Chronic Alterations in Serotonin Function: Dynamic Neurochemical Properties in Agonistic Behavior of the Crayfish, *Orconectes rusticus*

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ABSTRACT: The biogenic amine serotonin [5-hydroxytryptamine (5-HT)] has received considerable attention for its role in behavioral phenomena throughout a broad range of invertebrate and vertebrate taxa. Acute 5-HT infusion decreases the likelihood of crayfish to retreat from dominant opponents. The present study reports the biochemical and behavioral effects resulting from chronic treatment with 5-HT-modifying compounds delivered for up to 5 weeks via silastic tube implants. High performance liquid chromatography with electrochemical detection (HPLC-ED) confirmed that 5,7-dihydroxytryptamine (5,7-DHT) effectively reduced 5-HT in all central nervous system (CNS) areas, except brain, while a concurrent accumulation of the compound was observed in all tissues analyzed. Unexpectedly, two different rates of chronic 5-HT treatment did not increase levels of the amine in the CNS. Behav-

iorally, 5,7-DHT treated crayfish exhibited no significant differences in measures of aggression. Although treatment with 5-HT did not elevate 5-HT content in the CNS, infusion at a slow rate caused animals to escalate more quickly while 5-HT treatment at a faster rate resulted in slower escalation. 5,7-DHT is commonly used in behavioral pharmacology and the present findings suggest its biochemical properties should be more thoroughly examined. Moreover, the apparent presence of powerful compensatory mechanisms indicates our need to adopt an increasingly dynamic view of the serotonergic bases of behavior like crayfish aggression. © 2002 Wiley Periodicals, Inc. J Neurobiol 50: 276–290, 2002; DOI 10.1002/neu.10035

Keywords: serotonin (5-HT); 5,7-dihydroxytryptamine (5,7-DHT); compensatory mechanisms; agonistic behavior; crustacea

INTRODUCTION

Aggression is a key element in the natural behavioral repertoire of most decapod crustacean species (Berrill, 1978; Berrill and Arsenault, 1984; Karnofsky et al., 1989). Even in the absence of an obvious resource,

initial contact between two conspecifics is followed by a series of agonistic bouts that vary in length until one participant withdraws (Bovbjerg, 1953; Scrivener, 1971; Rubenstein and Hazlett, 1973; Bruski and Dunham, 1987). Encounters progress towards more intense stages of fighting in a step-wise fashion where decisions to escalate the encounter or retreat govern the temporal and probabilistic nature of subsequent behavior patterns (Huber and Kravitz, 1995; Huber et al., 2001a). In a majority of instances, physical superiority constitutes the primary determinant of fight length and outcome (Bovbjerg, 1956; Rubenstein and Hazlett, 1973; Pavey and Fielder, 1996; Barki et al., 1997; Vye et al., 1997). However, there are important

caveats to viewing this behavioral framework too simplistically. For instance, previous experience, in the form of social conditioning, diminishes the initial bias of morphological asymmetries in claw and body size (Daws et al., 2001). Moreover, as crayfish hierarchies differentiate, the aggressiveness and overall success of participants converge on distinct, linear ranks due to a process of self-assembly (Goessmann et al., 2000). Nevertheless, once social status becomes established, subsequent fighting dwindles and defeated animals avoid further contact with winners (Issa et al., 1999; Goessmann et al., 2000). The fundamental characteristics of this behavioral scheme closely match predictions of game theory (Maynard-Smith and Price, 1973; Parker and Rubenstein, 1981; Enquist and Leimar, 1983; Austad, 1989) and are thus ideal for quantitative behavioral analysis (Huber and Kravitz, 1995; Huber et al., 2001a).

Decapod crustaceans have proven to be useful models for exploring proximate mechanisms in aggression (Antonsen and Paul, 1997; Krasne et al., 1997; Yeh et al., 1997; Huber and Delago, 1998; Sneddon et al., 2000; Tierney, 2000; Doernberg et al., 2001). Initial studies illustrated that direct injection of substantial amounts of 5-HT into the hemolymph (i.e., blood) of lobsters and crayfish resulted in a posture resembling "meral spread"—a common display of dominant animals (Livingstone et al., 1980). Subsequently, a small population of 5-HT-containing neurons was identified (Beltz and Kravitz, 1983; Real and Czternasty, 1990) and has since been extensively characterized (Beltz and Kravitz, 1987; Ma et al., 1992; Ma and Weiger, 1993; Heinrich et al., 1999). A subset of these neurons has both central and peripheral effects on muscle flexion (Kravitz et al., 1984; Harris-Warrick and Kravitz, 1984), may enhance the occurrence of "dominant-like" postures (Ma et al., 1992; Kravitz, 2000), and is activated by command circuits that control tail flip—a common escape behavior (Hörner et al., 1997). Mediated through lateral giant neurons, the latter mechanism exhibits 5-HT-induced activity that varies inversely as a function of social status (Glanzman and Krasne, 1983; Yeh et al., 1996, 1997). Infusion of somewhat smaller amounts of 5-HT into freely moving lobsters and crayfish produces a renewed willingness to fight their larger, dominant opponents, and 5-HT reuptake is an important underlying mechanism for such behavioral reversals (Huber et al., 1997a,b; Huber and Delago, 1998). In crayfish and lobsters, chronic treatment with fluoxetine is accompanied by an expected increase in aggressive behavior, but the behavioral effects are less pronounced compared to those produced by acute 5-HT infusion, and they do not persist across the duration of treatment (Delago et al., in review).

Consistent with evidence in the crustacean species mentioned above, 5-HT is implicated in the neurochemical control of aggression throughout a broad range of taxa (Maas, 1962; Kostowski et al., 1975; Maler and Ellis, 1987; Blanchard et al., 1991; Raleigh et al., 1991; Reisner et al., 1996; Dyakonova and Schürmann, 1999; Lesch and Merschdorf, 2000; Larson and Summers, 2001). Attempts to explain such relationships have centered predominantly on 5-HT's presence or absence, and thereby its putative function in aggression and social dominance. Although often assumed to extend from evidence derived in acute experimental situations, the neurochemical mechanisms underlying long-term changes in aggressive motivation (Van De Poll et al., 1982; Jackson, 1991; Chase et al., 1994; Hollis et al., 1995; Whitehouse, 1997; Hsu and Wolf, 1999; Issa et al., 1999; Goessmann et al., 2000) have received comparatively little attention. Of the studies that have addressed such issues directly (e.g., Winberg and Nilsson, 1993; Kudryavtseva and Avgustinovich, 1998; Stribley and Carter, 1999), general agreement converges on the dynamic neurochemical processes that parallel long-term changes in aggressive state. Moreover, studies ranging from learning (Montarolo et al., 1986; Dale et al., 1987) and aggression (Yeh et al., 1996, 1997; Flugge et al., 1997; Kudryavtseva and Avgustinovich, 1998) to drug use (Robinson and Berridge, 1993; Bradbury, 2000; Koob and Le Moal, 2001) and depression (Hjorth and Auerbach, 1996; Blier and de Montigny, 1999) indicate an inherent, temporal complexity for studying the role of monoamines in behavioral plasticity.

In crayfish, the time course and potential reversal of 5-HT's aggression enhancing effects have not yet been thoroughly examined. Recent experiments with chronic fluoxetine treatments have underscored the need to carefully explore the time frame of serotonergic plasticity concomitant to changes in decapod aggressive motivation (Delago et al., in review). In the present article, we report experiments that examine the neurochemical basis of crustacean fighting behavior in the crayfish, *Orconectes rusticus.* Dyadic agonistic encounters were analyzed following different lengths of chronic exposure to silastic tube implants containing 5-HT, 5,7-DHT, or α-methyltryptophan (AMTP). The effects of 5-HT release were additionally explored by varying the rate of delivery. Following behavioral trials, changes in nervous system levels of 5-HT were measured with high performance liquid chromatography with electrochemical detection (HPLC-ED).

MATERIALS AND METHODS

Crayfish

Crayfish (*O. rusticus*) from the Portage River near Bowling Green State University were socially isolated in individual containers (Ø 0.17 m, 0.1 m depth) for at least 7 days prior to behavioral trials. Only intermolt males (5.0–26.6 g) with intact appendages were used. Individual containers were maintained in fiberglass trays (2.0 × 0.7 × 0.1 m) supplied continuously with freshly filtered and aerated water (20°C) from a main holding reservoir. All animals were fed twice a week and maintained under a 16:8 h light/dark cycle.

Pharmacological Treatments

Sections of silastic tubing (Ø 0.635 mm; Konigsberg Instruments, Inc.) were cut to a length of 15 mm and loaded with crystals of 5,7-DHT creatinine sulfate (FW: 403.4; Sigma D-0136 or Fluka 37970), AMTP (FW: 218.3; Sigma M-8377), 5-HT creatinine sulfate (FW: 387.4; Sigma H-7752), or left empty in the control group. All tubes were occluded at one end with 734 Flowable Sealant (Dow Corning). A fifth group (5-HT "slow") received tubes also loaded with 5-HT, but which had both ends of the tube sealed (compared to the 5-HT "fast" treatment that had only one end sealed). Differences in the rate of release between 5-HT treatments were confirmed by placing loaded tubes into 30 mL of 125 m*M* saline. Samples were taken over 1 week (ca. every 36 h) and assayed for 5-HT with HPLC-ED (see below). 5-HT remained intact within the tubes and was released continuously at stable rates in both 5-HT slow [$F(1, 4) = 11.01$; $p < .05$] and 5-HT fast [$F(1, 4) = 13.39$; $p < .05$] conditions. Rates of infusion were measured as 0.6 μg/h and 7.8 μg/h for 5-HT slow and 5-HT fast, respectively. Such treatments were expected to serve as a major challenge to aminergic systems. For instance, the total amount of serotonin infused (400 μg) over 4 weeks in the 5-HT slow condition exceeded natural levels (9.4 ± 0.85 ng) by 40,000 times.

Before implanting each tube, crayfish were anesthetized in ice for 10 min. An 18.5 gauge needle was used to make a small incision in the abdominal cuticle directly behind the most posterior ambulatory leg. With a pair of forceps, the silastic tube was inserted into the opening and guided into the thoracic body cavity. Subsequently, animals were placed in an observation aquarium and allowed to recover from the treatment. Within minutes, all animals exhibited normal activity and exploratory behavior, and no deaths resulted from such treatment. Animals were returned to their containers and maintained as previously described.

Profiles of Pharmacological Compounds

5,7-DHT is widely used to deplete 5-HT. It acts as a classical neurotoxin in vertebrates by destroying 5-HT-containing cells, presumably through a build up of free radicals produced by its intracellular actions with monoamine oxidase (MAO; Klemm

et al., 1979). However, neither MAO (Boadle and Blaschko, 1968; Dewhurst et al., 1972; Sloley and Goldberg, 1991; Sparks and Geng, 1992) nor 5-hydroxyindolacetic acid, the primary metabolite of 5-HT oxidation (Kennedy, 1978; Dubbels and Elofsson, 1989), appear to be present in many invertebrates, including crustaceans. Moreover, there is strong evidence indicating that invertebrate 5-HT neurons retain all functional membrane properties after treatment with 5,7-DHT (Lent and Dickinson, 1984; Cook and Orchard, 1993; Heinrich et al., 1999). Nevertheless, numerous studies of invertebrates have demonstrated that treatment with 5,7-DHT results in robust depletions of 5-HT (Livingstone et al., 1981; Lent, 1984; Lent and Dickinson, 1984; Glanzman and Krasne, 1986; Cook and Orchard, 1993; Sahley, 1994; Benton et al., 1997; Doernberg et al., 2001).

Likewise, AMTP has been used to evaluate the behavioral effects produced by depletion of 5-HT in the invertebrate CNS (Novak and Rowley, 1994; Dyakonova and Schürmann, 1999; Stevenson et al., 2000). AMTP leads to a general decrease in 5-HT synthesis by way of α-methylated amino acid substitutions (Sloley and Orikasa, 1988).

Quantitative Neurochemical Analysis

To verify the effects of pharmacological treatments we obtained neurochemical profiles for individuals with HPLC-ED (see Table 1). After the end of each behavioral trial (see below), the experimental animal was immediately anesthetized in ice for 30 min. The animal was then sacrificed and its entire ventral nerve cord (i.e., CNS) was removed in four sections: (A1–A6) all abdominal ganglia, (T1–T5) all thoracic ganglia, (SEG/CEG) subesophageal/circumesophageal ganglia, and (SUP) supraesophageal ganglion (i.e., brain). Tissues were placed into a 1.5 mL microfuge tube containing 200 μL 0.1 *N* perchloric acid, mechanically disrupted (Kontes motor driven pelleting tool equipped with a Teflon™ pestle), and cellular debris and denatured proteins were pelleted with a tabletop centrifuge (Capsulefug, 6200 RPM, 15 min). Twenty-microliter aliquots (diluted 10–100-fold with mobile phase) were separated on a C18 column (Spherisorb ODS2, 3 μm, 100 × 4.6 mm), with further details reviewed elsewhere (Panksepp et al., 2002).

The mobile phase contained 50 m*M* sodium phosphate (6.9 g/L monobasic anhydrous; FW: 120.0; Sigma S-0751), 5 m*M* heptanesulfonic acid (1 g/L sodium salt; FW: 202.2; Sigma H-2766), and v/v 16% MeOH and 4% acetonitrile as organic modifiers. The final solvent buffer was adjusted to pH 3.25 with concentrated phosphoric acid (ACS reagent; FW: 98.0; Sigma P-6560), filtered through a 0.22 μm filter, and operated at ambient temperature with a flow rate of 1.5 mL/min. Eluted compounds were detected electrochemically on a BAS LC-4C single-cell, amperometric detection system. The detector potential was set to 500 mV. Chromatograms were recorded with analog/digital converter (MacLab) and strip-chart software (MacLab Chart v3.3.3) on an Apple Macintosh computer. The size of eluting peaks was analyzed with chromatography software (MacLab Peaks v1.3.1). Identification of 5-HT was based on compar-

Table 1   Descriptive Statistics for Crayfish Used in Behavioral Trials and HPLC-ED Analysis

	5,7-DHT	AMTP	Control	5-HT "Slow"	5-HT "Fast"
Treatment					
Weight	13.6 ± 1.69 g	13.2 ± 1.64 g	8.7 ± 0.45 g	11.1 ± 2.20 g	13.1 ± 1.29 g
Duration	5–16 days	12–26 days	21–30 days	21–30 days	4–23 days
Behavioral trials					
n	8	6	5	4	5
# Interactions	100	65	63	26	65
HPLC-ED analysis					
n	8	8	35*	7	5

Numbers reported for weight are means ± standard error. In a few cases, crayfish were used for HPLC-ED analysis, but were not used in behavioral experiments. This accounts for the discrepancy in sample sizes (*n*) for treatment groups between behavioral and HPLC-ED analyses.

* Thirty additional animals that were not experimentally manipulated were included in the control group for a more accurate measure of standard crayfish CNS 5-HT content. There were no statistically significant differences between untreated and control animals, or between body weight and amine content.

ison with external standards by retention time under several different mobile phase conditions. Known standards were dissolved to a final concentration (5 pg amine/μL) in mobile phase from a stock solution each day. The detection limit was 3–5 pg 5-HT injected on column. Recovery rates were close to 100% and no further correction was applied. 5,7-DHT standards were also prepared as described above and included for the analysis of crayfish treated with this compound. Standards were injected every five samples, and at the beginning and end of each day.

Quantitative Behavioral Analysis

Following different lengths of drug exposure, treated crayfish were size-matched (i.e., < 5% difference in body weight) against randomly selected opponents (see Table 1). The behavioral analysis followed standard procedures used in our lab as detailed below (reviewed in Huber et al., 2001a). Both animals were placed in a Plexiglas™ observation tank (0.45 × 0.31 × 0.15 m) that received continuous flow of freshly filtered and aerated water through evenly spaced pairs of in/out holes. Initially, an opaque Plexiglas™ divider separated opponents for a 10 min acclimation period. The divider was then raised and each trial videotaped for 30 min (Sony DCR-TR7000 digital video camera). Design and size of this arena were guided by observations of agonistic interactions in the field. *O. rusticus* reach densities in excess of 50 individuals/m² at our collecting sites near Bowling Green, OH (unpublished observation) and are routinely observed fighting in shallow, shelterless pools with similar sizes and features to those used in the present study.

Behavioral measures were scored for all interactions that took place during 30 min trials. For each interaction the identities of the initiating and retreating animals, fight duration, maximum intensity reached, and the frequency of the highest intensity level (i.e., level 4) were scored. An interaction was defined as the point when the approaching animal came to within one body length of the opponent and its presence was overtly reacted to. Fighting intensity was coded on an ordinal scale: (0) no contest—one animal

retreats without challenge; (1) threat displays or "meral spread"—both animals contest the interaction without physically touching the opponent; (2) restrained use of claws—both animals contest and at least one individual is antenna whipping and/or pushing its opponent without grabbing; (3) clawlock—both animals contest and at least one individual uses claws to grab and wrestle with opponent; and (4) unrestrained combat or "strike and rip"—brief periods characterized by the attempt of one animal to tear an appendage from its opponent. When one animal retreated from its opponent by at least one body length and remained separate for at least 5 s, it was considered the end of the interaction.

Statistical Evaluation of Neurochemistry and Behavior

Analysis of variance was used to test for differences in all continuous variables (e.g., fight duration, frequency of intensity level 4, and 5-HT content of all CNS tissues). To reduce heteroscedasticity (i.e., inequality of variances among treatment groups) a logarithmic transformation was applied to 5-HT content. Posthoc comparisons were explored with Tukey-Kramer HSD tests.

Following the establishment of social relationships, winning crayfish become more likely to initiate while losers become more likely to retreat (Issa et al., 1999; Goessmann et al., 2000), and thus such measures will lack independence. This dominance-associated behavioral polarity is apparent within three interactions and remains stable during fighting periods (Goessmann et al., 2000). To eliminate such lack of independence in our analyses, we restricted our analyses to those interactions that occurred prior to "established dominance"—operationally defined as the first interaction after which the identity of both initiating and retreating animals remained unchanged in subsequent interactions. Differences in initiation, retreat, and number of fights reaching a particular maximum intensity were compared with G-tests, and overall significant effects were explored with Freeman-Tukey deviates (FTD).
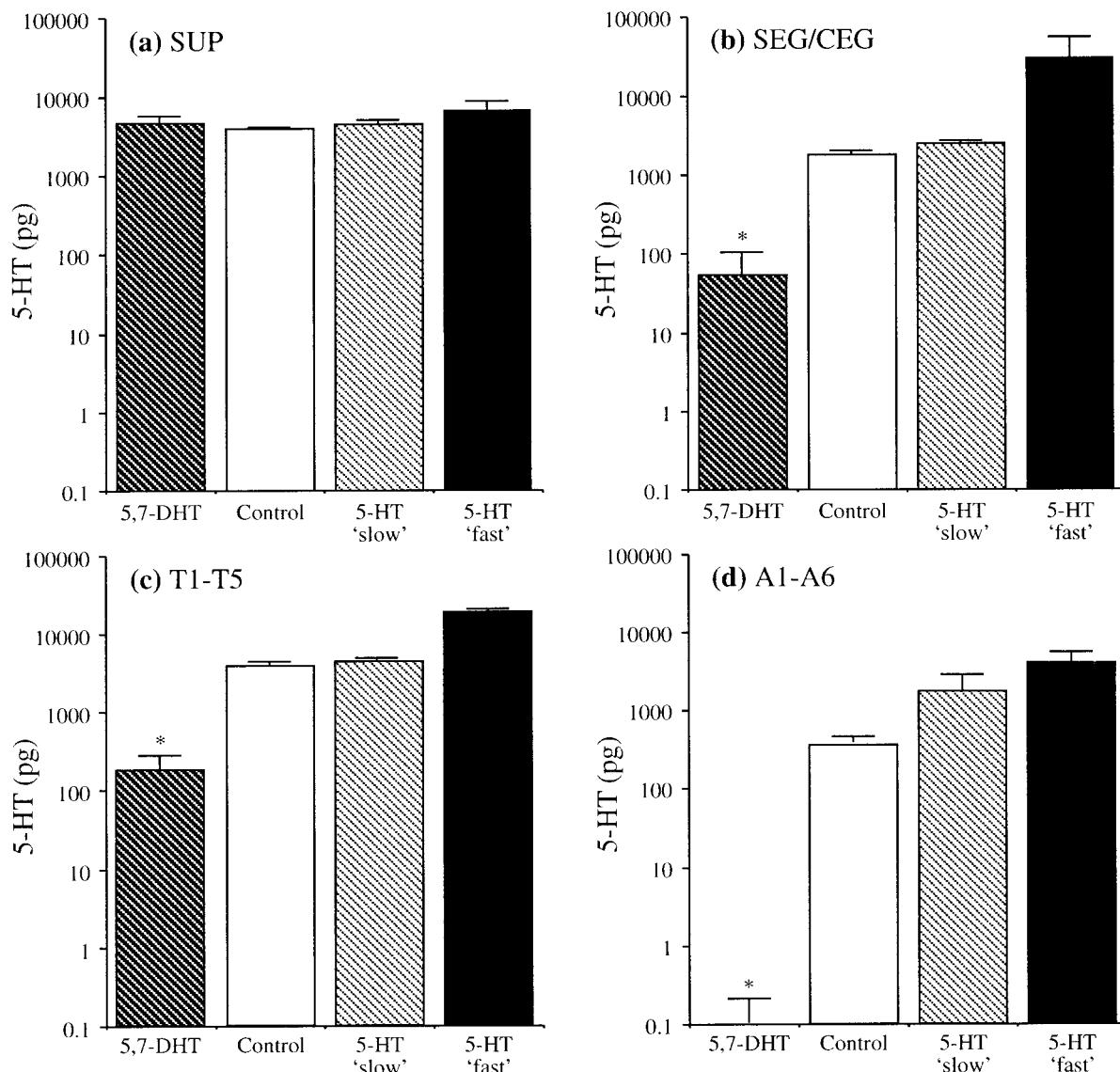
Figure 1   Graph of 5-HT content (means ± standard error) for different experimental groups plotted on log-scale: (**a**) SUP, (**b**) SEG/CEG, (**c**) T1–T5, and (**d**) A1–A6. Significant Tukey-Kramer HSD tests are indicated with asterisks. In the 5,7-DHT treated group, all tissues except SUP were significantly lower than controls.

Understanding how different behaviors covary as interrelated behavioral dimensions has proven useful in our previous studies of fighting behavior in decapod crustaceans (Huber and Kravitz, 1995; Huber et al., 2001a; Stocker and Huber, 2001; Schroeder and Huber, 2002). For example, when agonistic encounters between decapods last longer they inevitably escalate to higher intensities (Huber et al., 1997b; Huber and Delago, 1998; Goessmann et al., 2000). In this set of experiments, we used whole-model regression analysis to measure the effects of duration, treatment, and their interaction on maximum intensity. The resulting slope (i.e., the regression coefficient) provides an estimate for the average rate of escalation during a fight. Differences in the rate of escalating were evaluated by the interaction term (duration × treatment) and 95% confidence intervals of each regression coefficient were used to identify differences between experimental groups.

RESULTS

Neurochemistry

Significant differences in 5-HT (Fig. 1) were detected for A1–A6 [$F(4, 53) = 10.46$; $p < .001$], T1–T5 [$F(4, 51) = 34.95$; $p < .001$], and SEG/CEG [$F(4, 52) = 69.94$; $p < .001$]. 5,7-DHT treated animals had significantly lower levels of 5-HT in A1–A6,
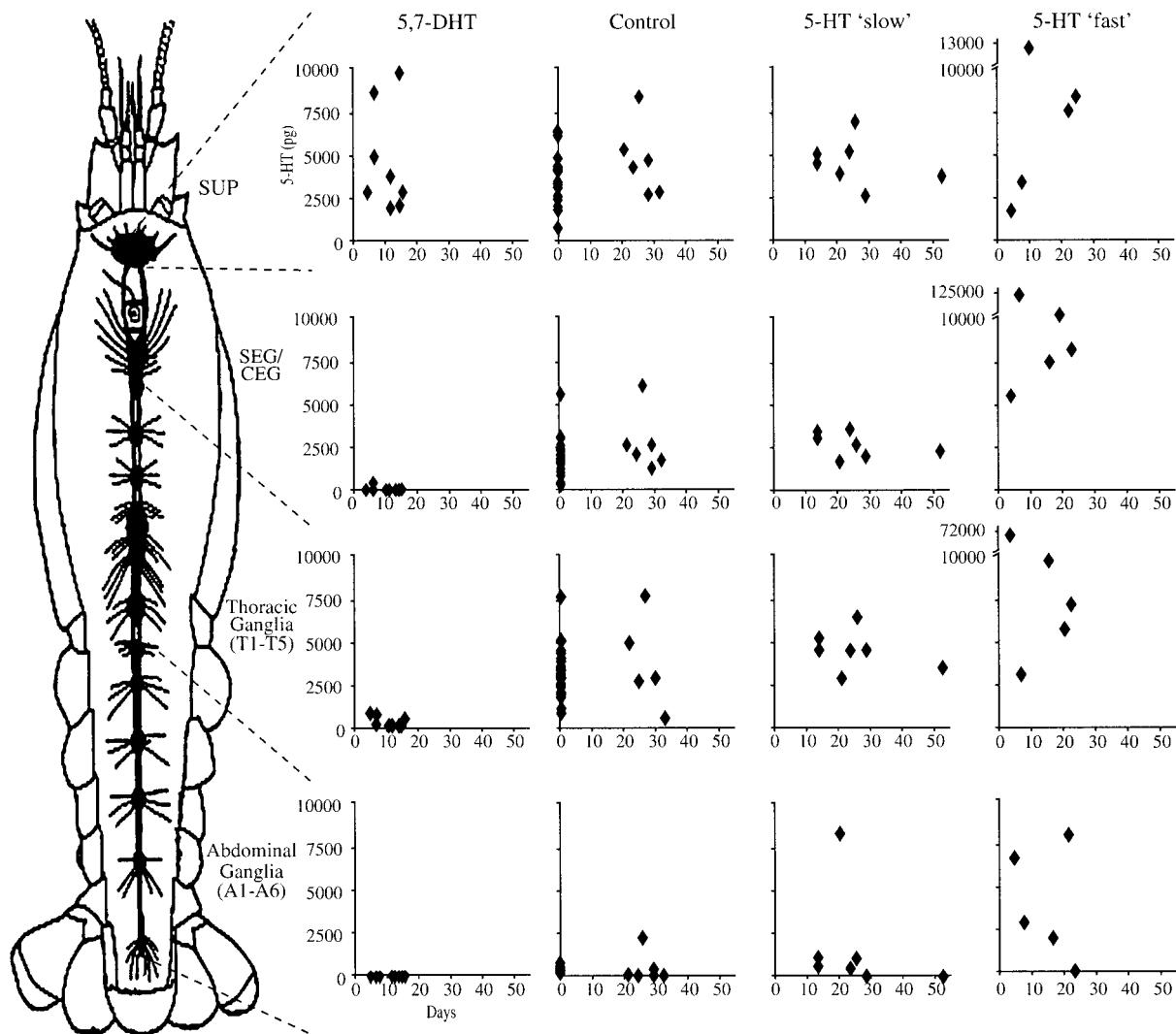
Figure 2 Scatter plots depict the amount of 5-HT (pg) as a function of treatment duration (days). Data points represent individual tissues with treatment groups arranged in columns and tissues arranged in rows.

T1–T5, and SEG/CEG compared to controls (p < .05 for all posthoc tests). 5-HT content in SUP was not altered by 5,7-DHT [$F(4, 52) = 1.54$; $p = .2$]. Results of AMTP treatment are not reported as it neither lowered 5-HT in CNS tissues (except abdominal ganglia) nor produced behavioral effects.

Depletion of 5-HT was evident in 5,7-DHT treated animals after 5 days of treatment and persisted for over 16 days (Fig. 2). No obvious qualitative pattern arose from different lengths of exposure to 5,7-DHT as affected tissues exhibited similar amounts of 5-HT depletion across the entire range of exposure periods. All 5,7-DHT treated crayfish contained considerable amounts of 5,7-DHT in every individual CNS segment (mean ± standard error): (A1–A6) 4.6 ± 1.24 ng, (T1–T5) 9.9 ± 3.89 ng, (SEG/CEG) 4.0 ± 1.02 ng, and (SUP) 2.0 ± 0.33 ng (Fig. 3). As 5,7-DHT

may serve as a 5-HT receptor ligand (see Discussion), the CNS of 5,7-DHT treated crayfish may have thus contained elevated amounts of "neuroactive" molecules [measures of 5-HT and 5,7-DHT combined $F(4, 51)$ 13.43; p < .001]. No significant differences in 5-HT content were found in CNS tissues that were exposed to 5-HT at different rates (see Fig. 1). 5-HT content was generally elevated after short periods of exposure to 5-HT fast tubes, but tended to return to control levels following longer treatment (see Fig. 2).

Behavior

Table 2 summarizes statistical tests performed on all behavioral measures. Significant effects were found
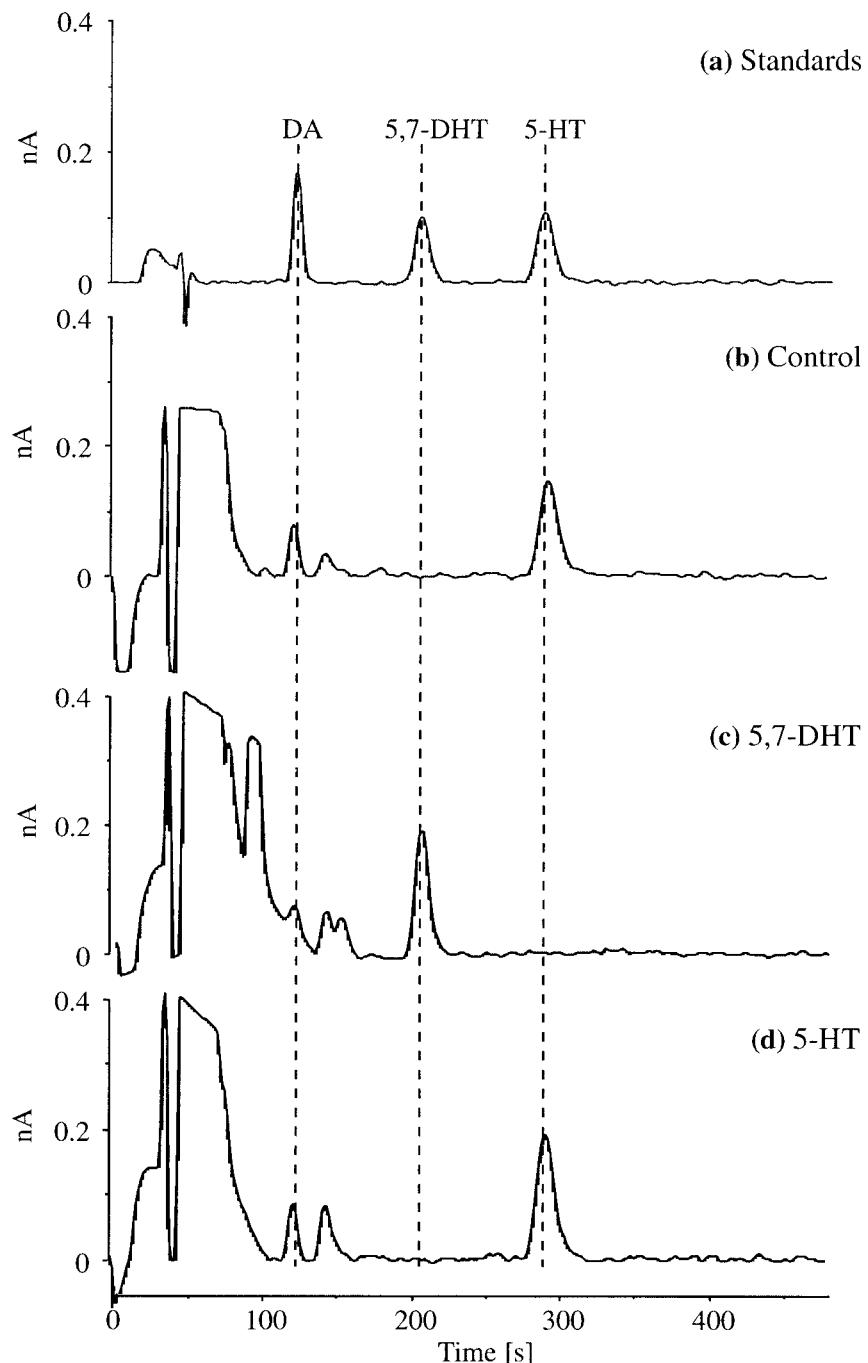
Figure 3 Sample chromatograms for (**a**) an external standard, T1–T5 tissue from (**b**) a control animal, and individuals treated with (**c**) 5,7-DHT and (**d**) 5-HT "slow." Substances are identified based on elution times across a range of mobile phase conditions: (DA) 116–120 s, (5,7-DHT) 198–202 s, and (5-HT) 290–294 s. Note the presence of a conspicuous peak (a likely 5-HT metabolite) that appears soon after the elution of DA.

for the identity of the initiating [$G(4) = 13.42$; $p < .01$] but not the retreating [$G(4) = 4.47$; $p = .4$] animal. Decreased probability ($FTD_{crit} = \pm 1.23$) to initiate fights was found in opponents of 5-HT slow

treated crayfish ($FTD = -1.54$). Moreover, the number of interactions reaching a particular fight intensity depended on experimental condition [$G(8) = 27.96$; $p < .001$]. Low fight intensities were observed in

Table 2 Results from Statistical Tests Performed on Behavioral Variables

Variable	Source	df	SS or -LLH	F or G	p
Initiate	-LLH	4	6.71	13.42	<.01*
	error	28	16.02		
Retreat	-LLH	4	2.23	4.47	.346
	error	38	27.47		
Duration	ANOVA	4	19000.80	1.06	.374
	error	314	1401742.90		
Max. intensity	-LLH	8	13.98	27.96	<.001*
	error	308	317.08		
# Intensity 4	ANOVA	4	0.53	0.44	.777
	error	314	92.92		
Whole model	duration	1	184.82	152.74	<.001*
regression	treatment	4	16.28	3.36	.01*
	dur. × treat.	4	39.25	8.11	<.001*

An α of <.05 was considered significant.

fewer instances than expected ($FTD_{crit} \pm 1.43$) in trials involving 5-HT slow treated crayfish ($FTD = -3.14$). No significant differences (Fig. 4) were found between treatment groups for fight duration [$F(4, 314) = 1.06$; $p = .4$] or for the frequency of unrestrained combat [intensity 4; $F(4, 314) = 0.44$; $p = .8$]. Moreover, to account for the high degree of interrelatedness between fight duration and intensity (Spearman's $r = 0.59$ and see below) MANOVA was performed. No significant differences in fighting behavior were apparent [Wilks' Lambda = 0.99, $F(4, 314) = 1.07$; $p = .4$].

Duration predicted the maximum intensity of interactions [$F(4, 309) = 152.74$; $p < .001$] and also accounted for a large amount of the total variation in fight intensity ($r^2 = 0.35$). In addition, significant treatment [$F(4, 309) = 3.36$; $p = .01$] and interaction [duration × treatment; $F(4, 309) = 8.11$; $p < .001$] effects were detected. The slope of each regression line, which measures the average rate of escalation, varied considerably across treatment groups. Compared to all other treatment groups, 5-HT fast escalated more slowly while 5-HT slow treated crayfish escalated more rapidly (Fig. 5).

DISCUSSION

The biochemical and behavioral effects reported in this article did not result in a single, clear pattern, and thus interpretation becomes complex when such levels of analysis are considered simultaneously. The present findings suggest changes in nervous system function that occurred at levels of organization outside the focus of our experimental design. Three po-

tential, albeit not mutually exclusive, explanations for the results of the present study are discussed.

Anatomical Location of Serotonin Neurons Involved in Decapod Aggression

Previous work implicates neurohormonal modulation as one mode of action for 5-HT in crustacean aggression (Beltz and Kravitz, 1987; Kravitz, 2000). 5-HT is released into the circulation from neurosecretory terminals originating in the first abdominal (A1) ganglion (Beltz and Kravitz, 1987), allowing it to potentially affect many areas concurrently (Pasztor and Bush, 1989; Glusman and Kravitz, 1982; Listerman et al., 2000; Santos et al., 2001). Mediated through an apparent change in activity of neurons involved in aggressive motivation, raising concentrations of 5-HT in the hemolymph fosters a decreased willingness to retreat in subordinate crayfish and lobsters (Huber et al., 1997a,b, 2001b; Huber and Delago, 1998).

In the current study, the collective lack of behavioral effects measured in individuals depleted of 5-HT in the A1 ganglion demonstrates that, at this site, decreasing levels of 5-HT does not by itself prevent the normal expression or utility of agonistic behaviors. Large reductions in 5-HT, however, were not found in all CNS tissues. For instance, 5-HT content in the supraesophageal ganglion was particularly resistant to chronic treatment as it was unaffected in all treatment conditions. HPLC/ED detected the presence of 5,7-DHT in every tissue, including the supraesophageal ganglion, suggesting that, relative to other CNS tissues, crayfish brain is resistant to such pharmacological intervention. It also indicates that failure
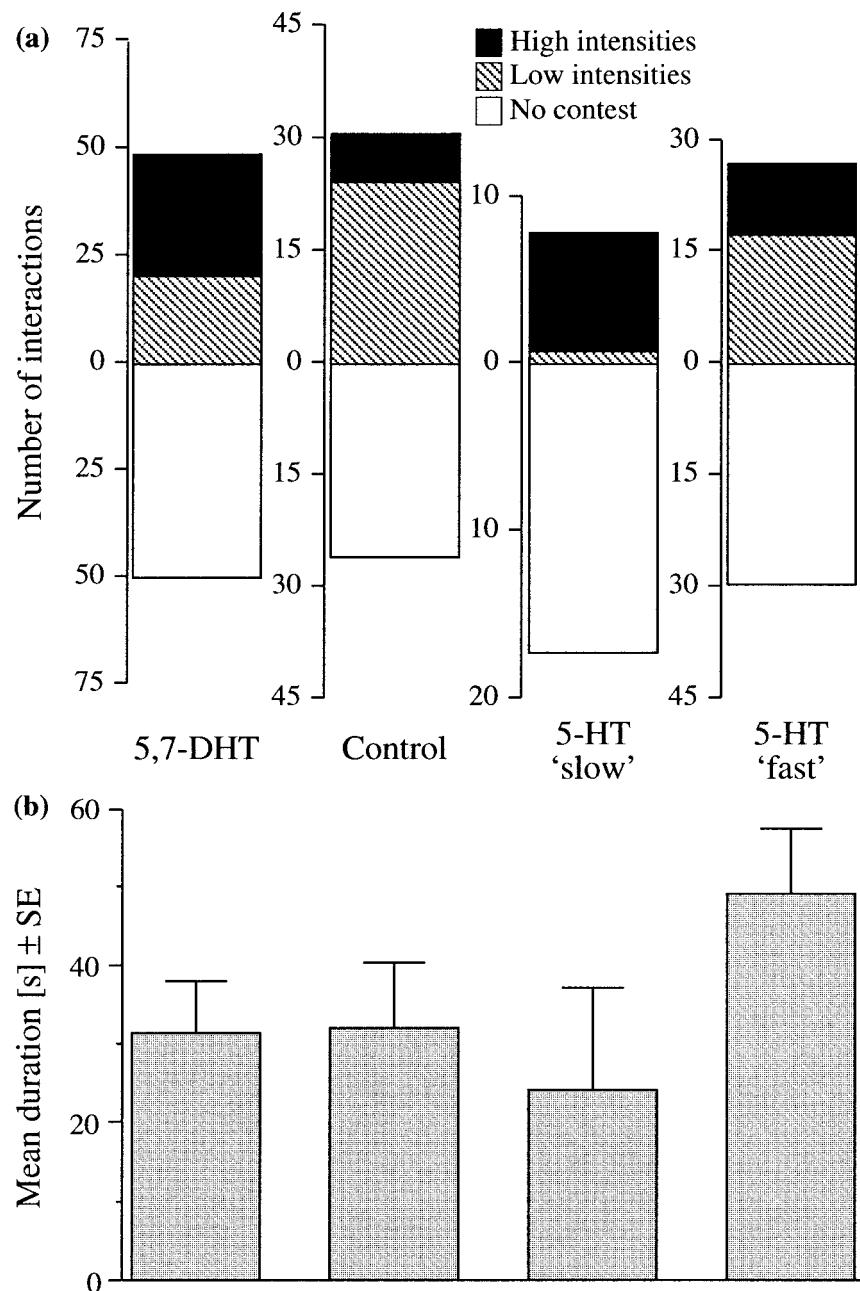
Figure 4 Measures of aggression were largely unchanged by experimental treatments. (**a**) Total number of interactions observed is plotted on the ordinate: (5,7-DHT) $n = 100$ in eight trials, (control) $n = 63$ in five trials, (5-HT "slow") $n = 26$ in four trials, and (5-HT "fast") $n = 65$ in five trials. The number of fights reaching a particular intensity is represented with different shades. Intensities were pooled to reduce empty cells in statistical analyses: (No contest) intensity 0, (low intensities) intensity 1 or 2, and (high intensities) intensity 3 or 4. (**b**) Average duration of interactions for experimental groups.

to alter 5-HT content in crayfish brain is not simply due to the existence of a functional blood brain barrier. Treatment may, however, have potentially induced changes at a variety of other levels including synaptic availability (Doernberg et al., 2001) or the

recruitment of non-5-HT neurons (Beltz et al., 1998; Musolf and Edwards, 2000).

5-HT neurons in decapod crustacean brain have been identified (Beltz and Kravitz, 1983; Sandeman et al., 1988) and studied (Sandeman and Sandeman,
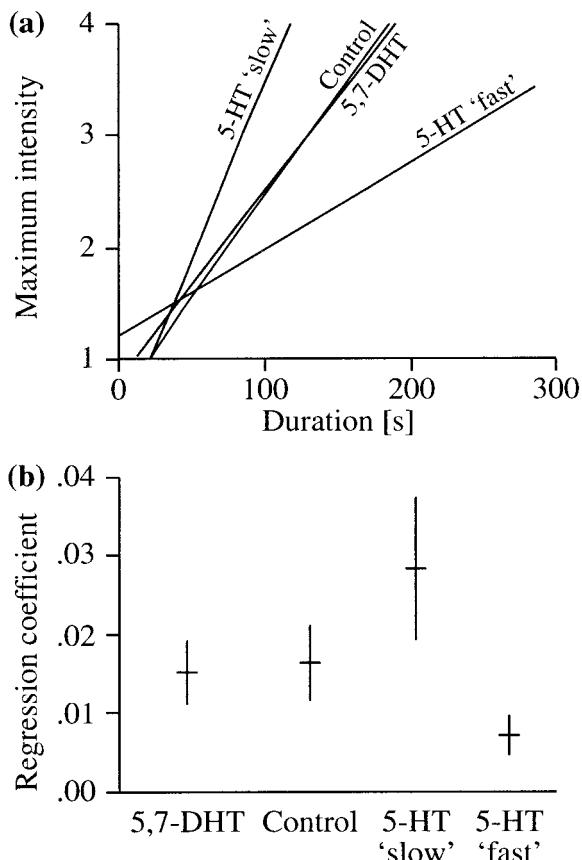
Figure 5 The slope (i.e., regression coefficient) of each regression line represents the average rate of escalation and provides an estimate for the maximum intensity of an agonistic encounter given a certain duration. (**a**) The linear regression functions for each experimental group were: (5,7-DHT) $y = 0.843 + 0.017\ x$, (control) $y = 0.668 + 0.018x$, (5-HT "slow") $y = 0.334 + 0.031x$, and (5-HT "fast") $y = 0.224 + 0.008x$. (**b**) Graph of regression coefficients of the linear function for experimental groups and their respective 95% confidence intervals.

1994; Benton et al., 1997), although their individual roles during agonistic encounters have not yet been explored. In our experiments, failure to alter 5-HT in the brain could account for the lack of behavioral effects found in 5,7-DHT treated crayfish. Key sites in the brain, such as the dorsal giant neurons (Benton et al., 1997; Benton and Beltz, 2001), are likely to be important during such situations—keeping in mind that complex behaviors like aggression presumably cannot be reduced to a single anatomical site or neurochemical substrate. Such "higher" brain areas may exert descending influence on postural systems or neurosecretory sites. Future studies are therefore needed to identify specific behavioral effects of 5-HT associated with the supraesophageal ganglion.

Does 5,7-DHT Have "Neuroactive" Properties in the Crustacean Nervous System?

Acute injection of 5,7-DHT into decapod crustaceans elicits a posture resembling the "dominant-like" stance characteristic of 5-HT injections (Livingstone et al., 1980; Glanzman and Krasne, 1986; Antonsen and Paul, 1997). Such results could be due to 5,7-DHT's releasing properties (Cook and Orchard, 1993) or an agonist-effect at 5-HT binding sites. At least at some synapses, however, different physiological effects result from application of 5-HT and 5,7-DHT. 5,7-DHT produces modest depolarization of specific neurons in the A1 ganglion (Doernberg et al., 2001), while 5-HT mediates comparatively stronger depolarizing activity followed by periods of prolonged inhibition (Heinrich et al., 1999). Nevertheless, 5,7-DHT treatment reduces 5-HT content in decapod CNS (cf. Doernberg et al., 2001 and this article). With regard to the present experiments, however, care must be invoked when interpreting the general lack of behavioral effects accompanying such depletions. Namely, is reducing 5-HT levels (with 5,7-DHT treatment) equivalent to depressing 5-HT function? The present results raise the caveat that nonspecific actions of 5,7-DHT, at least to an extent, may counterbalance its depleting effect on 5-HT content.

Compensatory Responses to Pharmacological Interventions

The complex array of physiological and behavioral results illustrated in this study indicate that no simple pattern links long-term changes in 5-HT levels and fighting behavior. For instance, neither 5-HT treatment significantly altered 5-HT content in the CNS, although such treatments produced opposite effects on the rate of fight escalation. Resistance in altering 5-HT levels due to chronic pharmacological intervention may result from the system's ability to maintain a homeostatic set point (i.e., a functional balance). The existence of compensatory mechanisms is consistent with similar reports of failure to disrupt 5-HT levels in the brain of a sea slug, *Tritonia diomedea* (Fickbohm et al., 2000), where 5-HT was uninfluenced by most pharmacological treatments although its precursors and metabolites were. The notion that "appropriate" levels of 5-HT are needed for a given site and time (Doernberg et al., 2001) corroborates the suggestion that crustacean 5-HT systems can compensate to maintain functional integrity. Moreover, compensatory feedback between monoamine systems and chronic biochemical intervention has been demon-

strated at the level of receptor turnover (Patel et al., 1996; Woo et al., 1996), synthesis (Stachowiak et al., 1986; Sivam, 1995), metabolism (Ase et al., 2000), reuptake (Gobbi et al., 1994; Pan et al., 2001), and synaptic availability (Lent, 1984; Hall et al., 1999). As is apparent from results in other invertebrate systems (O'Gara et al., 1991), the present experiments suggest that such mechanisms were not only activated, but were ultimately manifested at the behavioral level.

The behavioral effects associated with chronic 5-HT treatment (in this study) were unlike those that accompanied acute treatment with 5-HT (Huber and Delago, 1998). Decreased retreat, the main behavioral effect of acute 5-HT infusion, closely parallels the behavior of dominant crayfish during the initial periods of hierarchy formation (Goessmann et al., 2000). In contrast, slow 5-HT treatment produced a behavioral change (i.e., increased escalation) similar to what is found in dominant crayfish after several days of behavioral reinforcement (Issa et al., 1999; Goessmann et al., 2000). Such findings have implications for the formation of social dominance in crayfish. Namely, are compensatory mechanisms, similar to those apparent in 5-HT treated animals, also activated within natural contexts? Recent work supports such a possibility. For instance, CNS levels of 5-HT do not vary as a function of social rank (Huber et al., 2001b). Moreover, established dominance facilitates dynamic, synaptic alterations that occur at multiple time frames (Yeh et al., 1996,1997; Krasne et al., 1997). Rather than the absolute amount of 5-HT in the CNS, it is likely that magnitude, duration, and temporal pattern of release, as well as synaptic neuromodulator ratios, are critical for determining the resulting behavioral phenotype (Huber and Delago, 1998; Crider and Cooper, 1999; Listerman et al., 2000; Peeke et al., 2000; Sneddon et al., 2000; Kravitz, 2000; Teshiba et al., 2001).

CONCLUSIONS

As a motivational state, the term *aggression* is invoked as an intervening concept because it is of inherent utility for predicting a range of behavioral acts. The explanatory power afforded by such a concept, however, is diminished when each of its proximate mechanisms are viewed as static characteristics. Previous attempts to study "aggression" have centered on implementing pharmacological compounds with broad and often unknown actions (Olivier et al., 1989; Fuller, 1996), or "knocking out" one component of a particular system for a prolonged duration (Chen et al., 1994; Saudou et al., 1994; Cases et al., 1995). Despite the information derived from such approaches, the current status of our understanding is, in fact, that we understand very little about 5-HT's role in aggression without considering specific brain regions (Blanchard et al., 1991; Amstislavskaya and Kudryavtseva, 1997; Korzan et al., 2000), genetic history (Brunner et al., 1993; Cases et al., 1995), or social context (Ison et al., 1996; Yeh et al., 1997; Berton et al., 1999; Ferris, 2000). Moreover, the role of 5-HT in aggression may depend heavily on its interaction with other molecules (Ferris and Delville, 1994). A single neural substrate, such as amounts of a particular neuromodulator, is unlikely to explain the functional role of monoamine systems in behavior (Lederhendler and Schulkin, 2000). Similar perspectives have emerged from work on steroid hormones in developing insect (Henrich and Brown, 1995) and avian (Arnold and Schlinger, 1993; Ottinger et al., 1997) species, the effects of neuromodulators on network properties (Johnson and Harris-Warrick, 1990; Katz et al., 1994; Stevenson and Meuser, 1997), and environment-mediated alterations in neurochemical systems (Fox et al., 1997; Yeh et al., 1997; Soma et al., 2000). Such systems seem to *foster* adaptive behavior within the proper context (Kravitz, 1988).

Taken together, the biochemical and behavioral results of the present experiments make clear that although our goal was to measure effects of absolute 5-HT content on aggression, it is likely that we observed the behavioral effects caused by a complex cascade of neurochemical changes. Moreover, the present study underscores the importance of verifying the effectiveness and specificity of pharmacological substances in every system in which they are used. In conjunction with findings of Doernberg et al. (2001), an increasingly dynamic view of the neurochemical basis of aggression has emerged from recent work in crustacean species. A more productive framework for studying the interface between neuromodulators and behavior may be to view such molecules only as short-term modulatory components embedded within larger, dynamically organized systems that are critical for governing behavior. Chronic removal of one element will thus fail to cause a single, well-defined deficit, but rather it will reconstitute the entire neuronal environment. Accordingly, combining physiology and molecular biology with quantitative behavioral analyses is a principle goal for future research. In decapod crustaceans, such a combination offers a fruitful avenue for bringing behavioral analyses down to the level of individual neurons and thereby allowing for a more thorough understanding of causal mechanisms in aggression.

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