

Novelty stress and reproductive state alters responsiveness to sensory stimuli and 5-HT neuromodulation in crayfish

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Abstract

Sensory stimuli can produce varied responses depending on the physiological state of an animal. Stressors and reproductive stage can result in altered biochemical status that changes the responsiveness of an animal to hormones and neuromodulators, which affects whole animal behavior in relation to sensory stimuli. Crayfish serve as a model for examining the effects of neuromodulators at the neuromuscular junctions (NMJs) and for alterations in stereotypic behaviors for particular stimuli. Thus, we used crayfish to examine the effect of novelty stressors in males and the effect of being gravid in female crayfish to exogenous application of serotonin (5-HT). The responsiveness of neuromuscular junctions to 5-HT revealed that stressed as well as gravid crayfish have a reduced response to 5-HT at NMJs. The stressed crayfish were not fatigued since the basal synaptic responses are large and still showed a pronounced response to 5-HT. Using intact animals to examine a tail flip behavior, we showed that the rate of habituation in tail flipping to a strong repetitive stimulus on the telson is reduced in stressed males. Gravid females show no tail flipping behavior upon telson stimulation.

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1. Introduction

Stress in animals induced by environmental stimuli may arise in several forms. One such stress is novelty-induced stress by exposing animals to stressors that they are not normally provided in their natural environment. Examining how animals react to various stimuli when stressed allows insight into the physiological state and on-going processes of the animal. It is well known in humans that pharmacological agents show varied responses, which in some cases are due to the behavioral state (i.e., an excited or relaxed state) (Hicks et al., 1989).

In the field, chronic stress for example by a predator can result in altered behavior and the physiological well being of an animal. The wild animals held in captivity have been

intensely investigated for stress in reproductive status, dietary consumption and growth (Hediger, 1964; Romero and Wingfield, 1999; Uno et al., 1989). Laboratory conditions themselves can be stressful to animals taken from wild (Brown et al., 1970); however, there is merit to using controlled environmental conditions with a limited number of variables in order to investigate defined stressors on quantifiable behavioral and physiological responses to defined stimuli and pharmacological assessment.

Hormonal imbalance can result from endocrine response to particular stressors, such as extremes in the ratios of insulin/glucagon or levels in cortisol during physical exhaustion from skeletal muscle fatigue in vertebrates. Psychological stress is also known to play havoc on reproductive and glucocorticoid hormones in humans (Longo and Fabrizio, 2002; Maccari et al., 2003; Negro-Vilar, 1993). Thus, therapeutic drugs may show altered responses in such stressed individuals depending if a compound is an agonist or antagonists for the same receptors as the hormones or biogenic amines that have

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altered levels induced by the stress (Denney, 2001; Grigoriadis et al., 2003; Liebmann et al., 1998; Lutgendorf et al., 2000; Maccari et al., 2003; Soares et al., 2003). There is a plethora of information available in vertebrates on the pharmacological differences observed depending on the state of reproduction as a prime example of how an endocrine altered state can induce an altered physiological response to particular exogenously applied agents (Grigoriadis et al., 2003; Soares et al., 2003). In vertebrates, it has been demonstrated that aggressiveness of an individual is altered in mothers that are pregnant as well as in mothers raising young (Albert et al., 1993; Mayer and Rosenblatt, 1987). Such enhanced aggressiveness by mothers may also apply to invertebrates in general. For instance, it has been shown in lobsters that gravid mothers tend to be more aggressive in their posturing (Mello et al., 1999). Some behaviors may be a bluffing response as in to mock a defense to a predator or an opponent, versus true aggression in seeking out to attack an opponent (Listerman et al., 2000).

Crustaceans, particularly crayfish, offer unique characteristics to investigate the role stress plays on the responsiveness to induced behaviors and exogenously applied substances. Crayfish have several well characterized and established behaviors that can be used to examine the affects of acute or chronic stress. For example, the tail flip escape response is a model behavior since the neural circuitry and the muscles utilized are known (Edwards et al., 1999). This response, induced by a touch on the tail, is assumed to be used by the animal as a means to avoid predation. In addition, the circuitry shows plasticity in responsiveness between aggressive and submissive individuals (Yeh et al., 1996; Krasne et al., 1997). There is also a size dependence since large crayfish tend not to tail flip as compared to smaller crayfish, but this size dependence can be overridden by the behavioral state of the animal. Lang et al. (1977) first noted that when lobsters are induced to autotomize their chelipeds, a dominant animal will quickly switch to subordinate behavior and tail flip upon being touched on the tail. This phenomenon integrates well with the suggestion that dominant and submissive animals have altered responses to sensory stimulation of the telson. This size dependence and regulation by a behavioral state was also shown to occur in the crayfish, *Procambarus clarkii* (Kellie et al., 2001). The loss of chelipeds rapidly alters the tail flip behavior that can be maintained up to a week (Kellie et al., 2001). Likewise, stress might have an effect on the behavioral state thus influencing the tail flip elicited response.

It was proposed that serotonin (5-HT) might have a role in modulating the aggressiveness of lobsters and crayfish since some of the postures induced by injections of 5-HT are also observed when the animals display an aggressive stance during social interactions (Livingston et al., 1980). In crustaceans, the presence of 5-HT increases synaptic strength at neuromuscular junctions (NMJs) (Dudel, 1965; Fisher and Florey, 1983; Kravitz et al., 1980; Kupfermann, 1979; Southard et al., 2000; Sparks and Cooper, 2004), increases heart rate (Listerman et al., 2000; Florey and

Rathmayer, 1978) and enhances sensory neuronal activity (Strawn et al., 2000). 5-HT levels in the hemolymph of crustaceans also increase as a result of exercise in crustaceans (Sneddon et al., 2000). It remains to be determined if 5-HT levels are altered due to stress in crayfish. As previously shown in preparations from vertebrates (Fone et al., 1998; Hervás et al., 2001; Welner et al., 1989), chronically sustained 5-HT levels or receptor antagonist result in a compensatory negative feed-back regulation of target tissues. This was evident by a down-regulation in the responsiveness to exogenously applied 5-HT in crayfish (Cooper et al., 2001). The receptivity to exogenous application of 5-HT in animals in which the biosynthesis of 5-HT was blocked also showed an enhanced responsiveness, suggesting an up regulation of 5-HT receptors (Cooper et al., 2001). Since the neuromuscular junction of the opener muscle in the walking legs is very sensitive to exogenously applied 5-HT, it is now being used as a standard assay for pharmacology of 5-HT receptor subtypes (Tabor and Cooper, 2002) and cocktails of neuromodulators in crayfish (Djokaj et al., 2001).

Thus, in this study, we chose to investigate if novelty stressed crayfish show an altered responsiveness to a stimulus in a well defined behavioral assay. We used the tail flip response induced by a touch on the telson to compare stressed and non-stressed animals. In addition, we tested the responsiveness of the opener NMJ to exogenously applied 5-HT. We assume being gravid and with the eggs at the level of development in which they are attached on the exterior ventral abdominal surface of the crayfish (commonly referred to as being “in berry”) to be a natural stressor for female crayfish. Since animals in this state have altered behaviors, we examined the tail flip behavioral assay and the responsiveness to 5-HT application on the opener NMJ to examine if the state of being gravid showed altered physiological responses.

2. Materials and methods

2.1. Crayfish care

All experiments were performed on *P. clarkii* (Atchafalaya Biological Supply, Raceland, LA, USA). Animals were held in individual tanks within the laboratory for at least 2 weeks before being used for experimentation. The crayfish were fed dry fish food once a week. Prior to testing the animals were kept in isolation tanks within the test room for 24 h, which was lit 24 h a day with a dim indirect white light and returned to their isolation tanks after being tested.

2.2. Stress conditions

There were two groups of stressed animals: (1) gravid females and (2) novelty stressed males. Only males were used in the novelty stressed group in order to compare the

results obtained in this study to those in previously published reports in which only males were used. Control groups consisted of non-gravid females and males that were not stressed but were housed in the same conditions as in earlier studies utilizing males for comparisons. The gravid and non-gravid females were not exposed to novelty stress. Only the state of being gravid was tested for the female group. The groups were sized matched among the controls and experimental. The two male groups ranged from 3.4 to 11.6 g and the female groups ranged from 20 to 33.7 g. The female groups were larger than the males since the state of being gravid was found more often in large females.

The novelty stress protocol lasted four days. The animals were always kept as isolates in individual tanks. The first day of stress induction consisted of placing the animal's tank on a laboratory orbital shaker (Science ware orbital platform on a Corning PC-353 stirrer) for 24 h in the dark. The second day the animal's tank was placed under a radio playing hard rock music for 24 h in an overhead lighted room. The third day the animals were exposed to bright light produced by a standard desk lamp (60 W) illuminating the test tanks for 24 h. The light was placed 50 cm above the water surface of the tank. The crayfish had no escape from the light inside the tanks. The room had central air conditioning to maintain room temperature around 21 °C. The last day of the protocol, the crayfish were placed back on a orbital shaker for 24 h in the dark. The radio was used to simulate anthropogenic stressors such as engines or other noises.

2.3. Tail flip testing procedure

During the primary tests, the crayfish were moved from their isolation tanks to test tanks, which were 30×32×13 cm, at least 24 h before being tested. Each of the two test tanks sat adjacent to each other. The examiner remained still and as inconspicuous as possible, to reduce visual disturbances, while still having the ability to observe the crayfish. Testing followed a similar approach as Kellie et al. (2001). The crayfish received a tap from a glass rod on the tail once every 30 s. Testing continued until the crayfish failed to tail flip 20 consecutive times. After testing was complete the crayfish were returned to their isolations tanks and the water in the test tanks was replaced.

2.4. Dissection

The opener muscle of the first walking legs was prepared by the standard dissection (Southard et al., 2000). The entire opener muscle is innervated by a single tonic excitatory motor neuron (Atwood, 1976). Dissected preparations were maintained in crayfish saline (modified Van Harrevelde's solution: 205 mM NaCl, 5.3 mM KCl, 13.5 mM CaCl₂, 2.45 mM MgCl₂, 0.5 mM Hepes/NaOH, pH 7.4).

2.5. Evoked postsynaptic potentials

Intracellular muscle recordings were made with a 3 M KCl-containing microelectrode placed in a centrally located fiber in the opener muscle. A train of 10 pulses at 40 Hz was used to stimulate the motor axon. All events were measured and calibrated with the MacLab Scope software 3.5.4 version (ADInstruments). Stimulation was obtained by use of a Grass S-88 simulator and a stimulus isolation unit (Grass, SIU) with leads to a standard suction electrode.

Short-term facilitation (STF) was induced by a 40-Hz pulse trains. The STF was quantified by a facilitation index (FI) from the ratio of 10th EPSP amplitude to the 5th EPSP amplitude within each response train. The subtraction of one from the ratio is used so that if no facilitation occurs the FI will then be zero (Crider and Cooper, 1999, 2000). Even though the leg opener muscle in the crayfish is a muscle that is innervated by a single excitatory motor neuron there are still regional differences between properties of synaptic strength and muscle fiber phenotype among the distinct regions of the muscle. The first walking leg of crayfish is divided into three anatomical regions: distal, central and proximal (Iravani, 1965; Mykles et al., 2002). The nerve terminals on the central muscle fibers give rise to small excitatory postsynaptic potentials (EPSPs) as compared to the large responses measured in proximal fibers and the intermediate responses in the distal muscle fibers. The large EPSPs on proximal fibers in crayfish are due to more effective synaptic transmission per length of nerve terminal at low stimulation frequencies (Cooper et al., 1995). Differences in STF are present between these regions on the opener muscle (Linder, 1974; Günzel et al., 1993; Govind et al., 1994; Mykles et al., 2002; Crider and Cooper, 2000). Since the FI and degree of synaptic efficacy is unique to the three main regions of the opener muscle, we mapped responses in all three regions with multiple recordings during exposure to saline. This allowed comparisons among the three regions in stressed and non-stressed animals for basal conditions. After the electrophysiological mapping was completed, a central muscle fiber was monitored for its responsiveness to exogenous application to 5-HT (100 nM). The central muscle fibers display the largest increase in the EPSP amplitudes upon exposure to 5-HT (Crider, 1998), thus the rationale for monitoring this region among the stressed and non-stressed male groups as well as the gravid and non-gravid female groups.

3. Results

The opener muscle fibers can be divided into three groupings that are biochemically, physiologically and anatomically distinct. Based on morphology and physiology, there are inherent differences to distal, central and

proximal muscle fibers (Fig. 1A). A train pulse of 10 stimuli at 40 Hz is used in order to enhance the amplitude of the EPSPs by facilitation for quantifying the responses. Mykles et al. (2002) previously described the differences in myofibrillar protein isoform expressions as well as responses to facilitation of synaptic transmission between the regions for opener muscles in both crayfish and lobster. Crider and Cooper (1999, 2000) described issues concerning quantifying facilitation indices and overall difference in synaptic strength, and Cooper et al. (1995, 1996a,b) previously described differences in nerve terminal morphology and degree of synaptic efficacy per length of the nerve terminal for regions of the opener muscle in crayfish. Fig. 1 is a representative of the data set, as the absolute amplitudes are variable from preparation to preparation. The regional variability among preparations and across the opener muscle has been described as early as 1965 by Irvani. In this current study, we monitored the three regions for comparative measures in the amplitude of the EPSPs between the stressed and non-stressed males and between the gravid and non-gravid females.

Only the central muscle fibers were used to assay the effects of 5-HT (100 nM) on enhancing the amplitude of the EPSPs throughout the 40-Hz stimulus train. The amplitude of the 5th and 10th EPSPs were used for quantifying purposes. To obtain a measure of EPSP amplitude, the difference from the trough preceding the EPSP to the peak amplitude of the EPSP was used (Fig. 1B). As illustrated in Fig. 1B, the effect of 5-HT enhanced the amplitude of the EPSPs throughout the stimulus train. Since there is some time delay for a maximal effect of 5-HT in enhancing the EPSPs, the responses were plotted over time for each preparation as illustrated in Fig. 1C. After 5–10 min, the responses obtain a plateau and no further increase was noted; however, after about 20 min, a reduction in the amplitudes were sometimes observed. Thus, an average amplitude, over a 2-min period, was obtained after 8–10 min of exposure to 5-HT for calculating a percent change from an average baseline value prior to 5-HT exposure. The means of the percent change for the effects of 5-HT observed in each preparation were used to determine the overall percent difference among the various experimental groups.

3.1. EPSP responses in stressed and non-stressed males

The results indicate a trend in that the stressed males have an overall larger EPSP amplitude in the central muscle fibers for the 10th EPSP but not for the 5th EPSP (Fig. 2A). In addition, the influence of 5-HT had a pronounced effect in enhancing the amplitude of the EPSPs in the central fibers for the non-stressed (control) males (Fig. 2A). To normalize for the differences among preparations, a percent change in the 5th and 10th EPSP amplitude within a preparation to exposure of 5-HT (100 nM) was determined. The mean of the percent changes as shown in Fig. 2B depicts the large

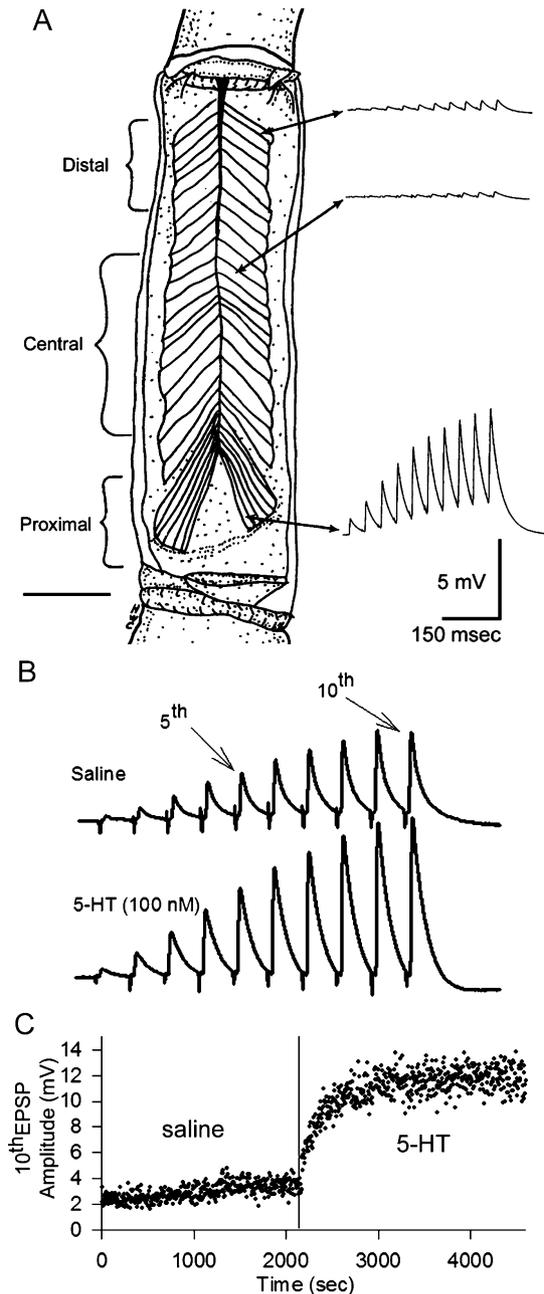


Fig. 1. (A) The opener muscle displays regional variation in the EPSPs. The largest amplitude responses are recorded from the very proximal tuft of muscle fibers and the smallest responses are observed from the central fibers with an intermediate size from the most distal fibers. A train pulse of 10 stimuli at 40 Hz is used in order to enhance the amplitude of the EPSPs by facilitation for quantifying the responses. Modified from Mykles et al. (2002). (B) The effect of 5-HT (100 nM) enhances the amplitude of the EPSPs throughout the 40-Hz stimulus train. For purposes of analysis, the amplitude of the 5th and 10th EPSPs are utilized. (C) The effect of 5-HT on the amplitude of the 10th EPSP is shown over time. The increase in the amplitude of the EPSPs is relatively quick with exposure to 5-HT. A plateau is usually reached after several minutes. In some cases, the plateau is maintained for 10–20 min before a reduction starts to occur. A representative response from a male crayfish is shown. The scale bars represents the traces shown in part A.

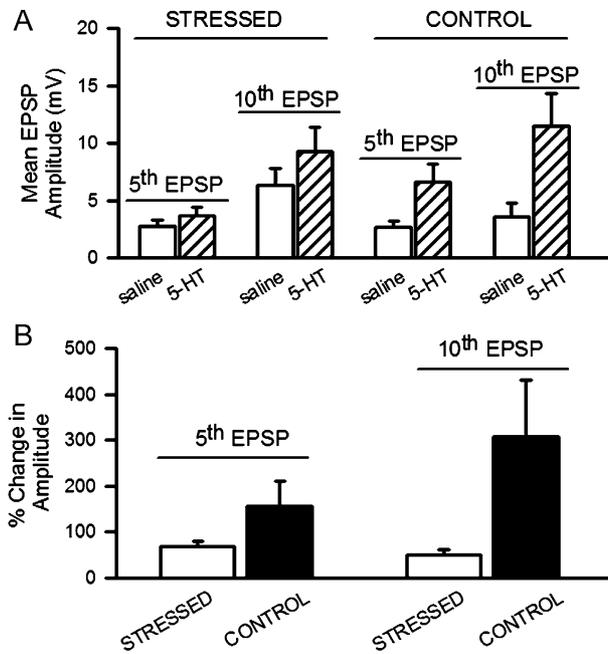


Fig. 2. The mean (\pm S.E.M.) of the 5th and 10th EPSP amplitudes within the response train are shown for the stressed and control groups. (A) During 5-HT exposure, the control group ($n=10$) showed a greater increase in the 5th and 10th EPSP amplitudes than the stressed group ($n=6$) (A , $P>0.05$, t -test). A percent change in the amplitudes for 5-HT observed in each preparation was used to determine the overall percent difference among the various experimental groups. (B) The percent difference for control males (non-stressed) to stressed males revealed a substantial difference ($P<0.05$, Student's t -test).

difference in control males as compared to stressed males. There is a significant difference between the stressed and non-stressed males for the percent change due to exposure of 5-HT in the 5th and 10th EPSP amplitudes (Fig. 2B, $P<0.05$, Student's t -test).

In comparing the regional differences across the opener muscle, there is not a significant difference in EPSP amplitudes in the proximal or distal fibers in stressed males as compared to the proximal or distal fibers of controls. The mean and standard error of the mean for the 10th EPSP of the 40-Hz stimulus trains within each group were compiled for the 3 major regions of the opener muscle (proximal, central and distal). There was a substantial amount of variation among individuals. However, to normalize for the variation, ratios among regions for each preparation were calculated and compared between groups. The proximal region is chosen as a reference for the ratios since this region has the smallest differences among the groupings and shows the least responsiveness to 5-HT (Mykles et al., 2002; Crider and Cooper, 2000). The control animals had a large variation in the responses obtained in the central muscle fibers, thus producing a much larger variation in the ratio of the proximal/central EPSP amplitudes as compared to the stressed group (Fig. 3). The non-stressed (control) males had a greater difference in the ratio of proximal/distal to the proximal/central as compared to the stressed group (Fig. 3, $P<0.05$, Student's t -test).

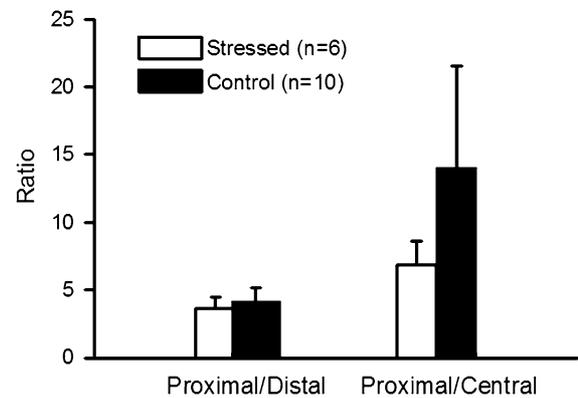


Fig. 3. The absolute amplitudes of the EPSPs within the various regions of the opener muscle were examined for a differentially affect by stress in both the male stressed group control group. There is a substantial amount of variation among individuals, which is also shown when comparing the ratios among regions for each preparation. The stressed males do not have as great a difference in the ratio of proximal/distal and proximal/central as compared to controls (A , $P<0.05$, Student's t -test).

3.2. EPSP responses in gravid and non-gravid females

As for the stress males the state of being gravid reduces the responsiveness to exogenous application of 5-HT in enhancing the EPSP amplitudes. Like for the male crayfish there is an inherent difference in the amplitudes between preparations so a percent change in the effect of 5-HT is used for comparisons. The mean of the percent changes for exposure to 5-HT (100 nM) is shown in Fig. 4 for the 5th and 10th EPSP amplitude within the 40-Hz 10-pulse trains. There is a significantly greater responsiveness to 5-HT in the non-gravid controls ($n=7$) as compared to gravid ones ($n=9$) for both the 5th and 10th EPSPs within the stimulus trains ($P<0.05$, Student's t -test). The gravid and non-gravid groups did not display a difference in the ratios in the EPSP amplitudes between the three regions of the opener muscle (data not shown).

In examining whether a difference existed in the degree of facilitation [(10th EPSP/5th EPSP)–1] between the

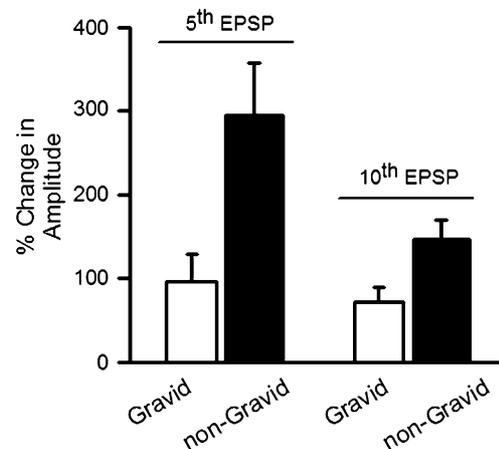


Fig. 4. The percent difference in the 5th and 10th EPSPs within the response train from saline to 5-HT (100 nM) exposure is greater for non-gravid as compared to the gravid females ($P<0.05$, Student's t -test).

various experimental groups (males: non-stressed and stress, females: gravid and non-gravid), the facilitation index (FI) was determined for each of the three regions. There was no significant differences for FI between the stressed and non-stressed males ($P=0.25$) and between the gravid and non-gravid females ($P=0.125$). Likewise there was no significant difference in the effects of 5-HT on FI between groups.

3.3. Tail flip behavior

The responsiveness of crayfish and lobsters to tail flip as a result of a stimulus applied to the dorsal aspect of the telson has been used as a measure of its responsiveness to external stimuli in a number of past studies (Bruski and Dunham, 1987; Fricke, 1986; Guiasu and Dunham, 1997; Copp, 1986; Pavey and Fielder, 1996; Lang et al., 1977; Kellie et al., 2001). Here, the tail flip response was used as an assessment of the whole animal status to stress or being gravid. Animals that tail flipped were marked as having a

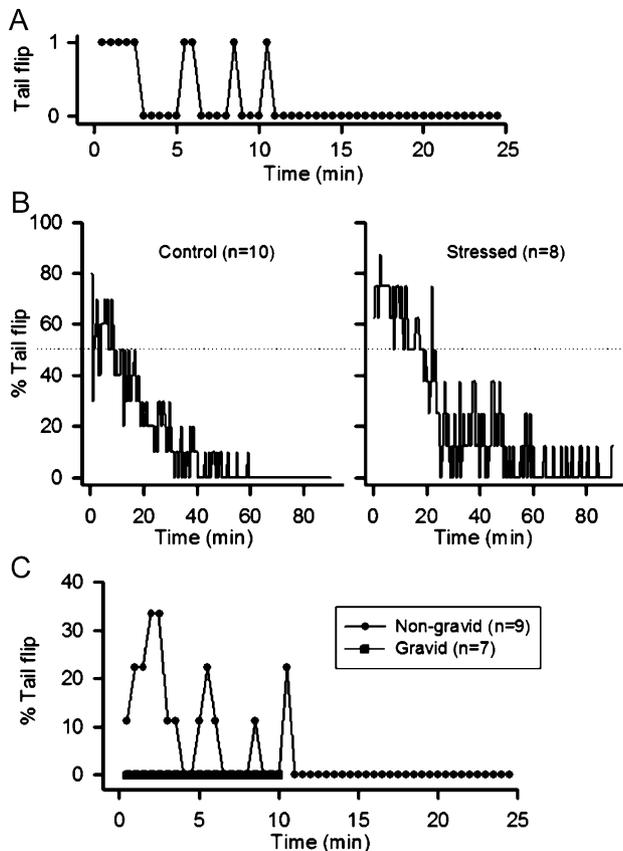


Fig. 5. A representative plot of the occurrences of tail flips for a non-gravid crayfish depicts a typical time until the animal habituates to the tail touching stimulus (A). A tail flip is marked with a '1' and a failure as a '0' over time. The stimuli are given every 30 s. Compiling the individuals within a group and determining a percent change revealed that control (non-stressed males) habituate sooner than stressed male crayfish (B). A dotted line at 50% tail flipping occurrence is shown to aid in comparisons between groups. Gravid females did not tail flip where as the non-gravid females took about 10 min to show a complete habituation (C). The female groups are significantly larger animals than the male groups used so direct comparisons are not applicable between the sexes.

response and those that did not as a failure in a response. In this study, we did not distinguish between a giant mediated and a non-giant mediated tail flip (Krasne and Wine, 1975) but rather if the animal tail flipped at all upon being forcefully tapped on the telson.

A measure of tail flipping over time depicts the rate of habituation for an individual animal as shown for a representative crayfish (Fig. 5A). If a crayfish tail flipped it was given a score of 'one' and if it failed a score of 'zero'. A representative response over time for a non-gravid female crayfish is shown in Fig. 5A. In order to compare between the groups, the individual responses were combined and a percentage in the number of animals that tail flipped were plotted over time. The percentages for both the stressed and non-stressed males (Fig. 5B) as well as the controls and gravid females (Fig. 5C) indicate that the stressed crayfish tended to tail flip more as compared to non-stressed males and that gravid females tail flipped less than non-gravid females. There is an overlap in the composite for the male non-stressed and stressed groups so individual plots are shown and a line at 50% of tail flipping behavior for ease in visual comparison. The gravid and non-gravid group is very clearly separated since the gravid crayfish did not tail flip. Since all the animals within the gravid group did not tail flip, the response index remained at zero throughout the trials and was only carried out for 10 min as compared to the non-gravid crayfish.

4. Discussion

The ability of an animal to react to environmental stimuli and to process cues for social interactions are dependent on the physiological state of the animal. Extreme examples are of an injured or an unhealthy animal, but even less drastic states, such as during mild stress, one would expect processing of sensory stimuli to be different as compared to non-stressed individuals (Basso, 2001). In this report, we demonstrate that male crayfish exposed to environmental novelty stress for 4 days have an altered responsiveness to neuromodulators at synaptic sites and altered behaviors to defined sensory stimuli. In addition, we have shown with female crayfish the state of reproduction results in a difference in synaptic physiology at neuromuscular junctions as well as responsiveness for the whole animal to an environmental stimulus.

The 4-day testing was the minimal amount of time during stress that we thought receptors would show altered sensitivity to application of exogenously applied 5-HT since in a previous study (Cooper et al., 2001) 4 days was the minimal time to observe changes to 5-HT sensitivity when agonists and antagonists were injected into the hemolymph of the animal. To maintain the novelty in the stimulus the stressful stimuli were altered daily (shaking, sound and light) and the 24-h exposures covered a full circadian cycle for the animal (Tilden et al., 2003).

The reduced responsiveness to exposure of 5-HT for the stressed male crayfish could have arisen due to a high level of endogenously released stress hormones, or possibly even 5-HT. If this occurred over the 4 days of stress the preparations might have a decreased sensitivity to an exogenous exposure at a receptor level and/or less activation of downstream processes involved with cellular transduction of the signal. Such a scenario was posed for the reduced responsiveness of neuromuscular preparations to 5-HT when preparations were previously treated with octopamine (Djokaj et al., 2001). This possibility appears to be very likely since the amplitude of the 10th EPSPs are larger within the response train for stressed animals (Fig. 2A, $P < 0.05$, Student's *t*-test). If the reduced responsiveness to 5-HT was due to fatigue of the NMJ due to the chronic stress then the amplitudes of the EPSPs should be reduced and more prone to depression within the 40-Hz 10-pulse stimulus train, which was not the case. In addition, the stressed animals would not have likely shown a reduced habituation to tail flipping if they were fatigued.

The possible exposure of NMJs to endogenously released biogenic amines over the few days of stress might well have altered the status of second-messenger cascades within the nerve terminals as well as the muscle (Betelle and Kravitz, 1978; Enyeart, 1981; Dixon and Atwood, 1989a,b; Goy and Kravitz, 1989; Beaumont and Zucker, 2000). 5-HT can also have an effect on hyperpolarization-activated cation channels within the presynaptic axon terminals (Beaumont and Zucker, 2000). The potential exists for prior exposure of octopamine interfering with the inducible response to 5-HT since octopamine can mediate its action through cAMP in arthropod tissues (Betelle and Kravitz, 1978; Groome and Watson, 1989; Thompson et al., 1990). Since it is known for crayfish and crabs that the presence or the recent presence of octopamine can alter the response of 5-HT (Djokaj et al., 2001), it is difficult to assess the multitude of possible scenarios for why novelty stress in the crayfish reduces the effectiveness of exogenously applied 5-HT. Since there is a myriad of potential neuromodulators in the intact animal that might act alone or in concert with several other neuromodulators, which includes biogenic amines (Christie et al., 1995) as well as peptides (Worden, 1998), only speculation exists at this time to what the mechanism is responsible for the reduced effects of 5-HT on the isolated NMJ after exposing the animal to novelty stressors. Recently, the effects of melatonin were assessed for behavioral and physiological effects in crayfish (Tilden et al., 2003), and it was demonstrated that melatonin can enhance the amplitudes of EPSPs at the crayfish opener NMJ. This is relevant to our study since one of the stressors was 24 h of light for the male crayfish. In addition, it is possible that the chronic stressors did not allow the animals to maintain their normal cycles of melatonin as compared to control crayfish maintained on a 12:12-h light cycle.

The initial indication that the state of being gravid altered responsiveness to 5-HT was conducted in 1997 (Crider, 1998). Since we had a recent stock of gravid animals, we were able to conduct the systematic studies presented in this report over a short period of time. There is sparse information on the endocrine state of gravid crayfish for titer levels for biogenic amines, catecholamines and hormones related to reproduction and growth (i.e., androgens, estrogens and vitellogenic compounds). However, a few reports exist which have made a good start in understanding the endocrine systems of crustaceans (Fingerman, 1995, 1997; Mattson and Spaziani, 1985; Rodriguez et al., 2002). Studies with vertebrates have advanced more rapidly in this area of stress on reproduction and other endocrine systems (Chemineau et al., 2003; Cheng et al., 2003; Maggioncalda et al., 2002; Uno et al., 1989) as well as direct actions on neuronal function (D'Arbe et al., 2002; Karst and Joëls, 2003; Miura et al., 2002; Touyarot et al., 2004). With so few investigations on this topic in crustaceans, it is not surprising that studies have not yet been conducted in addressing the role of biogenic amines affecting the behaviors of females within a gravid state not to mention the actions of biogenic amines at a cellular level during a gravid state. Considering that interacting gravid lobsters do tail flip in an aggressive manner (i.e., when holding on to an opponent and tail flipping to pull off an appendage) demonstrates that lobsters can tail flip while gravid (Mello et al., 1999). In the same study of lobsters, it was shown that there was no significant difference between gravid and non-gravid in the number of avoidance tail flips upon interactions. In combining the behavioral repertoire used in the earlier study, gravid lobsters were shown to be more aggressive. These results are in contrast to what we have shown in isolated crayfish with the tail tap stimulus provided by an experimenter. In the experiments that we conducted, there is no food reward for tail flipping which may be a compounding variable in the open field aggressive tail flips conducted with pairs of lobsters.

There are numerous reports suggesting that the aggressive state of lobsters and crayfish may indeed involve the serotonin based system, and it has been implied that higher systemic levels of 5-HT might be related to the degree of aggressiveness (see Huber et al., 1997; Livingston et al., 1980; Tierney and Mangiamele, 2001; Yeh et al., 1996). However, given that 5-HT receptors show down regulation in mammals (Fone et al., 1998; Hensler, 2002; Hervás et al., 2001; Welner et al., 1989) and likely in crayfish (Cooper et al., 2001) in addition to the fact that the crustacean cardiac ganglion is very sensitive to systemic levels of 5-HT (Listerman et al., 2000; Grega and Sherman, 1975; Wilkens and McMahan, 1992), it is unlikely that an aggressive crustacean would maintain a chronic high level of systemic 5-HT (Cooper et al., 2001, 2003a,b). This is also supported by the fact that lobsters with reduced 5-HT showed no differences in aggressive-

ness (Doernberg et al., 2001). Possibly an aggressive individual might be able to release a larger rapid bolus of 5-HT systemically or have an increased receptivity to 5-HT as compared to submissive individuals. One has to also keep in mind that depending on how 5-HT is applied to excised preparations (slow or rapid) opposing responses can be obtained (Teshiba et al., 2001). This again suggests down stream effects on receptors and/or cellular cascades may very likely be possible if systemic levels of 5-HT are modulated during chronic stress.

Experiments by Krasne et al. (1997) showed that the lateral giant reflex escape is less likely to occur in subordinate crayfish during social interactions. Since the stressed crayfish used in our study showed an enhanced response for tail flipping when induced by a tail tap (i.e., an escape response) the results are suggestive that subordinate crayfish in a social interaction may not be stressed or at least not stressed to the same degree induced by the novelty stress used in the present study. In relation to gravid crayfish, if one assumes they are behaviorally analogous to gravid lobsters, they would be classified as in an aggressive state and more likely to show a tail flip response for an induced lateral giant reflex. Since this was not the case, suggests there is a more complex nature to the problem. The field is now beginning to deal with the past commonly used reductionistic approach in examining a few compounding variables (i.e., gravid, non-gravid, aggressive, submissive, high or low systemic 5-HT) and addressing the highly complex issues around biogenic amines and crustacean behaviors (Cooper et al., 2003a,b; Kravitz and Huber, 2003; Marder and Thirumalai, 2002; Shuranova et al., 2003; Sneddon et al., 2000; Strawn et al., 2000; Teshiba et al., 2001). This integrative research area on neuromodulation and behavior is also very active with insect models (Baier et al., 2002; Blenau and Baumann, 2001; Dasari and Cooper, 2004; Li and Cooper, 2001; Monastirioti, 1999; Whitfield et al., 2003).

It will take this field sometime to investigate all the possible permutations of the various hormonal interactions on behavior and cellular physiology. Given the findings presented within this study also pertain to other animals, even humans, it would be informative to compile information in a comparative manner to examine trends and differences among species in relation to stress and altered responsiveness to neuromodulators.

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