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Research report

# Physiologically identified 5-$HT_2$-like receptors at the crayfish neuromuscular junction

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**Abstract**

The model synaptic preparation of the crayfish opener neuromuscular junction is known to be responsive to exogenous application of 5-HT. The primary effect of 5-HT is an enhancement of vesicular release from the presynaptic motor nerve terminal. 5-HT is known to act through an $IP_3$ cascade which suggests the presence of a 5-$HT_2$ receptor subtype; however, this is based on vertebrate 5-HT receptor classification. We examined this possibility by using a selective agonist and two antagonists of the vertebrate 5-$HT_2$ receptor subtypes. The antagonist ketanserin and spiperone reduce the responsiveness of 5-HT in a dose-dependent manner. The broad 5-$HT_2$ receptor agonist, α-methyl-5-hydroxytryptamine (α-Me-5-HT) enhances synaptic transmission, in a concentration-dependent manner, but it is not as potent as 5-HT. These results support the notion that a 5-$HT_2$ receptor subtype is present presynaptically on the crayfish motor nerve terminals. By knowing the types of 5-HT receptors present on the presynaptic motor nerve terminals in this model synaptic preparation, a better understanding of the mechanisms of action of 5-HT on vesicular release will be forthcoming. © 2002 Elsevier Science B.V. All rights reserved.

*Theme:* Neurotransmitters, modulators, transporters and receptors

*Topic:* Serotonin receptors

*Keywords:* Serotonin; Neuromodulation; Synapse; Crayfish; Presynaptic

## 1. Introduction

The crayfish neuromuscular junctions (NMJs) have been used as a model system for years to investigate properties of synaptic transmission [7–10,14–18,20–24,27,50,51]. This system allows one to monitor single vesicular events, with macroptach recordings at synapses on NMJ, thus the kinetics and mechanisms of vesicular release within identified neurons can be investigated [11]. This model system has served well in examining the role of neuromodulators on influencing the quantal parameters of synaptic transmission (i.e. *n*, the numbers of release sites and *P*, the probability of release) [16,20,44,45]. Serotonin has been known since the 1950s [20,23,24] to enhance synaptic transmission at the crayfish neuromuscular junction (NMJ) but only recently have the detailed mechanisms been investigated. An exposure of 100 nM 5-HT to the NMJs of the crayfish walking leg opener muscle results in primarily an enhancement of the probability of vesicular fusion during evoked transmission along with an increase in the number of vesicular release sites [29,44]. In crustaceans, since they have an open circulatory system, the 5-HT levels in the hemolymph bathe the NMJs of the skeletal muscles. 5-HT is released into the hemolymph by neurosecretory cells whose cell bodies are in the ventral nerve cord, but it is released from nerve endings in the 2nd thoracic roots and in the pericardial organs [3]. In addition, 5-HT titers vary in crustaceans depending on different physiological states, such as exercise [43]. Application of 5-HT in crustaceans not only increases synaptic strength at neuromuscular junctions [20,23,24,44,45], but also increases heart rate [25,37,49], and enhances sensory neuron activity [45]. Behaviorally, 5-HT levels in the hemolymph of crustaceans have also been implied to alter the assertiveness and aggression during social interactions [32–

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34,36,38], but this view is being reconsidered [19,37,41,45].

In order to advance this field of neuromodulation in relation to crustacean behavior it is imperative to know the types of receptors present and their mechanisms of action so that more refined pharmacological experimentation and manipulation can proceed to dissect the mechanisms involved in relation to altering synaptic transmission and ultimately behavior of the animal. In addition, it is now known that the absolute level of one particular neuromodulator (e.g. 5-HT) in the hemolymph is not informative enough since within the animal there is a multiplicity of neuromodulators which can determine the effects of any one neuromodulator [17]. It is likely that interacting second messenger cascades can either enhance or depress the actions of another neuromodulator. To determine what interactions are potentially occurring, the various receptor types and corresponding second messenger systems need to be established.

Recently, it has been reported that the functional responsiveness of 5-HT depends on the phenotypic state of the neurons [28], which are seasonally altered in the natural life history of the animal [39,40]. Altered receptivity to 5-HT in the crayfish has also been established when either 5-HT's endogenous production is reduced or when chronic pharmacological 5-HT agonists or antagonists are utilized [6,12]. These recent results imply that receptor density changes or possibly that a form of desensitization occurs as a result of chronic manipulations associated with receptor-mediated responses [6,12].

The varied responses to 5-HT in the invertebrates suggests a possible family of 5-HT receptor subtypes as in vertebrates. Recent pharmacological evidence indicates 5-$HT_2$ receptor types to be present in the crayfish nervous system [53], and an earlier study implies a 5-$HT_2$ receptor subtype to be present since an $IP_3$ cascade is indicated to be transducing the effects of 5-HT [18].

The purpose of this study is to elucidate, by pharmacological means, if the 5-$HT_2$ receptor subtype is one of the potential 5-HT receptor types present in the same preparation in which these earlier NMJ studies were conducted. This report sets the groundwork for investigation into the mechanism of 5-HT's action at a cellular level [13].

The results of this study have been presented in abstract form [12,13].

## 2. Methods

The methods are similar to those described earlier [6,8]. In brief, the following procedures were followed.

### 2.1. Animals

Mid-sized crayfish (*Procambarus clarkii*), measuring 8–10 cm in body length and weighing 20 to 36 g, 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

All chemicals tested were applied by fully exchanging the bathing medium of the preparation three times. The concentrations used were 100 nM, and 1 μM of the particular compounds of interest. The concentrations used are reported in the Results for each experimental paradigm. 5-HT was obtained from Sigma (St. Louis, MO, USA). The α-methyl-5-hydroxytryptamine (α-Me-5-HT), ketanserin, and spiperone were obtained from Tocris (Tocris Cookson Inc., Ballwin, MO, USA).

### 2.3. Dissection and physiology

The opener muscle in either the first or second walking legs, which is innervated by a single, purely-tonic excitatory motor neuron were used throughout these studies. The opener muscle was prepared by the standard dissection [9,21]. The tissue was pinned out in a Slygard dish for viewing with a Nikon, Optiphot-2 upright fluorescent microscope using a 40× (0.55 NA) Nikon, water-immersion objective. All dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205, NaCl; 5.3, KCl; 13.5, $CaCl_2.2H_2O$; 2.45, $MgCl_2.6H_2O$; 0.5, HEPES) adjusted to pH 7.4.

### 2.4. Excitatory postsynaptic potentials (EPSPs)

Intracellular recordings were performed with 30–60 MΩ 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 recorded at 10 kHz. All events were appropriately scaled to known values measured on an oscilloscope. The corrected scale was then adjusted with MacLab Scope software (version 3.5.4). A Grass S-88 stimulator and stimulus isolation unit (Grass), with leads to a standard suction electrode set-up, were used to stimulate the excitatory nerve in each preparation. The excitatory motor neuron was stimulated to induce short-term facilitation (STF) by giving a 40-Hz train of 10 pulses at intervals of 10 s [15,16].

A percent difference in the EPSP amplitudes was calculated in the following manner:

$$[|(\text{EPSP amplitude in saline}) - (\text{EPSP amplitude during exposure to compound})|/(\text{EPSP amplitude in saline})] \times 100 = \%\ \text{difference}.$$

A mean value was obtained over a 2000-s period for each trial, as depicted in Fig. 4A. This mean value was used for determining the average amplitude in each treatment within a trial for determining a % difference. The means and standard errors of the means were calculated from the % differences obtained from each preparation.

## 3. Results

This standard crayfish opener muscle preparation is depicted (Fig. 1) with representative excitatory postsynaptic potentials (EPSPs) in response to a 40-Hz stimulation train, consisting of 10 stimuli, before and after the exposure to 5-HT (100 nM). The 5th and 10th events (stars, Fig. 1) within the trains are used to index the influence of pharmacological agents on synaptic transmission.

5-HT exposure rapidly enhances the amplitudes of the EPSPs, and at the lower concentration the synaptic responses plateau within several minutes to a higher state of synaptic efficacy (Fig. 2A). With subsequent addition of a much higher 5-HT concentration (1 μM) the amplitudes of the EPSPs again rapidly increase (Fig. 2A). These results demonstrate that the preparations do not appear to be desensitized to 5-HT and that they are responsive within the range of 100 nM to 1 μM. The prolonged effectiveness of 5-HT at these concentrations suggests that there is no desensitization during the continued presence of the amine. The lobster opener muscle preparation also did not show desensitization to 5-HT in relation to muscle tension [52]. In addition, these responses serve as controls in examining the influence of various 5-HT agonists. Since there is variation in the initial amplitudes of the EPSP among preparations when only exposed to saline, the absolute changes observed during exposure of 5-HT are not practical for comparisons. Thus, a percent difference in the amplitude of the EPSPs was determined within each preparation relative to basal conditions during the initial saline exposure. As shown in Fig. 2B there is a significant increase ($n=5$, $P<0.05$ Wilcoxon rank sum test) in the amplitude of the 10th EPSP in response to both concentrations of 5-HT. In these experiments, the higher concentration was always proceeded by exposure to the lower concentration. The average in the plateau or an average of 20 values centered at a maximum change in the EPSP amplitudes were used to determine the mean response for comparisons within preparations.

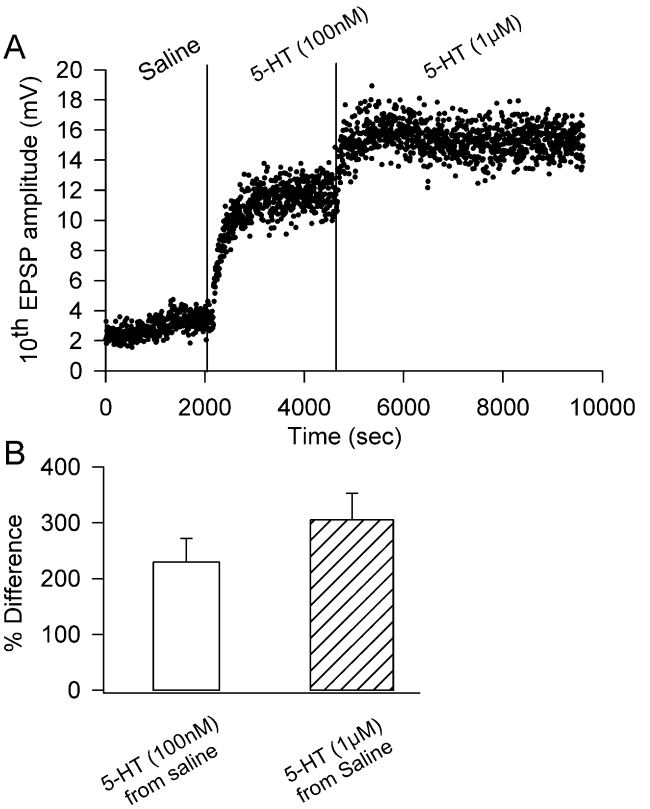


Fig. 2. Exposure to 5-HT results in a rapid increase in the amplitude of the EPSPs. (A) For later analysis of agonist and antagonist it was necessary to examine the influence of 100 nM 5-HT followed by 1 μM 5-HT. Note that after several minutes a plateau in the response is obtained with 100 nM before subsequent addition of the higher concentration. (B) In all cases there is a significant increase in the percent difference in the EPSP amplitudes with 100 nM and for 1 μM 5-HT ($n=5$, $P<0.05$ Wilcoxon rank sum test). The percent difference is measured relative to an average amplitude observed during saline exposure.

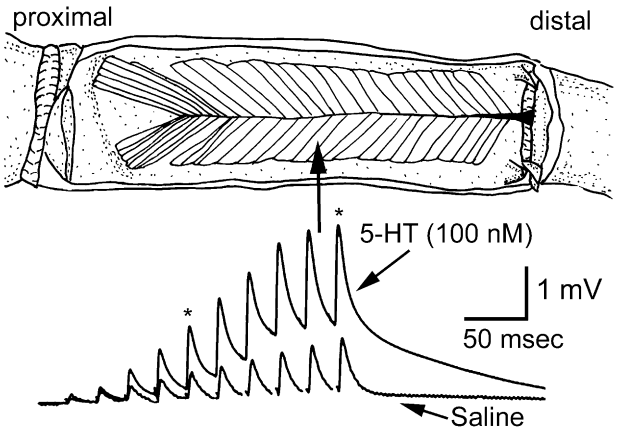


Fig. 1. A schematic of the opener muscle in the walking leg as seen from a ventral view with representative EPSPs responses to a train of 10 stimulation pulses given at 40 Hz before and after application of 5-HT (100 nM). It is readily shown that each of the EPSPs within the train are enhanced by the exposure to 5-HT. The 5th and 10th events (marked by stars) within the train of EPSPs were used as measures of responsiveness to the various pharmacological agents.

To examine if a 5-$HT_2$ receptor subtype is responsible for the effects detected in this preparation, selective 5-$HT_2$ antagonists were used to determine if they could partially block the responses normally initiated by 5-HT. Previous studies suggest that a vertebrate equivalent of the 5-$HT_2$ receptors is responsible for the enhanced evoked synaptic transmission [18]. The activation of cellular phosphatidyl

inositide turnover is associated with 5-$HT_2$ receptors in vertebrate subtypes [2]. In addition the antagonists ketanserin and spiperone have the greatest affinity ($pK_i$) for the vertebrate 5-$HT_{2A}$ subtype as compared to the 5-$HT_{2B}$ and 5-$HT_{2C}$ subtypes (see review in Ref. [2]). Given that it is not known what 5-HT receptor subtypes exist in the crayfish, we started with the most likely candidates based on cellular cascades to 5-HT exposure [18] and physiological responses [44]. Exposure of the NMJ to ketanserin or spiperone did not have any adverse effects on evoked synaptic transmission (Figs. 3A and 4A), and both agents reduced the influence of 5-HT at low and high concentrations. This antagonistic action is readily evident when comparing Figs. 2B with 3B and 4B. The effects of these antagonists and mixed cocktails of antagonist with 5-HT were consistent between preparations. However, the responses of individual preparations varied, so percent changes were again used for quantitative purposes. In each paradigm, the antagonist reduced significantly the response to 5-HT ($n=5$ for each agent and for each 5-HT concentration, $P<0.05$ Wilcoxon rank sum test).

The broad 5-$HT_2$ receptor agonist, α-methyl-5-hydroxytryptamine (α-Me-5-HT) enhanced synaptic transmission in a concentration-dependent manner but was not as potent as the native 5-HT at each concentration ($n=5$ for each concentration, $P<0.05$ Wilcoxon rank sum). Exposure to 100 nM α-Me-5-HT slightly increased the EPSP amplitudes (Fig. 5A), whereas the higher concentration of 1

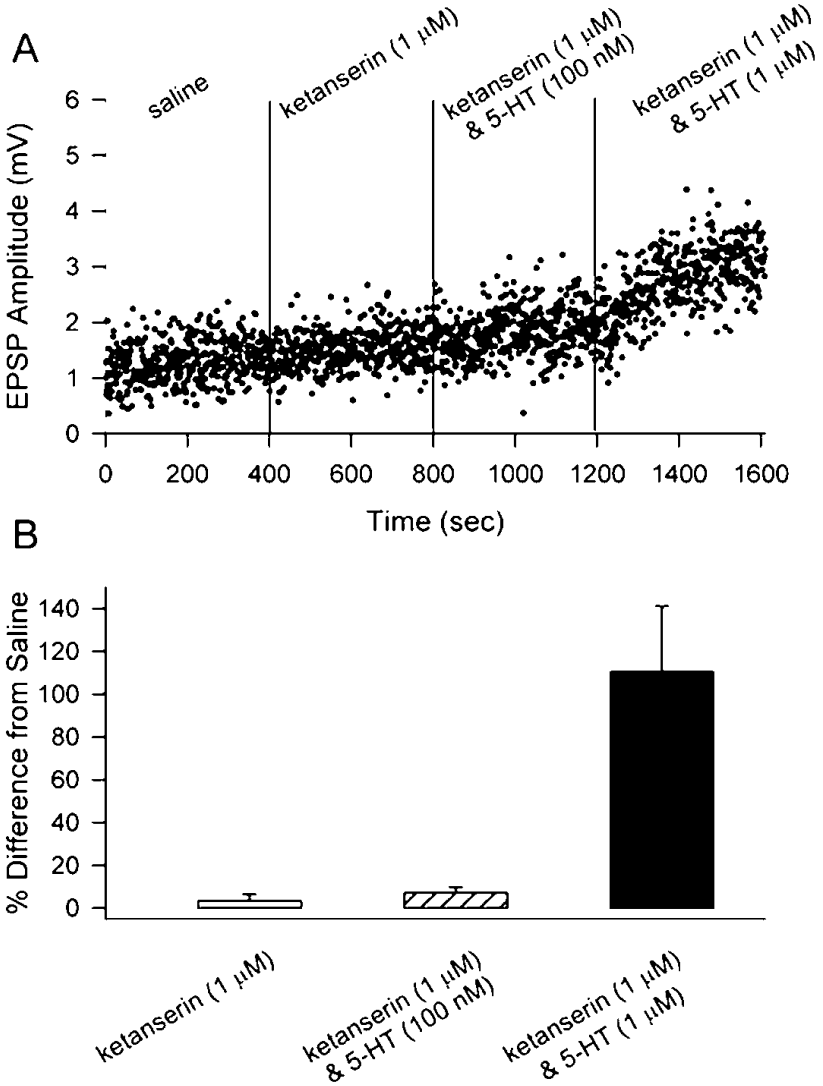


Fig. 3. Application of the 5-$HT_2$ receptor antagonist, ketanserin, had no effect on the synaptic transmission, but did decrease the responsiveness to 5-HT. (A) The representative responses in the amplitudes of the 10th EPSPs, within a 40 Hz train of 10 events, are illustrated throughout the exposure of ketanserin (1 μM), followed by ketanserin (1 μM) and 5-HT (100 nM). Note the normal responsiveness to 5-HT is blocked. A subsequent application of ketanserin (1 μM) and a higher concentration of 5-HT (1 μM) resulted in an increase in the amplitudes. (B) In all cases the same trends were observed as indicated ($n=5$, $P<0.05$ Wilcoxon rank sum test). The percent difference is measured relative to an average amplitude observed during saline exposure.

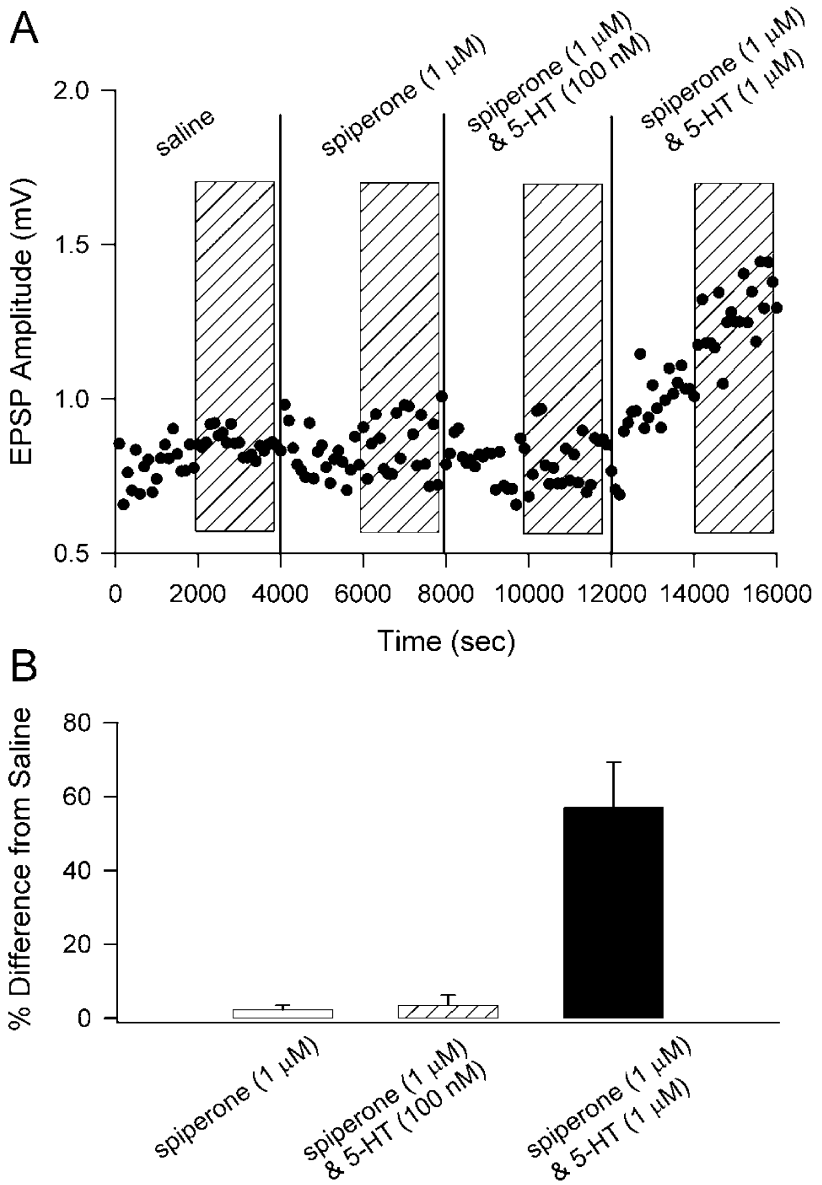


Fig. 4. Following the same protocol as for the 5-$HT_2$ receptor antagonist, ketanserin, another 5-$HT_2$ antagonist, spiperone, was used. Spiperone also had no significant effect on evoked synaptic transmission, but did decrease the responsiveness to 5-HT as observed for ketanserin. (A) The representative responses in the amplitude of the 10th EPSPs, within a 40-Hz train of 10 events, are illustrated throughout the application of spiperone (1 μM), followed by spiperone (1 μM) and 5-HT (100 nM). Note as with ketanserin the normal responsiveness to 5-HT is blocked. A subsequent application of spiperone (1 μM) along with a higher concentration of 5-HT (1 μM) resulted in an increase in the amplitudes. In this representative response every 10 events were averaged. The hatched boxes represent the period in which a mean value was obtained for comparisons among treatments. (B) In all cases the same trends were observed as indicated ($n=5$, $P<0.05$ Wilcoxon rank sum test). The percent difference is measured relative to an average amplitude observed during saline exposure.

μM (Fig. 5B) produced a larger response (Fig. $5B_2$). The application of α-Me-5-HT combined with 100 nM 5-HT produced a further enhancement of synaptic transmission but only when the preparation was exposed to the lower concentration of α-Me-5-HT (most right bar of Fig. $5B_1$ and $B_2$). As with the earlier paradigms the percent differences with respect to saline exposure are used for comparisons.

## 4. Discussion

In this study we have shown that the crayfish opener NMJ is responsive to exogenous application of 5-HT and that a selective agonist and two antagonists of the vertebrate 5-$HT_2$ receptor subtypes are functional in this invertebrate preparation. These results support the notion that a 5-$HT_2$-like receptor subtype is present presynaptical-

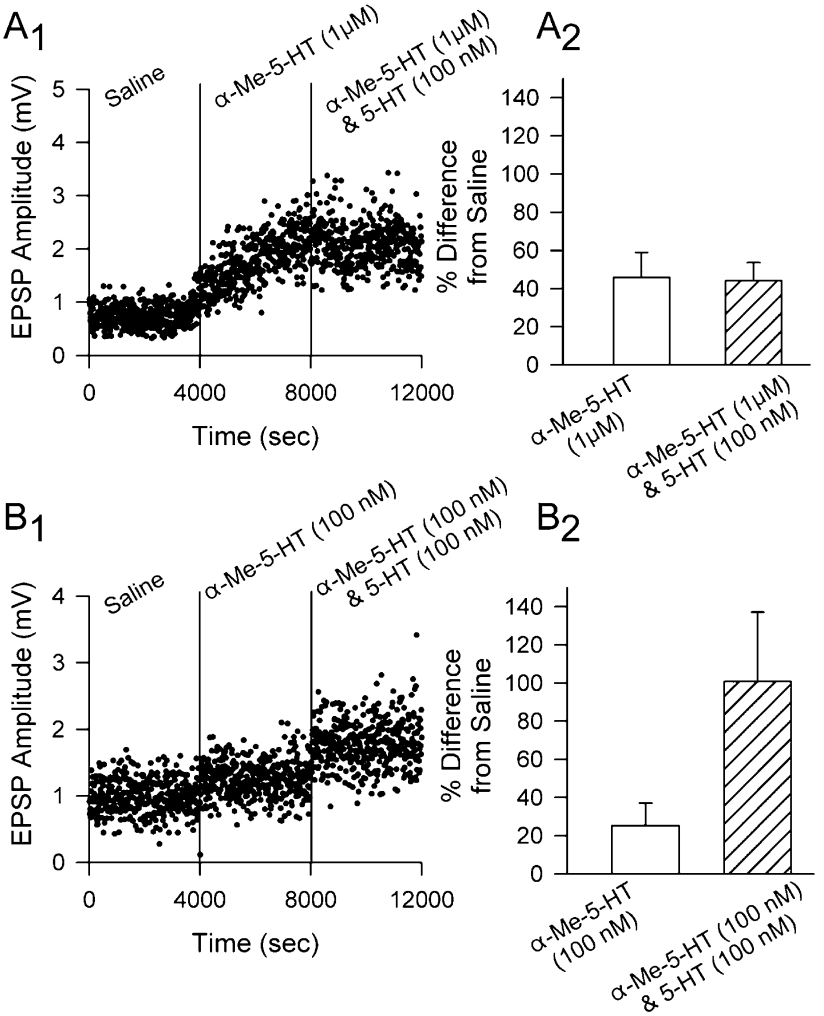


Fig. 5. The broad 5-$HT_2$ receptor agonist, α-methyl-5-hydroxytryptamine (α-Me-5-HT) enhances synaptic transmission at the crayfish NMJ. Representative responses are shown for the amplitudes of 10th EPSPs within a 40-Hz stimulation train of 10 events for a high, 1 μM, ($A_1$) and a low, 100 nM, ($B_1$) concentration of agonist. Similar trends were observed in all preparations for both the high ($A_2$) and low ($B_2$) agonist concentrations ($n=5$, $P<0.05$ Wilcoxon rank sum test). Addition of 5-HT in the presence of the low concentration of agonist further enhanced transmission, which was not always the case when previously exposed to the higher agonist concentration. The percent difference is measured relative to an average amplitude observed during saline exposure.

ly on the crayfish motor nerve terminals. Previous evidence that injecting a blocking agent of the intracellular phosphatidyl inositide cascades caused a reduction in the effects of 5-HT [18] concurs with the present findings.

To further divide the potential 5-$HT_2$ receptor subtypes in this system, as in vertebrate models, cloning of the receptors is needed for better comparative analysis and functional studies within the crayfish preparations. From the reports in a recent review of 5-HT receptors in invertebrates (*Drosophila*, mollusks and nematodes) there appear to be many similarities to the vertebrate receptor subtypes based on pharmacology, but also the need is stressed for molecular cloning of the receptors in order to provide a more precise classification scheme [47]. Our preliminary pharmacological assessment at crustacean neuromuscular junction also agrees with the vertebrate pharmacological classification. However, the 5-$HT_2$ agonist did not mimic to the same extent the actions of 5-HT for a given concentration which suggests that either multiple 5-HT receptor subtypes are present or that α-Me-5-HT is not as potent as 5-HT for the 5-$HT_2$-like receptors at the crayfish NMJ. Since the effects of the antagonists could be overridden by higher concentrations of 5-HT they may function as a competitive-antagonist and/or all the receptors were not blocked by the concentrations of antagonists used. Further studies in pharmacological binding assays are needed to resolve such issues. In the lobster, the monoamine pharmacology of agonist and antagonist did not correspond to the receptor specificities as defined in vertebrates [4].

In studies monitoring the synaptic currents produced by individual vesicular events and subsequent quantal analysis before and after 5-HT exposure, it is apparent that 5-HT increases the probability of a vesicular event to occur

during an evoked stimulation in tonic motor nerve terminals in the opener muscle [44], the superficial flexor muscle [45] and the tonic and phasic terminals of the leg extensor muscle [42]. Considering the results of this study and those of Dixon and Atwood [18] it is likely that the mechanisms of action of 5-HT within the nerve terminal are via an $IP_3$ cascade which would lead to the activation of various protein kinases. As suggested in Southard et al. [44], 5-HT could induce the phosphorylation of molecules such as Munc-18-1 or synaptophysin or even NSF. These scenarios are possible considering that phosphorylation of the first two proteins has been shown, in vitro, to inhibit their ability to interact with the SNARE proteins, and that they may regulate the interaction of syntaxin for Munc-18-1 and synaptobrevin for synaptophysin (see Ref. [44]). Other proposed mechanisms of action are that 5-HT may lead to phosphorylation of synapsins to increase the pool of free vesicles for docking or possibly actions on calcium influx and release from internal stores [10]. More detailed studies are currently underway to elucidate these potential mechanisms with injections of $IP_3$ analogs into the motor nerve terminals and altering calcium dynamics within the terminals before and after exposure to exogenous 5-HT [13].

As in the CNS of vertebrates, the expression of 5-HT receptors on the crayfish motor nerve terminals might be altered in response to receptor activity or neuronal function. For example it is known in vertebrates that a down-regulation of $5\text{-HT}_{2c}$ receptors is induced by an increase in whole animal activity, which also enhances 5-HT release [5]. In crayfish, if the neural activity of phasic motor neurons is enhanced by electrical conditioning of the neurons, the net synaptic transmission is reduced, but the terminals show a greater sensitivity to 5-HT neuromodulation [28]. In a very recent study [6], in which a pharmacological agent was used to reduce endogenous 5-HT production there was a tremendous increase in the sensitivity to the application of 5-HT, while on the other hand chronic applications of a $5\text{-HT}_2$ receptor agonist/antagonist (*m*-CPP) produced a decrease in responsiveness to exogenous application of 5-HT. On going studies in our lab suggest that *m*-CPP may also be inhibiting synaptic transmission by yet an unknown mechanism. The compound *m*-CPP in rats acts as a $5\text{-HT}_1$ and $5\text{-HT}_2$ receptor agonist and can down-regulate the number of receptors [26]. Also, *m*-CPP acts as an antagonist for cloned human $5\text{-HT}_{2B}$ receptors [46]. The precise mechanism of action in regulation of the 5-HT receptors in other systems has not yet been elucidated.

It is established in the vertebrates that agonists or antagonists being bound to a receptor can alter the levels of functional receptors [26,31,35,48] and that there can be selectivity in the alteration based on the receptor subtype [26,30,54]. So as in the vertebrates, the 5-HT receptors in crayfish may undergo up- and down-regulation which might be controlled by alteration of their expression levels which results in differences in the densities on the cell surfaces [1]. Thus, it is necessary to understand which types of 5-HT receptors are present within this system to understand the mechanism of 5-HT action, the regulation of receptor function in relation to neuromodulation of synaptic function, and the role of neuromodulators in behavior.

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