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Review Article

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Serotonin and Synaptic Transmission at Invertebrate Neuromuscular Junctions

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The serotonergic system in vertebrates and invertebrates has been a focus for over 50 years and will likely continue in the future. Recently, genomic analysis and discovery of alternative splicing and differential expression in tissues have increased the knowledge of serotonin (5-HT) receptor types. Comparative studies can provide useful insights to the wide variety of mechanistic actions of 5-HT responsible for behaviors regulated or modified by 5-HT. To determine cellular responses and influences on neural systems as well as the efferent control of behaviors by the motor units, preparations amenable to detailed studies of synapses are beneficial as working models. The invertebrate neuromuscular junctions (NMJs) offer some unique advantages for such investigations; action of 5-HT at crustacean NMJs has been widely studied, and leech and *Aplysia* continue to be key organisms. However, there are few studies in insects likely due to the focus in modulation within the CNS and lack of evidence of substantial action of 5-HT at the *Drosophila* NMJs. There are only a few reports in gastropods and annelids as well as other invertebrates. In this review we highlight some of the key findings of 5-HT actions and receptor types associated at NMJs in a variety of invertebrate preparations in hopes that future studies will build on this knowledge base.

Key words: synapse, reserve pool, readily releasable pool

GENERAL BACKGROUND OF 5-HYDROXYTRYPTAMINE AND RECEPTORS

5-Hydroxytryptamine (5-HT, serotonin) is a common biogenic amine found in both vertebrates and invertebrates as well as in plants [1, 2]. The precursor to 5-HT, tryptophan, is likey important in the early evolution of life and perhaps the early presence of tryptophan is a reason for 5-HT to be potentially the first neurotransmitter noted with the development of a nervous system [2]. 5-HT acts as both a neurotransmitter and neurohormone and as a potent modulator of neurons and various tissues in many animal species [3]. Generally 5-HT actions are elicited by transmembrane G protein coupled receptors (GPCRs), which then activate or inhibit different intracellular second messenger cascades. 5-HT receptors from some organisms have been classified based on sequence or pharmacology [4, 5]; for example in the vertebrates, 7 families (5-HT_{1-7}), 14 subtypes have been identified, whereas in *Drosophila* four 5-HT receptors named 5-HT_{1Adro} 5-HT_{1Bdro} 5-HT_{2dro} 5-HT_{7dro} [5-10] have been classified. 5-HT receptors appear to be present on invertebrate presynaptic nerve terminals and on muscle membranes; receptors of a cricket (*Gryllus domestica*) mandibular muscle have a similar pharmacological profile as a 5-HT_2- like receptor subtype [11]. Profiling the 5-HT receptor subtypes directly on skeletal muscle within invertebrates is an area of research that is lacking. The 5-HT_4 and 5-HT_7 receptors are shown to have alternate splice variants which increase the number of receptor subtypes and may

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alter the selectivity to pharmacological agents [12]. In addition, 5-HT_2 receptors can have different RNA-edited isoforms [13, 14].

With the use of the genetically modifiable model *D. melanogaster*, a number of studies have examined over-expression and under-expression of receptors subtypes on the effects of development, behavior and physiology as well as the general actions of 5-HT in *D. melanogaster* [7, 15-19]. Based on physiological and pharmacological studies in crustaceans there may be a larger number of 5-HT receptors present than in *D. melanogaster* [20-26]. Two receptors types have been cloned and characterized in crustaceans [9, 27, 28] and in a pond snail [29]. A 5-HT receptor 5-HT(apAC1) has been cloned, sequenced and characterized in *Aplysia* sensory neurons [30]. 5-HT receptors are being cloned in a variety of invertebrates and surely more will be forth coming with the rapid development in genomic sequencing abilities. There are a plethora of reports on the effects of 5-HT for sensory and central neurons as well as on behaviors in invertebrates which are worthy of multiple reviews. However, for this brief review we focus on the physiological effects of 5-HT at the skeletal neuromuscular junctions in some of the key model invertebrates. The invertebrate neuromuscular junctions (NMJs) are very diverse across species and within species in structure and function [31-37]. The recent majority of reports on structure and function of NMJs are of *D. melanogaster* due to the genetic approaches and manipulations being utilized [38-41].

WHY FOCUS ON NMJS?

The synaptic communication between neurons and target cells depends on the specialized anatomy and physiology of the synapses [42]. The regulation and modulation of neurotransmitter release is the basis of chemical synaptic transmission. For nervous systems to function properly, the efficacy of synapses are finely regulated and adjustable to respond to changing circumstance and requirement. Too high or too low synaptic input both result in inappropriate communication of target cells. Both pre- and postsynaptic factors can influence the synaptic strength. The amount of neurotransmitter released and the sensitivity of the postsynaptic membrane both are important for measuring synaptic strength. Each step in the process of synaptic transmission can be the target of many factors that lead to alteration of synaptic strength. For example, the phosphorylation state of SNARE proteins that are involved in vesicle docking, or the density of active zones where transmitter is released, can influence the number of quantal units released per impulse (presynaptic mechanism). Postsynaptically, the number of active receptors, the postsynaptic input resistance, the area and the ultrastructure of subsynaptic reticulum, all can alter the effectiveness of quantum release and thus influence synaptic strength.

The ease in accessibility to the synaptic sites at NMJs allows one to record intracellular or very close to synapses by extracellular recordings (focal macropatch over a varicosity) in order to minimize cable properties in signal decrement [43-45]. Such signal loss occurs with recordings in a neuron cell body to measure synaptic function in the dendritic trees. The localized recording over a NMJ allows one the ability to measure properties of single and multiple vesicular quanta for very precise quantal analysis (occurrences, size and shape) to index synaptic function [46-49]. In addition, invertebrate NMJs are relatively stable for hours in a minimal saline at room temperature as compared to mammalian NMJs. Since most muscles in invertebrates are innervated by relatively few motor neurons, for the most part, they are identifiable anatomically and physiologically from preparation to preparation [50, 51]. Since the fine structure and detailed quantal analysis is feasible for many invertebrate NMJs, the acute and chronic actions of modulators on structure and function can be examined for their mechanistic actions [52-54].

INSECTS

Given such a diverse group of animals within the class Insecta, it would not be surprising to find a wide range of anatomic and physiologic profiles in the innervation of skeletal NMJs. For example, the innervation of the genital chamber of the female cricket, *Acheta domestica*, shows 5-HT-immunoreactive nerve terminals that contact the muscle fibers which likely releases 5-HT in a type of volume transmission over the muscle as there are no defined synapses [55]. Such 5-HT containing nerve endings are also present in earthworm skeletal muscles [56]. However, no serotonin is associated with the oviducts or the innervation to the oviducts in the locust [57]. Earlier studies did not elucidate if the effect of 5-HT was directly on the presynaptic terminal or on the muscle but reported overall changes in force of muscle contraction. In a locust leg muscle, 5-HT produces an overall decrease in force development [58] but the mechanism of action still needs to be determined. It is suggested that in some of the earlier studies with insects, the high concentrations of 5-HT used may indeed block synaptic transmission by impeding the postsynaptic receptors [36, 58].

Despite the intense investigations in synaptic structure and plasticity in *D. melanogaster* related to genetic and mutational manipulations, there are few reports on the modulation of synaptic efficacy by peptides or modulators at the skeletal NMJ [54, 59-64]. As for the influence of 5-HT at the NMJ, the scantiness

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of studies is likely due to the mild effects observed by using 5-HT itself as well as pharmacological agonists/antagonists of 5-HT receptors. However, application of 5-HT to the intact larval CNS does enhance the drive of motor neurons (MN) [17]. The most commonly studied *Drosophila* neuromuscular junctions are those in the most prominent ventral longitudinal abdominal muscle fiber muscles 6 and 7 [65], which have the simplest innervation pattern among the *Drosophila* body wall muscles. Both electrophysiological and morphological studies imply that each of these two muscles is innervated by only 2 axons [66, 67]. Application of 5-HT to these NMJs appears to slightly depress synaptic strength [68,69]. We are not aware of any attempt to investigate actions of 5-HT on adult skeletal NMJs. However, with the recent advent of designer receptors exclusively activated by designer drugs (DREAD) in motor neurons allows one to examine mechanisms of activating second messenger cascades as if receptors for modulators existed on presynaptic nerve terminals or on the muscles themselves [70, 71].

CRUSTACEANS

The NMJs in crustaceans offer many advantages for addressing mechanism of action in modulation of synaptic efficacy at NMJs, but crustaceans do fall short in being able to genetically modify the properties for investigations. Potentially approaches with RNAi might be practical to address more species-specific manipulations in synaptic function in a variety of crustaceans [72-75]. The same physiological and anatomical advantages of the *Drosophila* NMJs apply for the crustaceans, but in addition, the wide range in known diversity in synapses within crustaceans makes them attractable for comparative studies in commonalities of mechanisms in low- and high-output synapses or ones that facilitate or depress rapidly [31, 32, 76]. The parallels to vertebrate central synaptic physiology of phenomenon described at crustacean NMJs are likely one reason of continual interest to a wide variety of researchers investigating synaptic transmission. In addition, the historical contribution of crustaceans in synaptic physiology is unsurpassed [77-80]. The ability to combine direct structure and function in defined labeled synapses offers the ability to unravel synaptic structural complexity with function [31, 32, 43-45, 81].

It was demonstrated as early as 1954 that 5-HT enhances synaptic transmission at the crustacean NMJs [82, 83] and that the effect was likely a presynaptic enhancement of mean quantal content came afterwards [84]. The 5-HT that modulates most crustacean skeletal NMJs does so through the exposure of hemolymph. 5-HT is released from nerve endings in thoracic roots and from the pericardial organs into the hemolymph [85]. Thus, 5-HT is accessible to all the exposed NMJs. The excitatory as well as inhibitory NMJs are enhanced in transmission by 5-HT [86, 87]. The quantal effects are explained by increased probability of vesicular fusion during evoked transmission likely caused by an increase in the number of vesicular vesicles being docked and possibility their sensitivity of fusing due to enhanced Ca^{2+} sensitivity or presence of free Ca^{2+} within the terminals [88]. However, several studies have shown that a presynaptic rise in free Ca^{2+} is not substantial enough to account as a primary mechanism of 5-HT's action [86, 89-91]. Since there is a steep rise in sensitivity to Ca^{2+} for enhancing synaptic efficacy at crustacean NMJs [92] a low release from internal stores may account well enough for part of the effect [20]. This notion of an internal release of Ca^{2+} is also supported by experiments conducted by Glusman and Kravitz [91] in which they showed that a calcium-free bath, along with EGTA and high $MgCl_2$, 5-HT could still cause spontaneous release of transmitter for lobster NMJs. The enhanced spontaneous and evoked fusion events relates to an increase in 'n' (number of sites) and 'p' (probability of release) to explain the enhanced 'm' (mean quantal content; m=np) after exposure to 5-HT [69, 93, 94]. An interesting observation, but not yet explained mechanistically, is that 5-HT produced an effect with low or zero extracellular calcium at a crayfish NMJ but 5-HT's effect depended on extracellular sodium concentration [89].

Low- and high-output NMJs in crayfish and crab show differential responses to 5-HT [95-97]. This could be due to the larger reserve pool of vesicles in tonic (low-output) terminals than the phasic (high-output) terminals and the fact that higher-output synapses in crustaceans have more complex synapses containing more active zones in close apposition on synapses than lower output synapses [45, 98-100].

NMJs investigated in lobster and crab revealed similar findings to those of the crayfish. 5-HT also enhances both excitatory and inhibitor NMJs that have been examined in *Homarus americanus* (lobster) [101, 102]. 5-HT also promotes the force of nerve-evoked contractions of the gastric mill muscle of the crab, *Cancer borealis* [103].

The differential responses and cellular mechanism of 5-HT's action at crustacean NMJs is likely accounted for by the density and receptor subtypes on the presynaptic terminals. Vertebrate $5\text{-}HT_2$-like receptors were physiologically identified for *Procambarus clarkii* at NMJs [21-24, 69]. Since this subtype of receptor has been sequenced in a crab and crayfish [28] these may be the subtypes present at the NMJs; however the blockers for the vertebrate $5\text{-}HT_2$-like receptors could not block the entire 5-HT enhancement of synaptic enhancement [24]. Also $5\text{-}HT_2$ agonists did not mimic the responses fully at the crayfish NMJ [24], so

potential affinity in binding 5-HT and pharmacological agents differ in crustaceans to vertebrate subtype receptor analogs. The pharmacology of monoamines in the cardiac ganglion of lobsters also does not mimic vertebrate classifications [104]. Care needs to be taken in assuming the pharmacology of mammalian 5-HT receptors applies to invertebrates [22].

Given there is at least some pharmacological and sequence similarity to vertebrate 5-HT_2 receptor subtype present in crayfish and that injection of an IP3 analog (adenophostin-A) in the presynaptic motor nerve terminals enhances release [20], a potential mechanism is that 5-HT receptors on the presynaptic membrane mediate activation of G coupled receptors which leads to activation of phospholipase C (PLC) which in turn produces 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [105]. The production of IP3 can directly result in Ca^{2+} release from internal stores (i.e., ER) through IP3 receptors on the ER [106].

Since vertebrate 5-HT_2 receptor family activates phospholipase C (PLC) [9] a similar receptor activated cascade is possible at the crayfish NMJs, Such mechanisms are established in other systems [105, 107, 108] and given that caffeine and ryanodine actions are in concurrence with IP3 receptors potentially on the ER in crayfish presynaptic motor nerve terminals [20] we have to consider this mechanism as a likely possibility. The rise is Ca^{2+}, even a slight rise, could activate calmodulin and in turn activate CaM-Kinase (CaM-K), which can lead to phosphorylation of proteins such as synapsin. The possibility is that vesicles would then be able to leave the tethers to the cytoskeleton and dock to the presynaptic membrane, which is also a phosphorylation step [109-111]. The increased docked vesicles could be subjected to the calcium influx and release from internal stores [112]. This would account for the increase in the occurrence of spontaneous quantal events and enhanced evoked responses with 5-HT exposure. In the invertebrate *Aplysia*, it was shown that exposure of neurons to 5-HT results in phosphorylation of synapsins [113]. cAMP was also suggested to be involved in 5-HT action [30, 114-116). cAMP has been shown to activate Protein Kinase A (PKA) which then can lead to phosphorylation of transcriptional factors such as CREB. Such action can regulate synthesis of proteins used in synaptic transmission [117-119]. It has also been suggested that the cAMP and calmodulin pathways may work together and promote transcription [120]. When phosphatases are inhibited at the crayfish NMJ the effect of 5-HT is enhanced, thus demonstrating the significance of phosphorylation [121] which is known to occur with exposure to 5-HT at crustacean NMJs [122].

In a recent study addressing the potential mechanisms of 5-HT, as well as stimulation of the motor nerve terminal, in recruiting vesicles from a reserve pool (RP) to a readily releasable pool (RRP) within the presynaptic nerve terminals of crayfish NMJs, we developed a model to account for the observations and previous reports. In a current study, we inhibited the packaging of glutamate by blocking the vesicular glutamate transporter (VGlut) with the drug bafilomycin A1 (BA) [123-125]. In this way, the rapidly recycling vesicles within the RRP will be empty with repetitive stimulation. However, if the RP is spared from being recruited by low frequency stimulation and if they are already packaged with transmitter, prior to exposure to BA, then 5-HT should be able to recruit these RP vesicles to the RRP and synaptic transmission restored temporarily. This is exactly what was observed indicating that the RP and RRP can be physiologically differentiated into distinct functional groups and that 5-HT recruits the RP into action [126, 127]. To deplete or use up the packaged RRP vesicles, continuous stimulation was provided since the opener NMJ preparation is low-output and fatigue resistant. A high frequency of 40 Hz was used for comparative purposes to 20 Hz continuous stimulation. As expected, preparations stimulated at 40 Hz depressed faster than the ones stimulated at 20 Hz and there was a reduced effect for the 40 Hz stimulated preparations to exposure of 5-HT. This suggests that a higher stimulation frequency is able to recruit some of the RP to the RRP. This is illustrated in a model (Fig. 1). To address if PLC is an intermediate step within the cascade of events activated by 5-HT mediated responses, we used a PLC inhibitor (U73122) and an inactive analog (U73343) to serve as a negative control [128]. We found that the treatment of U73122 caused a significant decrease of 5-HT effect on synaptic transmission. This result confirmed the involvement of PLC signaling cascade in inducing the enhancement of synaptic transmission by 5-HT at a different physiological condition. There are observations in other preparations that indicate the presence of two distinct vesicle pools: RRP and RP. In the cat superior sympathetic ganglion, Prado et al. [129] separated the two pools by electrically stimulating the nerve to deplete the RRP of acetylcholine, and then recruit RP vesicles by tityustoxin. Using FM 1-43 dye, the two pools have been identified in a temperature-sensitive mutant *Drosophila* line, *shibire*, and later in WT [130, 131]. However in our study with the crayfish NMJ, a novel approach with bafilomycin A1 was used together with continuous stimulation to deplete the RRP, and then 5-HT was applied to recruit RP vesicles and the recruitment involves a PLC signaling cascade. A mechanistic illustration is detailed in Fig. 1.

There does not appear to be a substantial direct effect on crustacean skeletal muscle to account for an increase in EPSP or IPSP amplitude due to an increase in input resistance of the fibers [82, 114, 132, 133]. A small increase in input resistance, by exposure to 5-HT, accounts for a slight increase in the EPSP

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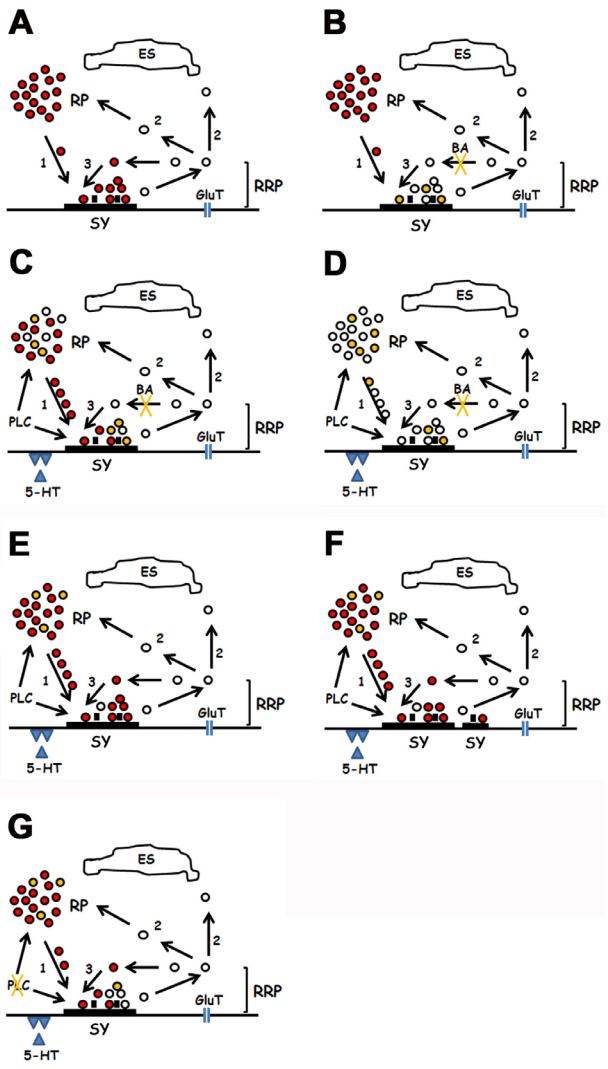

Fig. 1. Schematic illustration of 5-HT in recruiting vesicles from a reserve pool. (A) Two vesicle recycling pathways have been proposed. In a resting synapse, vesicles in RP slowly join in to the RRP (1), and then recycle back to RP either through or bypass endosome (2). This is called slow recycling loop. However, in an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP (3) which is named quick recycling loop. Recycling vesicles are refilled with glutamate to be able to participate in the coming synaptic activities. (B) In an active synapse treated with Bafilomycin A1, vesicles in RRP can be used up in time with stimulation because recycling vesicles can no longer be refilled. Synaptic depression occurs sooner than the one without Bafilomycin A1 treatment. (C) If 5-HT is added after depression, 5-HT possibly activates PLC signaling cascade and recruits RP vesicles to revitalize the synaptic transmission in a fast manner. (D) In time, synaptic depression occurs again because most RRP and RP vesicles are empty. Yellow colored vesicles represent partially full of glutamate. (E) Even when RRP vesicles are not depleted by Bafilomycin A1, 5-HT can also recruit RP vesicles into RRP via one possible mechanism (PLC). (F) It is also possible that 5-HT can activate silence synapse most likely in low-output terminals. (G) The PLC activation of 5-HT effect is confirmed with PLC non-selective inhibitor. RP, reserved pool; RRP, readily releasable pool; SY, synapse; GluT, glutamate transporter; BA, Bafilomycin A1.

amplitude for superficial flexor muscle fibers of crayfish [94]. More substantial alteration in input resistance can occur in crustacean neurons due to 5-HT exposure [134] so there could be some effect on the presynaptic motor nerve terminals.

In comparison to the smooth muscle in the intestine of vertebrates, the muscles of the crayfish hindgut are striated with gap junctions and generate intrinsic pacemaker activity [135, 136]. Application of 5-HT [137] and octopamine [138] to GI tract increases the frequency and strength of contractions. 5-HT and dopamine are highly concentrated in CNS and GI tract and they are directly responsible for the peristalsis and muscle contraction [137, 139].

ANNELIDS

The leech has served as a model organism in neurobiology for many years [140] but few studies have directly focused attention at NMJs in the leech and even fewer on the effects of 5-HT in synaptic transmission at NMJs. However, studies have examined the effect of 5-HT on the drive of motor neurons and innervation patterns [141-147]. 5-HT exposure has a relaxing effect on skeletal muscle in the leech [148] and enhances muscle force and work production during locomotion and feeding [149]. This is physiological relevant since Retzius neurons do directly innervate skeletal muscle in the leech and these cells do release 5-HT [144, 150-152]. In the earthworm and polychaete (*Sabellastarte magnifica*) muscle contraction is reduced by 5-HT [153, 154] which lead to the idea that 5-HT might be acting as inhibitory transmitter in these preparations [155].

GASTROPODS

A few studies with gastropods have been approached for the direct effect of 5-HT at the NMJ. 5-HT produces facilitation for an evoked response in buccal muscle within *Aplysia* [156]. The presynaptic actions of 5-HT is to enhance transmitter release [157]. Like for some of the actions in annelids, 5-HT can also produce muscle relaxation and reduce force in *Aplysia* [158]. Such effects on muscle contraction and force maybe dependent on 5-HT concentration and the species studied, since in *Aplysia brasiliana* 5-HT increases a Ca^{2+} influx that promotes muscle contraction used for swimming [159].

OTHER INVERTEBRATES

In a sea urchin (*Parechinus*), 5-HT apparently had no effect at the NMJ [160]. However in a sea cucumber (*Apostichopus*

japonicas), 5-HT inhibited evoked contractions induced by acetylcholine and there appears to be 5-HT innervation directly to muscles of the body wall [161].

SUMMARY

Although headway has been made in describing the various actions of 5-HT at NMJs in invertebrates, the cellular mechanisms of these actions are still lacking. Additional pharmacological and molecular profiling in a variety of invertebrate preparations will increase our knowledge of both the uniqueness and similarities among the invertebrates. As history has taught us in physiology, and in particular neurobiology, what is learned in invertebrate preparations paves the way to new views and mechanistic cellular understanding of complex processes within the vertebrates.

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