the generation of epilepsy (Xu et al., 2013) as they are thought to be capable of producing a persistent current associated with neuronal bursting and hyperexcitability (Klein et al., 2004). The upregulation of $Na_v1.1$ and $Na_v1.6$ in iron-induced epilepsy is consistent with findings in a number of studies. In kainic acid-induced epilepsy, altered expression of $Na_v1.1$, 1.2 and 1.6 was reported by Qiao et al. (2013). In the rodent model of genetic absence epilepsy, upregulation of $Na_v1.1$ and $Na_v1.6$ was found in the epileptic cortex (Klein et al., 2004). In the spontaneously epileptic rats, and tremor rats, upregulation in the expression of $Na_v1.1$, 1.2, 1.3, 1.6 of the hippocampus was reported (Xu et al., 2013). Zhu et al. (2016) and Blumenfeld et al. (2009) have found increased expression of $Na_v1.6$ in kainic-acid and electric-shock-induced models respectively. Cheah et al. (2013) have reported a time-course dependant rise in the expression of $Na_v1.1$ in Dravet syndrome mice with susceptibility to seizures. The observed increased expression of the sodium channels in the current study may or may not be causally related to epilepsy generation in the present experiments. It is thought that both Nav1.1 and Nav1.6 may be involved in the generation of epilepsy since they are capable of providing necessary persistent sodium current associated with neuronal bursting and hyperexcitability (Klein et al., 2004), that is essential in the generation of epilepsy. However, direct evidence in this regard is lacking.

In the current study, changes in the expression of $Na_v1.1$ and $Na_v1.6$ occurred both in the cortex (the region of seizure onset) as well as in the hippocampus. In the iron-induced model of epilepsy, the focal epileptiform activity spreads from its site of origin (i.e., the epileptogenic focus at the iron-injection site in the cortex) into the entire cerebral cortex and various subcortical structures (Moriwaki et al., 1992; Sharma and Singh, 1999; Sharma et al., 2007), and independent neocortical and hippocampal foci may develop and coexist in traumatic-injury induced epilepsy (D'Ambrosio et al., 2004, 2005). Thus, the occurrence of changes in the expression of $Na_v1.1$ and $Na_v1.6$ in the hippocampus also may be associated with the development of epileptiform activity.

The data derived from the present experiments showed that the curcumin treatment markedly suppressed the electrical seizure activity indicating that curcumin significantly protects against seizures. This is consistent with previous findings showing curcumin's effectiveness in suppressing behavioural seizures in several models of epilepsy (Jyoti et al., 2009; Mehla et al., 2010; Choudhary et al., 2013). In most of the

previous studies (as cited in the Introduction) of curcumin's anti-epileptic action, its effects were monitored only on behavioural seizures. In the present study, however, its antiepileptic effect has been demonstrated on electrographic seizure activity.

Another aim of the current study was to examine the effect of curcumin on the increased expression of $Na_v1.1$ and $Na_v1.6$ in iron-induced epileptic state. Curcumin treatment in normal rats did not affect the expression of $Na_v1.1$ and $Na_v1.6$ in the cortex and the hippocampus. In the curcumin-treated epileptic animals, the expression of only $Na_v1.1$in the cortex was decreased to near control levels. Curcumin did not affect both cortical and hippocampal $Na_v1.6$, and hippocampal $Na_v1.1$ in the epileptic animals. Therefore, circumin treatment appears to counter epileptogenesis-associated alteration only in $Na_v1.1$ in the cortex and thus curcumin's antiepileptogenic effect in the iron-induced epilepsy model may involve its action only on cortical $Na_v1.1$.This is of interest since seizures originate in the cortex only (Mc Namara, 1994).

Curcumin's action on a variety of voltage-gated channels has been demonstrated (Zhang et al., 2014). It has been shown to inhibit glutamate release from synaptosomes by suppressing presynaptic voltage-gated Ca^{2+} Channels (Lin et al., 2011). Curcumin has also been shown to modulate glutamate transporter-1 (GLT-1) (Zhang et al., 2014) which is essential for maintaining a low extracellular glutamate concentration necessary for preventing glutamate's excitatory action at synapses. In the iron-induced epilepsy, glutamatergic mechanisms seem to be involved in epileptogenesis (Mishra et al., 2013). Curcumin has been found to block $K_v1.4$ potassium channel in bovine adrenal gland cells (Liu et al., 2006), and voltage-dependent K (+) channels in rabbit coronary arterial smooth muscle cells (Hong et al., 2013). Thus, curcumin's action on voltage-gated channels in iron-induced epilepsy model is plausible.

Recently (Nelson et al., 2017), in view of curcumin's poor pharmacokinetic/pharmacodynamic properties, low efficacy in some disease models, and instability in biological settings, its pharmacological efficacy has been doubted. In the electrically-induced status epilepticus rat model, curcumin failed to protect against seizures (Drion et al., 2016), and it was also found in these experiments that free curcumin in blood was not detectable after dosing. It, thus, appeared that curcumin's effect were model dependent. There have been several studies where distinct significant antiseizure effects of curcumin have been found in a variety of experimental models of epilepsy, and in the present study also the seizure-suppressing effect on electrographic seizure activity has been clearly demonstrated.

In summary, the present study demonstrates that the expression of sodium channels $Na_v1.1$ and $Na_v1.6$ is upregulated in iron-induced epilepsy, and curcumin's antiepileptic action is associated with down-regulation of $Na_v1.1$ in the cortex. The result also show that curcumin greatly reduces the electrical seizure activity.

Disclosure

None of the authors has any conflict of interest to disclose.

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