Research report

Altered responsiveness to 5-HT at the crayfish neuromuscular junction due to chronic p-CPA and m-CPP treatment

Robin L. Cooper*, Rachel J. Chase, Jami Tabor

Thomas Hunt Morgan School of Biological Sciences, University of Kentucky, Lexington, KY 40506-0225, USA

Accepted 23 July 2001

Abstract

Serotonin (5-HT) levels in the hemolymph of crustaceans has been implied to alter aggressiveness which influences social interactions. The activation of IP3 as a second messenger cascade within crayfish motor neurons in response to application of 5-HT, suggests that the 5-HT receptor subtypes on the motor neurons are analogous to the vertebrate 5-HT2A receptors. Based on evidence in other systems, it would be expected that chronically sustained 5-HT levels in aggressive individuals would result in a compensatory negative feed-back regulation and/or that target tissues would diminish their sensitivity to high levels of circulating, free 5-HT. We addressed the issue of up- and down-regulation in the sensitivity of the responsiveness to exogenously applied 5-HT at the NMJs of crayfish in which the animals have altered endogenous 5-HT levels. Injections of the 5-HT1 and 5-HT2 vertebrate receptor agonist, 1-(3-Chlorophenyl) piperazine dihydrochloride (m-CPP), for 1 week resulted in a decreased responsiveness to application of 5-HT. The compound p-chlorophenylalanine (p-CPA) blocks the enzymatic synthesis of 5-HT and following 7 days of p-CPA injections, a super-sensitivity to exogenous application of 5-HT for both tonic and phasic neuromuscular junctions (NMJs) was observed. However, acute applications of p-CPA and m-CPP, followed by extensive saline washing, did not reveal any altered receptivity to 5-HT application. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Serotonin receptors

Keywords: Serotonin; Neuromodulation; Synapse; Crayfish; Presynaptic

1. Introduction

It is well established that receptors to hormones or neuromodulators undergo up- and down-regulation via alteration of their expression levels and/or densities on cell surfaces [1]. Altered cellular activity, as well the action of agonists or antagonists being bound to a receptor can induce regulation in the levels of functional receptors [24,29,36,55]. For example it has been shown that 5-HT1A receptors will demonstrate desensitization when either an agonist or antagonist is present [28]. Clozapine, an antipsychotic in humans, has a dual action on 5-HT receptors. It can upregulate 5-HT7 receptors while down-regulating 5-HT6 receptors [59]. Even naturally induced down-regulation of 5-HT2c receptors can be induced as a result of exercise [8]. The 5-HT1 and 5-HT2 receptor agonist 1-(3-Chlorophenyl) piperazine dihydrochloride (m-CPP) has been observed in rats to down-regulate the number of receptors [24]. In addition, m-CPP is thought to block the 5-HT transporter resulting in the acute increase in levels of 5-HT within the extracellular fluid in the central nervous system (CNS) of rats [22]. The precise mechanism of action in regulation of the 5-HT receptors has not yet been elucidated.

In crustaceans, the presence of 5-HT enhances synaptic strength at neuromuscular junctions (NMJs) [15,37,38,52,53], increases heart rate [23,40,56], and enhances sensory neuronal activity [53]. 5-HT levels in the hemolymph of crustaceans have also been implied to alter the assertiveness and aggression during social interactions [30–32,50]. Pharmacological examination indicates that m-CPP has agonistic functions in crayfish altering sensory...
drive into the animals ventral nerve cord [58]. Based on evidence in other systems, mentioned above, one would suspect that chronically sustained 5-HT levels would result in a compensatory negative feed-back regulation and/or that target tissues would diminish their sensitivity to high levels of circulating, free 5-HT.

The purpose of this study was to address the issue of up- and down-regulation by examining the sensitivity in the responsiveness to exogenously applied 5-HT at the neuromuscular junctions of a crustacean (e.g. crayfish) in which the animals have had either reduced endogenous 5-HT levels by enzymatic inhibition in the synthesis of 5-HT or have had a chronic presence of an agonist/antagonist in their hemolymph. Reduction of the systemic levels of 5-HT in the crayfish is likely to result in up-regulation of 5-HT receptors, since these animals normally contain 5-HT in their hemolymph (open circulatory system). In contrast, chronic high levels of the 5-HT agonist m-CPP, would likely result in a down-regulation of the 5-HT receptors so that exogenous application of 5-HT would show reduced responsiveness as compared to sham injected animals. We demonstrate alterations in the physiological sensitivity to exogenous application of 5-HT at the crayfish neuromuscular junction after altering levels of the endogenous production of 5-HT and exposure of a 5-HT receptor agonist. The results of this study have been presented in abstract form [12].

2. Methods

2.1. Animals

The initial chronic studies, which involved the high dosages of m-CPP and p-CPA, were conducted in crayfish in the range 10.5–25 g. Since a number of these crayfish usually died within 3 days, larger mid-sized crayfish were used with a lower dosage for the chronic studies. The physiological results these animals are reported. These animals measured 8–10 cm in body length and weighed 28–36.4 g. All the crayfish used were (Procambarus clarkii) and were obtained from Atchafalaya Biological Supply Co. (Raceland, LA). Animals were housed in an aquatic facility within the laboratory in individual tanks, and were fed fish food pellets every 3 days. Only male crayfish in their intermolt stage were used.

2.2. Pharmacology

To manipulate the potential receptivity of the animal to exogenous application of 5-HT, two separate drugs were chronically injected into the animals’s hemolymph. One compound, p-chlorophenylalanine (p-CPA; Sigma) depletes 5-HT levels by binding irreversibly to tryptophan hydroxylase. The other compound, 1-(3-Chlorophenyl)piperazine dihydrochloride (m-CPP; Sigma), acts as a 5-HT1 and 5-HT2 receptor agonist in rodents [17,35,45], although it acts as an antagonist for cloned human 5-HT2B receptors [54]. All injections were given through the articulating membrane between segments A1 and A2 of the ventral abdomen.

Initial testing was performed to determine the dose of the compounds which did not cause death. The daily injection of 10 mg/250 l of saline/animal of p-CPA caused death of each animal within 3 days. Reducing the amount to 5 mg/250 l of saline/animal resulted in 50% death of the animals after 7 days. All animals survived 2 weeks when injected with the 2.5 mg/250 l of saline/animal for both p-CPA and m-CPP. These animals measured 8–10 cm in body length and weighed 28–36.4 g. This dosage and range in animal size was then utilized throughout the experiments mentioned in this report.

2.3. Dissection and physiology

Two groups of muscles were used for these studies. One muscle was the opener muscle, which is innervated by a single, purely-tonic excitatory motor neuron. The second muscle was the dorsal extensor lateral (DEL1) muscle of the deep abdominal extensor muscles, which is purely phasic in its motor nerve innervation. For a review in innervation and phenotype of these muscles, see Ref. [39].

The opener muscle of the first walking legs was prepared by the standard dissection [20]. The cuticle was cut along the length of the propus, between the attachments points of the opener and closer muscle fibers. The tissue was pinned out in a Sylgard dish for viewing with a Nikon, Optiphot-2 upright fluorescent microscope using a ×40 (0.55 NA) Nikon, water-immersion objective.

The ventral surface of the deep abdominal extensor muscles was exposed by removing the ventral side of the abdomen after cutting along the length of the abdomen between the deep extensors and the deep flexors on each side [13]. The dorsal half was pinned down and residual flexor muscle removed. This allows for excellent visual identification of the deep extensor lateral muscles (DEL1 and DEL2). The DEL1 muscles were used for physiological studies and protein analysis. The nomenclature of these muscles follows the work of Ref. [51].

All dissected preparations were maintained in crayfish saline, a modified Van Harreveld’s solution (in mM: 205, NaCl; 5.3, KCl; 13.5, CaCl2, 2H2O; 2.45, MgCl2, 6H2O; 0.5, HEPES) adjusted to pH 7.4 [10]

2.4. Excitatory postsynaptic potentials (EPSPs)

Intracellular recordings were performed with 30–60 M resistance microelectrodes filled with 3 M KCl. Responses were recorded with a standard intracellular electrode amplifier (Axoclamp 2A, Axon Instruments). Electrical signals were recorded onto VHS tape and on-line to a Power Mac 9500 via a MacLab/4s interface. EPSPs were
distinctly different regions, central and proximal, that have was expected that even higher-output phasic nerve termi-
chronically treated animals
Effects of
3.1. Effects of 5-HT on tonic NMJs of p-CPA and m-
CPP chronically treated animals
The ventral surface of the opener muscle has two distinctly different regions, central and proximal, that have pronounced differences in postsynaptic responses even though a single motor neuron innervates the entire muscle. These two regions also show differences in STF [15,16], and the terminal varicosities on the proximal muscle fibers are known to have a more complex structure than those on the central fibers at the morphological level. The structural differences are thought to account for the higher synaptic efficacy of the proximal varicosities [9,14]. In the present study, EPSP measures were restricted to the proximal region of the opener muscle in order to obtain larger initial responses (Fig. 1A, arrow). The amplitude of the 10th EPSP responses of the response train was used in this study to ensure a measurable value before and after treatment with 5-HT.

Representative amplitudes of EPSPs within a ten-pulse stimulation train before and after 5-HT (100 nM) application are shown in Fig. 1. It is readily apparent that the amplitude of the EPSPs increase in response to 5-HT. In all cases 5-HT results in an enhancement of the EPSP amplitudes.

The effect on the EPSP amplitude when exposed to 5-HT is rapid, as illustrated in Fig. 1B. In Fig. 1B an average of 20 repetitive trials for the 5th and 10th EPSP amplitudes is shown. The facilitation index revealed that overall synaptic transmission is depressing the nerve terminal. enhanced by 5-HT and that 5-HT does not alter facilitation
sections within the stimulated segment [26]. Single stimuli were given to the motor nerve every 10 s to avoid depressing the nerve terminal.

The percent difference in the responses was determined by comparing the average amplitude in the EPSP prior to the exposure of one compound (X) to another (Y) by the following manner: \[
\frac{[(\text{amplitude during exposure to X} - \text{amplitude during exposure to Y})]}{\text{(amplitude during exposure to X)}} \times 100 = \% \text{ Difference}
\]
A standard of 100 nM 5-HT (Sigma) in saline was freshly made before each experiment from a frozen 1 mM stock. The maximum response was used as a measure of 5-HT effects.

3. Results
Exogenous application of the neuromodulator 5-HT (100 nM) to excised neuromuscular preparations of the crayfish produces a larger increase in synaptic responses for tonically innervated slow muscles than for phasically innervated fast phenotype muscles [16,33,47]. This general phenomenon is also illustrated in this study with the tonically-innervated opener muscle and the phasically-innervated DEL1 muscle.

3.1. Effects of 5-HT on tonic NMJs of p-CPA and m-
CPP chronically treated animals
The high-output tonic terminals on the opener muscle fibers resulted in dramatic alterations in 5-HT sensitivity. It was expected that even higher-output phasic nerve termi-
Fig. 2. Chronic exposure to m-CPP or p-CPA altered the synaptic responsiveness of the neuromuscular junctions to 5-HT. Trains of 40 Hz containing 10 events were delivered every 10 s. The amplitude of the 10th EPSP responses were measured from the trough to the peak before and after 5-HT (100 nM). The mean (± S.E.M.) of the percent differences for the sham (vehicle), m-CPP, and p-CPA groups (n = 6) are shown. The responses during the plateau were measured for comparisons. The star denotes a significance (P < 0.05; Mann-Whitney non-parametric test) compared to the sham group.

Fig. 1. A schematic of the walking leg opener muscle as seen from a ventral view (A). The proximal bundle of muscle fibers, as indicated by the arrow, are the fibers innervated by high-output motor terminals and were used throughout this study. Representative preparations are shown in which EPSPs were recorded with a train stimulation of ten identical stimulus pulses given at 40 Hz before and after application of 5-HT (100 nM). It is readily observed that each of the EPSPs within the train are enhanced by the exposure to 5-HT. An average of 20 trains of EPSPs were used to reduce the noise in the representative traces. (B) The EPSP responses plotted over time demonstrates the quick effect of 5-HT in increasing the amplitude of the response in a preparation not previously treated with m-CPP or p-CPA. In this representative experiment, the 5th and 10th EPSP amplitudes are shown. No significant change in the facilitation index (FI-see Methods) could be observed among the preparations for 5-HT exposure (C). Averages of ten trials were taken for each representative value.

3.3. Direct effects of m-CPP and p-CPA

To examine the acute actions and the reversibility of m-CPP and p-CPA, direct acute applications of these compounds were performed on the opener muscle. Crayfish are approximately 30% volume by mass [25,27]. Thus, the estimated concentrations from chronic injection studies were determined to be approximately 1.5 mM m-CPP and 2.1 mM p-CPA. These same concentrations of m-CPP and p-CPA were made in crayfish saline and directly applied to the opener muscle. Small reductions in the EPSP amplitudes could be observed during the acute exposure (10 min) to p-CPA; but upon addition of 5-HT...
Fig. 3. A schematic of the innervation pattern and layout of the abdominal deep extensor muscles for stimulation and recording from the DEL1 muscle (A). The viewing is from the ventral side towards the dorsal aspect of abdominal segments 3 and 4 (A3, A4). The most medial muscles are the deep extensor medial (DEM) muscles with a spiral fiber pattern. The more lateral muscles are the DEL1 and DEL2. The most lateral bundle of fibers is the superficial extensor lateral muscle (SEL). The DEL1 and DEL2 are fast muscles in phenotype with phasic innervation. There is a selective segmental crossing in innervation to the DEL1 from segment 2 to the DEL1 muscles in segment 3. This allows selective monitoring of a single excitatory motor neuron to DEL1 in a more caudal segment then from the segment in which multiple motor neurons are stimulated. A representative EPSP trace for the DEL1 muscle is shown in the lower right corner (modified from Refs. [26,51]). (B) In chronically treated animals with m-CPP or p-CPA the responsiveness to exposure of 5-HT is different as compared to the sham treatment. The mean (±S.E.M.) of the percent differences in the EPSP amplitude for the sham (n = 6), m-CPP (n = 6), and p-CPA (n = 6) groups are shown. The star denotes a significance (P < 0.05; Mann–Whitney non-parametric test) compared to the sham group.

(10 or 100 nM) in the presence of p-CPA, normal EPSP enhancement to 5-HT was observed as shown in a representative example for the 10th EPSP amplitudes (Fig. 4A). This trend was observed in all five preparations (Fig. 4B, P < 0.05 Wilcoxon rank sum test). Since the p-CPA resulted in a small reduction in the EPSP amplitudes, a percent difference for the 5-HT with p-CPA effect to that of the p-CPA alone was determined (Fig. 4C). This percent difference is much less than that for the exposure of 5-HT in chronically treated animals (Fig. 2).

Substantial decreases in the EPSP amplitudes were observed in the opener muscle (Fig. 5A) and the DEL1 muscle (data not shown) upon exposure of m-CPP. The responses do not mimic those for application of 5-HT in respect to increasing synaptic strength, as an 5-HT2A
receptor agonist would be expected to do. If the preparations are exposed to 5-HT (100 nM), the preparations retain their responsiveness to 5-HT, although the response is not as robust as when 5-HT is applied without prior exposure to m-CPP. In further acute trials in which a more substantial washing of the preparation with saline was preformed following the m-CPP exposure (i.e. five or more times of completely exchanging the bathing medium), the EPSP amplitudes were able to recover to baseline conditions (Fig. 5A). In such cases the subsequent exposure to 5-HT resulted in an enhanced response of the EPSP amplitudes. This suggests that m-CPP can be washed off the preparation. Similar trends were observed in five out of five preparations (Fig. 5B). The extensive washing with saline after exposure to m-CPP was essential before any effect of the subsequent exposure to 5-HT would result in significant increases in the EPSP amplitudes. The percent change in the EPSP amplitudes from the saline wash to the 5-HT treatment is similar for both the p-CP A and the m-CPP treatments (Figs. 4C and 5C). Thus, the acute exposure to p-CP A and m-CPP, after thorough saline washing, has not affected the responsiveness to 5-HT. The acute studies supports the observations that the chronic exposures to the compounds significantly alters the NMJs responsiveness to 5-HT.

4. Discussion

In this study we have shown that NMJs of phasic and tonic motor nerve terminals are responsive to exogenous application of 5-HT (100 nM). Since 5-HT is a biogenic amine within the animal’s hemolymph, it is not surprising that it has a neuromodulatory effect on the various types of neuromuscular junctions. Recently it has been shown [15,52,53] that the effect is primarily presynaptic in increasing the probability of vesicular release. The lower initial output of the NMJs of tonic motor neurons may partially account for the fact that the tonic NMJs demonstrated a greater percent enhancement of synaptic efficacy when compared to phasic NMJs for a given concentration of 5-HT. Injections of the 5-HT1 and 5-HT2 vertebrate receptor agonist, m-CPP, for 1 week resulted in a decreased responsiveness to application of 5-HT. In contrast, chronic exposure of the enzymatic blocker to 5-HT synthesis, p-CP A, resulted in a supersensitivity of both tonic and phasic NMJs.

The fact that acute application of m-CPP decreased the EPSP amplitudes does suggest that m-CPP does not have a strict 5-HT2 subtype agonist action, but that different 5-HT receptors may be present such as a vertebrate analog of a 5-HT3 subtype that may inhibit cellular responses. In addition, the results show that m-CPP can depress glutaminergic transmission. In the vertebrates, m-CPP has many actions, such as increasing the extracellular concentrations of 5-HT in the particular brain regions, which is thought to be a result of reversing the 5-HT transporter [22]. It is also noted that m-CPP can increase the extracellular concentrations of dopamine [22]. Human experimentation has indicated that m-CPP has 5-HT receptor agonist/antagonist actions and that such broad actions cause the ‘serotonin syndrome’ that can be induced from a single oral dose of m-CPP [35]. We are not aware of any study that directly examined the role of m-CPP on neuronal function, such as blocking ion channels, or m-
CPP having a direct antagonistic effect on glutaminergic receptors. However, that does not mean that there is no effect on vertebrate glutamate receptors. Glutaminergic receptors at the crayfish NMJ are of an quisqualate-type with rapid sodium conductance [19,48], so it is worth investigating the actions of m-CPP on analogous vertebrate receptors.

Since alterations in the physiological effects to exogenous application to 5-HT are observed due to chronic pharmacological interventions, binding assays will need to be performed to determine if receptor density has changed and/or if another form of desensitization is occurring. There are a number of potential scenarios to explain the results observed in this study. For example, desensitization may occur by receptor down-regulation by a reduction in receptor synthesis or expression. Alternatively, 5-HT receptors may be internalized by the function of arrestin and possible association of dynamin by clathrin-coated pits. A more recent mechanism thought to effect 5-HT responsiveness is the uncoupling of the G-proteins by phosphorylation of the receptors via protein kinases and subsequent binding of arrestin to physically separate the receptor and its associated G-protein (Hensler, personal communications; University of Texas at San Antonio). It is also established that in humans suffering from depression that 5-HT2A receptor as well as the linked IP3 signaling transduction pathway are altered in expression [21]. Such concomitant events of receptor regulation and alteration of transduction signaling may well be taking place in our chronic experimentations with m-CPP in one direction and the opposite direction with the p-CPA studies, but further studies will be needed to examine this issue.

In vertebrates it is suggested that a down-regulation of 5-HT2c receptors is able to be induced by activity in the form of exercise which can stimulate 5-HT release [8]. Recently, it has also been demonstrated that exercise in a crabs results in a humoral elevation of 5-HT [50]. This suggests that whole animal activity is likely to induce a modulation of neural activity and could be linked to motivational states [34,57]. It is well established in crayfish that activity, either by electrical conditioning of the neurons or seasonal activity in the field, results in phenotypic changes of phasic motor nerve terminals to a tonic-like state [43]. Transformed phasic to tonic-like nerve terminals are more varicose in nature, and the mitochondria show an increase in cross-sectional area [42]. Physiologically, the transformed terminals have a reduced initial EPSP, and upon repetitive stimulation, will demonstrate fatigue resistance [5–7,13,44]. More important to this study is that the transformed nerve terminals demonstrate an increased sensitivity to 5-HT neuromodulation [26]. The enhancement in the percent change of the EPSP amplitude by 5-HT is 99% greater for transformed, tonic-like nerve terminals than non-conditioned phasic neurons [26]. These results are in keeping with the general finding that purely tonic motor neurons are more sensitive to 5-HT than are phasic motor neurons in the enhancement of the postsynaptic response, as we have shown in this study as well as for the extensor muscle in the crayfish walking leg [47]. It is plausible that the heightened chronic activity of the phasic neuron may have altered 5-HT receptor density or subtype expression. Previous reports [30–32,41] have implied that in crustaceans the aggressive individuals have a higher chronic 5-HT level. One report to date [50] has actually measured, using high-performance liquid chromatography (HPLC) techniques, the biogenic amine levels in paired, aggressive and submissive crabs and has shown a higher 5-HT level in the aggressive individuals. Such studies are very intriguing since it has now been shown that in crayfish depleted of 5-HT, aggressive tendencies are unchanged [46]. It does appear unlikely that aggressive individuals would maintain a chronically higher level of free 5-HT circulating within the hemolymph (or other neuromodulators for that matter), since receptivity to 5-HT would be expected to be altered over time. In addition, since dominance is generally size related among a pair of crayfish, one would expect a very dynamic response for rapidly-altered social conditions. For instance, a dominant male in one dyad would likely change quickly to being submissive in another dyad pairing with a much larger opponent. This suggests rapid modulation within an individual’s social state; and if such states can be hormonally altered, this may in part regulate the crayfish’s ‘fight or flight’ response [49], as would be expected for an autonomic nervous system in the vertebrates and less likely by chronically altered levels of neuromodulators. In addition, it is very likely that more than one neuromodulator is responsible for a behavioral state, but that the ratio of combinations of substances will shape a behavioral state of the whole animal. In fact, recently it was demonstrated that the neuromodulator octopamine has antagonist actions to 5-HT effects in enhancing synaptic transmission [18]. One needs to be cognizant that there are numerous neuromodulators working in concert to exert their influence on synaptic function, which ultimately shapes the behavior of the animal.

Acknowledgements

Appreciation is expressed to Mr Austin Cooper and Dr Wendi Neckameyer (St. Louis University School of Medicine) for editorial comments and to Dr Julie G. Hensler (University of Texas Health Science Center at San Antonio, TX) for helpful experimental suggestions. Funding was provided by NSF grants IBN-9808631 (RLC), NSF-ILL-DUE 9850907 (RLC) and an undergraduate training fellowship from Howard Hughes Medical Institute (RJC) as well as a G. Ribble Fellowship for undergraduate studies in the School of Biological Sciences at the University of Kentucky (RJC and JT).
References


[18] S. Djkaj, R.L. Cooper, W. Rathmayer, Effects of octopamine, serotonin, and cocktails of the two modulators on synaptic transmis-


