Manipulation of Crayfish (*Astacus astacus*) Aggression by Administration of 5-Hydroxytryptophan and Perfluoro-5-Hydroxytryptophan

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Abstract

In a prior study using Florida orb-web weaving spiders (*Nephila clavipes*) as biological screens for possible hallucinogenicity of new imaging compounds, we observed what appeared to be elevated aggressiveness after oral administration of perfluoro-5-hydroxytryptophan (PF-5HTP). Huber and his collaborators have demonstrated that serotonin infused into the hemolymph of subordinate crayfish will decrease the likelihood they will withdraw from attacks by a dominant opponent. Subordinates renewed willingness to engage in combat appears to be mediated by uptake and subsequent release of 5-HT from serotonergic terminals in this species, as this behavior can be modified by the co-administration of a Prozac, which blocks the uptake of serotonin. Serotonin also produces distinct motor effects when administered to crayfish, exciting flexor muscles. We are currently developing perfluorinated compounds for magnetic resonance imaging studies. One of the many questions of interest to us in this line of inquiry is what happens *in vivo* when multiple fluorine atoms are attached to a neurotransmitter precursor such as 5-HTP. To be a feasible imaging agent, PF-5HTP must be taken up and concentrated in serotonergic neurons. The utility of the compound would be heightened if it converted *in vivo* into PF-5HTP. When we administered 5-hydroxytryptophan (5-HTP) and perfluorinated 5-hydroxytryptophan (PF-5HTP) to crayfish, they displayed behaviors similar to those induced in this species by administration of serotonin.

Introduction

In a prior study using *Nephila clavipes* as biological screens for potential hallucinogenicity of new imaging compounds, investigators observed elevated aggression after oral administration of perfluoro-5-hydroxytryptophan (PF-5HTP)[1] Elevated aggression in other invertebrate species, such as lobsters and crayfish, can be produced by administration of 5-hydroxytryptamine (5-HT), commonly known as serotonin.[2,7] The effect that serotonin produces on readily observable, overt behavior in crayfish makes it feasible to use behavioral analysis of this species as a rapid, inexpensive and sensitive biological screen for pharmacological effects of novel 5-HT agonists and antagonists.

The biosynthesis of serotonin begins with the amino acid L- tryptophan which is converted by the enzyme tryptophan hydroxylase into 5-hydroxytryptophan (5-HTP) as shown in *Figure 1*. The 5-HTP is then changed by Aromatic L-Amino Acid Decarboxylase (AACD) into 5-hydroxytryptamine (5-HT). Saturation of tryptophan hydroxylase is the rate limiting factor in this synthesis, and an increase in 5-HTP normally leads to a linear increase in 5-HT. Our collaborators have tagged the 5-HTP molecule with multiple fluorine atoms to produce a probe for magnetic resonance imaging. The tag, attached to the indole ring of PF-5-HTP molecule resonates in a magnetic field with sufficient signal-to-noise it could be detected in MRI if the probe bioaccumulates in the vesicles of serotonergic neurons. It would increase the utility of the novel compound if it is converted into perfluoro-tagged serotonin (PF-5HT) *in vivo*.

We used crayfish as a biological screen for the novel imaging compound because the effects of serotonin on their behavior is well characterized. Co-administration of fluoxetine with 5-HTP blocks these serotonergic effects on crayfish behavior, which provides strong evidence that when 5-HT is administered to crayfish, it is taken-up and released from their neurons to produce the resulting changes in behavior. Crayfish have an open circulatory system, and compounds injected diffuse through hemolymph, reaching the 120 serotonergic cells of this crustacean nervous system. Behavioral biology courses frequently use crayfish aggression in laboratory exercises because it is so well characterized,[8] making this a useful pharmacological model (*See Appendix A for details*).

We hypothesized that administering the precursor of serotonin, 5-HTP, to crayfish would mimic serotonergic effects. If pharmacological effects could be demonstrated by administering 5-HTP to crayfish, it would be possible to use this species to test the bioavailability of the novel probe PF-5-HTP. If an injection of PF-5-HTP mimics the *in vivo* effects of a comparable injection of 5-HTP, it would provide strong evidence that this novel imaging compound is taken up into neurons. An injection of phosphate-buffered saline solution (PBS) is not expected to change behavior. The C$_6$F$_5$ tag used in this study was covalently bound onto the aromatic ring of 5HTP as a heptafluorobutyryl. It is extremely unlikely that it could come off of the 5HTP molecule *in vivo* without subjecting the crayfish to lethal temperatures.

Methods

Housing

Medium sized crayfish were purchased from Carolina Biological Supply and housed in aquariums with pumps and filters upon arrival at Marist College. Tanks were maintained at room temperature. Tap water, pretreated to remove chlorine, was changed twice during the 11 week study. Up to 8 crayfish were housed in each 20 gallon tank, and 4 were housed in each 10 gallon tank. Tank dividers were used to decrease the number of crayfish sharing space, and broken 3-inch clay pots were provided as hiding places. Sex of crayfish was determined and individuals were marked with a colored code of three dots on their carapace. For the first 4 weeks after arrival, crayfish were allowed to habituate to their environment and tank mates. Live feeder goldfish were added after one week to simulate a more natural aquatic environment. Crayfish captured and ate these feeder fish and began molting. Several died during this process, apparently falling victim to their cannibalistic and opportunistic tank mates. Four pairs of crayfish used in this study were housed and cared for by honors biology students at Pine Bush High School.

Injections

Pairs of crayfish were observed by placing them together in a neutral "fight" tank to establish a dominance hierarchy between the pair prior to injections. The order of injections was counterbalanced so half of the pairs were randomly selected to receive phosphate-buffered saline (PBS) -> 5-hydroxytryptophan 5-HTP -> perfluoro-5-hydroxytryptophan PF-5-HTP. The other pairs were injected in the sequence PBS -> PF-5-HTP -> 5-HTP. To control for the effects of injections, dominant crayfish in each pair received a placebo injection of PBS at the same time that the subordinate was injected with compound dissolved in PBS. Injections of subsequent compounds were spaced at least 24 hours apart. As a control for potential pharmacological effects of trace amounts of 5-HTP in the PF-5HTP sample (which was 99 percent pure), we injected several crayfish with a low dose of tryptophan.

Prior to receiving injections, crayfish were placed in an icebath for 10 to 20 minutes to immobilize them. Upon removal from the icebath, the crayfish were immediately injected with a dose of 100 mg/kg of compounds 5-HTP or PF-5HTP in a vehicle of 100 µl of PBS. Fluid was injected into crayfish using a 1/2 cc. 30-gauge micro-fine insulin syringe. The site of the injection was located ventrally and slightly off centerline (to avoid the ventral nerve cord) at the junction of the cephalothorax and the abdomen as shown in *Illustration 2*. This site was empirically selected after we injected crayfish with a green food coloring. It survived, but visibly changed color. The crayfish were placed in isolation tanks for 10 minutes before being placed together into the fight tank for observation. Both the dominant and subordinate crayfish were observed for 40 minutes. Trained observers recorded ethograms on data sheets marked in 10 second intervals. The ethogram key is described in *Appendix A*.

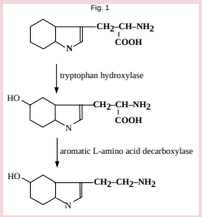
Results

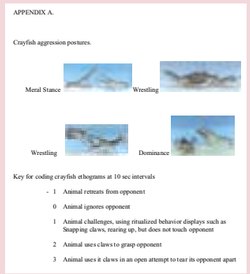
Cumulative aggression indices were plotted for each crayfish for the 40 minute post-injection observation period. Representative plots are shown in graphs of Encounters 1-4. We found that injecting 5-HTP or PF-5HTP made subordinates less likely to retreat and more likely to face their aggressor. When injected with either PBS or low doses of tryptophan, subordinates retreated from dominant crayfish. Dominant crayfish also became more aggressive when injected with 5-HTP or PF-5HTP. This increase in aggression was observed several minutes after the injection and soon subsided.

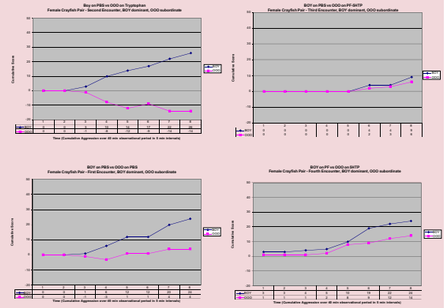
Pair BOY vs OOO

Female crayfish Blue-Orange-Yellow (BOY) and female crayfish Orange-Orange-Orange (OOO) were injected with PBS for their first encounter shown in Graph 1. BOY emerged as the dominant crayfish of the pair in this first encounter. For the second encounter, OOO was injected with a low dose of tryptophan. Her negative cumulative score demonstrates her retreat to avoid BOY. BOY maintains about the same level of aggression as she displayed during this pair's first encounter. For the third encounter, OOO was injected with PF-5HTP. Compared with her prior behavior, OOO is more willing to confront, and less likely to retreat from BOY. In response to this new behavior from OOO, BOY reduces her displays of aggression. For the final encounter between this pair, OOO was injected with 5-HTP and BOY was injected with PF-5HTP. Both crayfish become more aggressive than in prior encounters.

Fig. 1

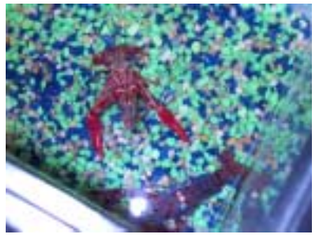






The Crayfish Injection Site

The Crayfish That Overdosed



PF-5HTP Overdose

Illustration 3 shows the fate of the first crayfish injected with PF-5HTP. This smaller-than-average male displayed unusual postures for several minutes, including the pronounced dominance stance associated with infusion of 5-HT. He then appeared to overdose on the compound. In this photograph, the previously dominant crayfish of the pair has retreated into the corner to avoid his tank mate.

Huber's results

To confirm we were observing the same behaviors as those described in the literature as being elicited by administration of serotonin to crayfish, we wanted to provided a sample of our compounds to another laboratory for independent confirmation. Dr. Huber, an experienced crayfish biologist reported, "the crayfish definitely looked like a critter who is getting serotonin, it looked like it took much longer for the effect to build up after the start of infusion and it is all very consistent with the idea of 5htp converting to serotonin."

Disscussion

In this study we found that a single injection of 100 mg/kg of PF-5-HTP mimicked the behavioral effects of serotonin on crayfish. The perfluoro-tagged version of the molecule had similar behavioral effects on both aggression and posture in crayfish. This provides support for the hypothesis that both 5-HTP and PF-5HTP are taken up into neurons and converted to serotonin. Research by other investigators has demonstrated that co-administration of fluoxetine and serotonin blocks the effects of the serotonin, most likely by preventing its uptake for subsequent release from terminals. The crayfish we observed appeared to be under the influence of stimulation of serotonin receptors.

In future studies we intend to infuse crayfish with novel imaging probes, thereby increasing the duration of the effects of the compounds on their behavior patterns. We believe that crayfish can serve as useful, inexpensive, rapid biological screens in neuroscience.

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