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ORIGINAL PAPER

Study of epileptiform activity in cerebral ganglion of mud crab *Scylla serrata*

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Abstract An attempt is made to induce in mud crab (*Scylla serrata)* epileptiform activities that resemble the generalized epileptic seizures. Cerebral ganglion of crab was exposed in situ, to a convulsant drug pentylenetetrazole (PTZ) 100 mM, for induction of seizures. Also, crabs were pretreated with antiepileptic drug viz sodium valproate (120 μmol/l) to inhibit epileptiform activities. The surface electrical discharges of cerebral ganglion were recorded using Unkelscope (MIT, USA) in control as well as experimental animals. The cerebral ganglion of crab showed a pattern of high cerebral electrical discharges after PTZ treatment compared to control. The sodium valproate promoted sedative action in control and prevented PTZ-mediated epileptiform discharges. Glutamate and GABA contents in cerebral ganglion were assayed. Glutamate level increased (31.45%) during PTZ treatment with concomitant decrease (43.93%) in GABA. Sodium valproate had no effect on glutamate concentration, but it decreased GABA by 24.75%. The present study shows that epileptiform activities can be induced in crabs.

Keywords Crab · Pentylenetetrazole · Sodium valproate · Glutamate · GABA

Introduction

Invertebrate systems are useful for understanding processes in the central nervous system (CNS). To understand the basic mechanisms of epilepsy, it is essential to know how populations of neurons interact and how the physiological processes in individual neurons are altered. Owing to an easy access to invertebrate neurons, it is possible to examine the basic mechanisms controlling neuronal excitability and to predict the electrophysiological mechanisms that may be present in central nervous system (Lewis et al. 1986).

Epilepsy is a common neurological disorder evidenced by the occurrence of spontaneous seizures with diverse etiologies (Liu et al. 2009). Although the causes of epilepsy are many, the fundamental disorder is secondary to abnormal synchronous discharges of a network of neurons and can be caused by either abnormal ionic conductance or alterations of neuronal membranes, or an imbalance between excitatory and inhibitory neurotransmitters. Epilepsy is characterized by intense electrical discharges in localized brain areas, associated with a change in glutamate (Meldrum 1994; Urbanska et al. 1998) as well as ϒ-aminobutyric acid (GABA) levels (Heinemann and Hamon 1986).

Invertebrates have often been used as an experimental model for studying the effect of convulsant and anticonvulsant agents at the cellular level for understanding the cellular and neurophysiological mechanisms of epilepsy (Janahmadi et al. 2006). It is known that pentylenetetrazole (PTZ) induces a pattern of electrical discharges in molluscan neurons, resembling the paroxysmal depolarization shift (PDS) of epilepsy observed in mammalian neurons (Goldenshohn and Purpura 1963; Matsumoto and Ajmone-Marson 1964; Sugaya et al. 1973). PTZ is used experimentally to study expression of seizures and to identify pharmaceuticals that control them (Janahmadi et al. 2006).

Earlier, attempts were made to study mechanism of epilepsy using invertebrate animal models such as gastropod *Helix pomatia* (Altrup et al. 1992) at a single neuron

The authors declare that the experiments comply with the current Institutional ethical laws.

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level, *Caenorhabditis elegans* (Williams et al. 2004) at an organismic level i.e., whole body movement. However, there is a growing interest in invertebrate neurobiologist for the crustaceans as they are large and have well-organized nervous systems and a typical behavioral pattern ranging from reflexes to complex social interactions (Sandeman et al. 1992). In the present work, we have made an attempt to study the epileptiform activity in the cerebral ganglion of mud crab *Scylla serrata*. Here, we hypothesize that cerebral ganglion of mud crab *Scylla serrata* possesses the structure necessary for development of seizure, which is suppressed by Sodium valproate, an antiepileptic drug commonly used for human epilepsy treatment. Here, we describe electrophysiological (surface electrical recordings) activities and changes in the concentrations of glutamate and GABA that occur in crab cerebral ganglia on exposure to pentylenetetrazole, a common convulsing agent and an antiepileptic drug such as sodium valproate.

Materials and methods

Experimental animal

Mud crab, *Scylla serrata*, is a common edible crab found in the coastal region of India. Adult male and female mud crabs ranging in weight from 100 to 120 g with a blood volume of approx. 15 ml and carapace width of 6.0–8.0 cm were collected from the field and maintained in the laboratory on a 12:12 h light–dark cycle, in glass tanks (10 animals each) filled to a depth of 4 cm with artificial sea water, pH 7.4–7.6. The water temperature was maintained between 22 and 24°C. Experiments were carried out on crabs after a minimum of 1 week of acclimatization to the laboratory conditions. Experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, USA, and Institutional Animal Ethical committee regulations.

The behavioral changes of animal were recorded, kept in aquarium tank having water and large stones, besides their behavioral changes during treatment were recorded when they were exclusively out of water. The behavioral changes in such conditions were minutely observed for any difference under the two conditions.

Solutions

Crab physiological saline (Cooke et al. 1989): NaCl 440.0 mmol, KCl 11.3 mmol, $CaCl_2$ 13.3 mmol, $MgCl_2$ 26.0 mmol, Na_2SO_4 23.0 mmol, HEPES 10.0 mmol, pH adjusted to 7.4 with NaOH. Pentylenetetrazole was obtained from Sigma Chemical Co, (US), while sodium valproate tablets were obtained from a local pharmacy. PTZ (100 mmol/l) was prepared in crab physiological saline (CPS), and sodium valproate (120 μmol/l) was also prepared in CPS.

Experimental design

All drugs were injected into the infrabranchial sinus using a 22-gauge needle inserted through the membrane between the cephalothorax and the coxa of the fifth leg, in order to study the behavioral change as well as for biochemical study, whereas, for electrical recording, drug was applied directly onto the desheathed ganglion. The needle used for infrabranchial injection was of such a length that the release of the drug was within millimeters of the heart and thus was distributed through the circulation (Wood et al. 1995).

PTZ and sodium valproate were screened in preliminary experiments designed to record behaviors correlating with the doses and duration of administration. The test concentrations of PTZ chosen for analysis were based on concentrations known to evoke epileptic behaviors in similar experiments on other animals like *Helix pomatia* and rodents. Threshold concentrations are defined as the minimum dose needed to evoke a behavioral component (Fig. 1). The concentration of sodium valproate used in other studies was converted to milligrams of drug per gram of average body weight, and equivalent doses were given to mud crab. The volume injected into both the treated and control animals was 0.5 ml (V/V).

Each animal was anesthetized by chilling (Allodi et al. 1999) before the cerebral ganglia were desheathed. Recordings were done in vivo on desheathed ganglion at 24°C temperature.

Electrophysiological recording

The surface electrical recordings from the desheathed cerebral ganglia were obtained from the treated and

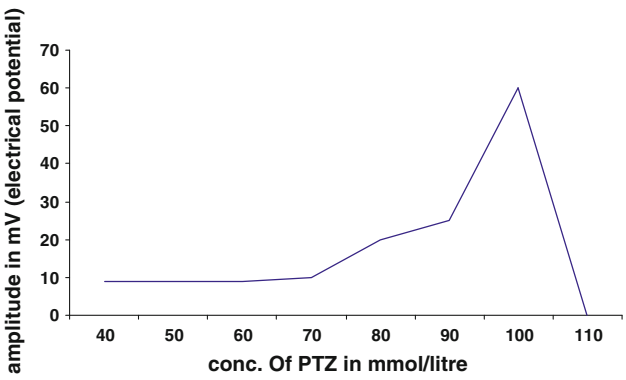

Fig. 1 Graph showing effect of concentration of PTZ on amplitude. Number of crabs used = 5; amplitude in mV indicates electrical potential developed on the crab ganglion

controls under normothermic conditions. The electrical properties of the cerebral ganglia were recorded by placing platinum electrodes (0.35 mm diameter and resistance 0.1 Ω) on the surface of the ganglia and connecting them to the computerized Unkelscope (MIT, USA; version 1984, Bhat and Desai 1998). Source for vertical trace used was analog 0, with a span of 50 mV full scale. Span on horizontal trace used was 100 s full scale, and the sample rate was 0.1 s at 5 Hz. For each experiment, 5 animals were used. The readings were taken only when they steadied relatively and consistently showed the same pattern, i.e. after 10 min of drug administration. Recordings were made for 100 s.

Glutamate and GABA assay

Ganglia from both control (treated with crab saline) and experimental crabs (treated with PTZ, second set treated with sodium valproate alone, and third set with sodium valproate and PTZ) were homogenized in 0.25 M sucrose. Protein-free homogenates were prepared adding 10% TCA followed by centrifugation at 3,000 rpm for 15 min. For the development of fluorophores, 0.1 ml of supernatant was taken and mixed with 0.2 ml ninhydrin (14 mM solution in 0.5 M carbonate buffer, pH 9.95). The mixture was incubated at 60°C; after 30 min, the tubes were cooled and contents were mixed with 2.5 ml of copper tartrate reagent. After 25 min, GABA concentration was measured spectrofluorophotometrically using excitation wavelength (377 nm) and emission wavelength (451 nm).

The glutamate was assayed by running a parallel procedure with another 0.1 ml supernatant. The protein-free homogenate was incubated with 0.2 ml ninhydrin solution at 60°C for 30 min, cooled, and then mixed with sodium tartrate reagent. The summated absorbance of both glutamate and GABA was obtained spectrofluorophotometrically at excitation and emission wavelengths of 377 and 451 nm, respectively. The absorbance of glutamate was determined by subtracting the absorbance of GABA from the respective summated absorbance (Nayak and Chatterjee 2001).

The amount of glutamate and GABA was determined from a standard curve using standard solution of glutamate and GABA containing 1, 2, and 3 mg/ml.

Statistical analysis

All experimental data are presented as the mean ± SD for biochemical assay and mean ± sem for electrical recording. Student's *t*-test was used to analyze the results using Graphpad Software. $P < 0.05$ was considered significant.

Results

Behavioral changes

PTZ treatment induced tonic–clonic seizures in crab; particularly, onset of frequent body jerks followed by brief loss of balance, oozing of froth from the mouth, rapid movements of antennules as well as eyestalks. Whereas, sodium valproate-treated ones showed sluggish/sedate behavior and even after PTZ injection they did not exhibit epileptiform activity demonstrated by those treated with PTZ alone (Table 1). The sedated crab shows drowsiness i.e., on stimulation/proding shows sluggish movement of limbs and eyestalks, while the resting ones run away and show rapid eyestalk movement. The antennular movements of PTZ-treated crabs were faster than those observed in controls and valporate-treated ones. Whether in water or outside the water, behavioral changes were similar. Reversal of PTZ effect was observed after 30 min of treatment.

Electrophysiological changes

Surface electrical recordings of cerebral ganglia showed abnormal synchronous electrical discharges. Control crabs' ganglia bathed with crab saline exhibited normal neuronal electrical discharges in the range of −20 to +20 mV (Fig. 2a). PTZ (100 mmol/l)-treated crabs (Fig. 2b) showed ganglionic electrical discharges in the range of −50 to +50 mV.

Sodium valproate (20 mg/kg body weight) promoted a decrease in electrical discharges of cerebral ganglia in comparison with those of controls, and the electrical discharges were in the range of −30 to +20 mV (Fig. 2c) but

Table 1 Crabs showing the behavioral responses, in control, PTZ-treated, sodium valproate-treated, and sodium valproate + PTZ–treated

Behavioral responses	Control ($n = 10$)	PTZ-treated ($n = 10$)	Sodium valproate-treated ($n = 10$)	Sodium valproate + PTZ–treated ($n = 10$)
Body jerks	−	+	−	−
Brief loss of balance	−	+	−	−
Oozing of froth from mouth	−	+	−	−
Rapid movements of antennules and eyestalks	−	+	−	−
Sedation	−	−	+	+

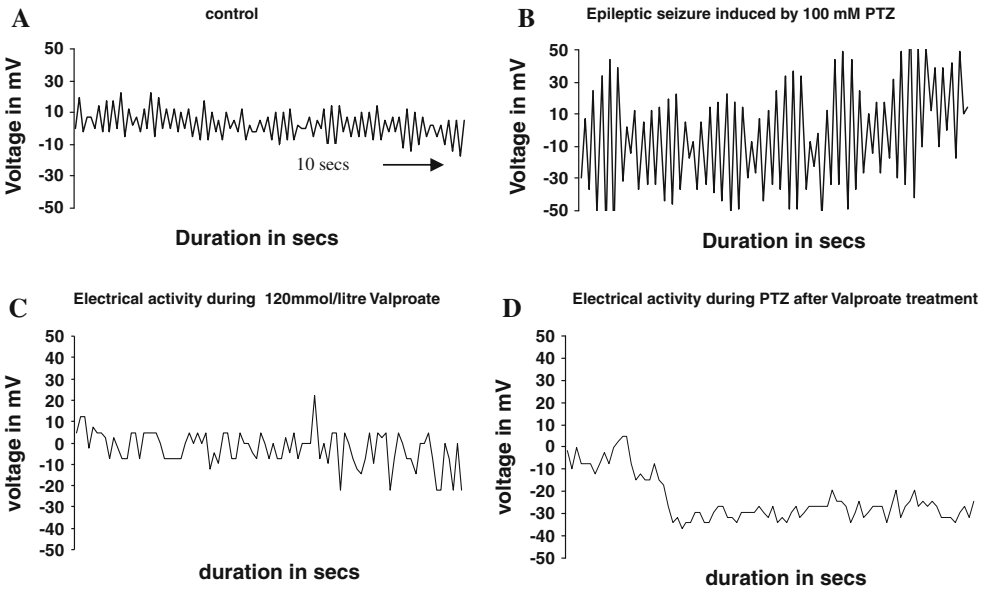

Fig. 2 Surface electrical recording of cerebral ganglion of crab *Scylla serrata* (Control and experimental), recorded for 100 s, but a representative segment of 10 s is shown. **a** Control, **b** Seizure induced by 100 mM PTZ, **c** electrical activity with 120 µmol/l valproate treatment, **d** electrical activity during PTZ after valproate treatment. The crabs take 30 min to recover from the drug effect

Table 2 The amplitude in mV and frequency of electrical recordings—data representing the mean ± SEM

	Amplitude in mV	Frequency
Control ($n = 5$)	8.71 ± 0.44	48.2 ± 0.86
100 mM PTZ ($n = 5$)	55.12[a] ± 1.36	46.4[b] ± 0.48
120 µM sodium valproate ($n = 5$)	13.59[a] ± 0.65	24.8[a] ± 0.35
Sodium valproate + PTZ ($n = 5$)	8.13[b] ± 0.19	23.4[a] ± 0.63

[a] Represents the value to be statistically significant

[b] Represents statistical insignificance

with reduced spike frequency as shown in Table 2, and cerebral ganglia of crabs exposed, 20 min after pretreatment of sodium valproate to similar dose of PTZ, showed lower electrical discharges in the range of −40 mV to +10 mV (Fig. 2d) with reduced spike frequency, indicating a protective action of sodium valproate against PTZ-induced seizures.

Glutamate and GABA concentrations

Glutamate and GABA concentrations of controls were equivalent to 4.81 ± 0.98 and 0.74 ± 0.01 mg/gm wet weight of tissue, respectively. Exposure to 100 mmol/l dose of PTZ elevated glutamate concentrations of brain by 31.45% and decreased GABA concentration by 43.93% (Fig. 3). However, exposure of crabs to sodium valproate alone effected no significant change in glutamate levels; however, it induced a significant increase (24.75%) in GABA level. The crabs pretreated with sodium valproate and then exposed to PTZ after 20 min showed a significant increase (31.03%) in glutamate level without any change in GABA level with respect to controls.

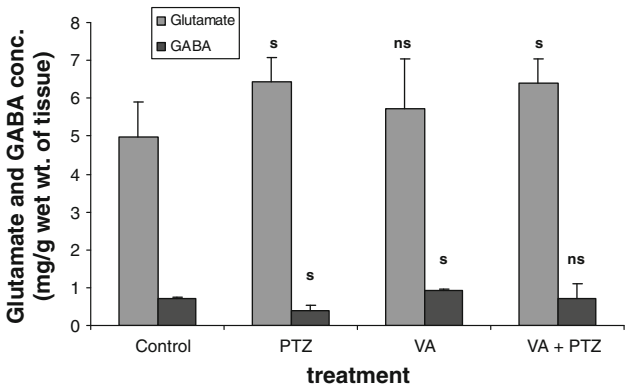

Fig. 3 Glutamate and GABA level in cerebral ganglion of crab *Scylla serrata*. PTZ—pentylenetetrazole, VA—sodium valproate. Data represents mean ± SD. Significant difference was calculated by Student's *t*-test. *P* value is calculated with reference to control. $n = 5$

Discussion

A basic phenomenon of epilepsy consists of typical neuronal discharges induced in the nervous systems of many organisms. The present work shows convincingly that crab cerebral ganglia are able to produce epileptiform electrical discharges. Epileptic electrical discharges of invertebrate ganglia are easy to investigate at the level of single neuron. The buccal ganglia of *Helix pomatia* were studied to understand the mechanisms underlying epilepsy (Altrup et al. 1992). Speckmann and Caspers (1973) used pentylenetetrazole (PTZ) to promote epileptic electrical discharges (EED) in isolated neurons of *Helix pomatia,* while Altrup et al. (1991) used hypnotic drug etomidate for producing EED in mollusks.

The present work shows that with induction of EED, normal electrical rhythms are altered and new epileptic rhythms appear. The behavioral changes in crabs like fast running and sudden loss of postures, vigorous movements of eyestalks and antennules, frequent body jerks, and oozing of froth from mouth followed by brief loss of posture after PTZ treatment are quite comparable to tonic–clonic epileptic behaviors in rats (Maciejak et al. 2009). Besides, human epileptic patients exhibit oozing of froth from mouth and brief loss of postures along with frequent body jerks. Therefore, the epileptiform behavior of crab is partly comparable to the human generalized seizure pattern.

The altered rhythms of neuronal discharges are nothing but paroxysmal depolarization shifts (Goldenshohn and Purpura 1963; Matsumoto and Ajmone-Marson 1964). It is known that 40 mmol/l dose of PTZ can induce epileptic discharges in mollusks (Altrup et al. 1992). PTZ promotes volleys of electrical discharges of high frequency and amplitudes. The control crabs exhibited normal neuronal firing in the range of approximately between −20 mV and +20 mV. The rate and pattern of volleys of electrical discharges induced by PTZ in crabs indicate the onset of epileptiform activities. PTZ produces large swings from positive to negative wherein more positive effect indicates hyperexcitability, while negative recording indicates hyperpolarization, indicating membrane potential have gone below the value of resting membrane potential. Further, they indicate involvement of excitatory circuits for progression of epilepsy (Khalilov et al. 2003).

Millimolar concentrations of PTZ, the most extensively used epileptogenic drug, have been shown to affect a variety of ion-selective channels and neurotransmitter receptors (Hartung and Hermann 1987; Madeja et al. 1991; Bloms et al. 1992; Bloms-Funke et al. 1994; Madeja et al. 1996). These effects can lead to enhanced excitability of nerve cells by membrane depolarization or to a hyperpolarization that suppresses small depolarization and allows re-initiation of seizure activity (Madeja et al. 1996). Here, we have demonstrated that 100-mM concentration of PTZ increases the glutamate level above that of control. Glutamate is a source of energy in oxidative phosphorylation and acts as a building unit for brain protein synthesis as well as an excitatory neurotransmitter. In addition to that, glutamate has several specific functions. It is a detoxifying agent that binds NH_3 to form glutamine, a precursor for GABA, an inhibitory neurotransmitter and proline synthesis (Cooper et al. 1996). Therefore, it has been extremely difficult to dissociate the role glutamate plays in neuronal metabolism and as a precursor for GABA from its possible role as a neurotransmitter. The present investigation indicates that the elevations in electrical activities could be due to elevations in the levels of glutamate and a reduced level of GABA, suggesting the role of altered glutamate and GABA in induction of epilepsy (Freitas et al. 2007). Controls show glutamate level equal to 4.81 ± 0.98 mg/gm wet weight of tissue indicating as a requisite level for a normal functioning of cerebral ganglion, but larger increase in glutamate indicates release of glutamate on administration of PTZ, which leads to hyperexcitation of neurons leading to epileptiform activity. Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system, but it has been established that an excessive neurotransmission at glutamatergic synapses, mediated mainly by the *N*-methyl-D-aspartate (NMDA) receptor type, is greatly involved in epilepsy and neurodegeneration (Choi 1988; Meldrum 1991; Tapia et al. 1999). Sodium valproate reverses the effect of PTZ. The sodium valproate-mediated declines in electrical discharges of neurons of both the control and PTZ-administered crabs indicate involvement of GABA. Sodium valproate is known to increase GABA in the brain tissue (Arroyo 2004). Sodium valproate increases synaptosomal GABA concentrations through the activation of the GABA-synthesizing enzyme glutamic acid decarboxylase (Arroyo 2004). In addition, sodium valproate inhibits GABA catabolism through inhibition of GABA transaminase and succinic semialdehyde dehydrogenase (Arroyo 2004). Sodium valproate also inhibits the excitatory neurotransmission mediated by aspartic acid and glutamic acid (Arroyo 2004). Furthermore, valproate reduces cellular excitability through modulation of voltage-dependent sodium currents (Vreugdenhil and Wadman 1999). Valproate has shown efficacy in animal models of absence, partial, and generalized seizures induced by chemical (bicuculline, PTZ, picrotoxin, strychnine, quinolinic acid), electrical (maximal electroshock), or sensory stimuli (photic). Valproate prevents the development of kindled seizures and thus has an antiepileptogenic effect (Silver et al. 1991). The prevention of epileptic activities in the form of synchronized electrical discharges as well as change in the behavioral pattern and suppression of glutamate rise by valproate, (although it does not decrease glutamate significantly) suggests a condition similar to that observed in experimental rodent models and human epilepsy. The present research indicates that valproate alone promotes rise in GABA but PTZ probably suppresses valproate ability to promote rise in GABA. But the negative trace indicates that the factors other than GABA are suppressing the action of PTZ. Hence, it is necessary to further investigate the levels of other inhibitory neurotransmitters in the cerebral ganglion.

The present work also shows the presence of glutamate and GABA in crab brain with possibility of presence of their receptors on the neurons. Khalilov et al. (2003) reported that for propagation of seizures, excitatory neural circuits are essential, and the PTZ-mediated induction of

seizures suggests the existence of such circuit in crab brain. The expression of behavioral pattern, synchronous abnormal electrical discharges by the brain of crab, and abolishment of these responses by the antiepileptic drugs indicate the suitability of crab for studying epileptiform activities. Since the CNS of crab is a simple ganglionic tissue comprised of neurons, it would be possible to study the effects of epilepsy and antiepileptic drugs at cellular level quite easily when compared with the complex rodent brain.

Therefore, it is concluded that the cerebral ganglion of mud crab, *Scylla serrata*, can be used for studying epileptiform activities and physiological as well as biochemical changes associated with it. The present study indicates that the crab neurons have the requisite paraphernalia for producing volleys of electrical discharges associated with epilepsy. With caution, the information obtained from invertebrate studies could be applicable to higher systems.

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

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