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Serotonin alters decisions to withdraw in fighting crayfish, *Astacus astacus*: the motivational concept revisited

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Abstract The biogenic amine serotonin is thought to play an important role in aggression in many species, including man. This paper summarizes experimental approaches which attempt to link this neuromodulator with fighting in a crayfish model for which the complex agonistic behavior is well characterized. Based on a quantitative analysis of fighting we demonstrate that the infusion of small amounts of serotonin into freely-moving crayfish alters fighting behavior by specifically interfering with the timing of a treated animal's decision to withdraw from an encounter. In the presence of added serotonin, fights last considerably longer compared to controls, but no changes were detected in the rules of escalation, the likelihood of initiating an interaction, or its eventual outcome. Attempts to dissect the underlying neuronal mechanisms pharmacologically hinged on fluoxetine as a potent inhibitor of serotonin re-uptake. Although no behavioral changes were associated with acute infusion of fluoxetine alone, in combination with serotonin it effectively prevented the previously observed fight-enhancing effects. Our data strongly support the significance of functional amine re-uptake mechanisms for behavior and continued use of this invertebrate model should prove a promising route to unravel further the complex bases of aggression.

Key words Biogenic amine · Fluoxetine/Prozac · Aggression · Agonistic behavior · Motivation

Abbreviations *5HT* 5-hydroxytryptamine · *DFA* discriminant function analysis · *PCA* principal components analysis

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Introduction

The term motivation collectively refers to all reversible, short-term alterations in behavior not associated with fatigue or learning – irrespective of the underlying mechanisms. The use of such an intervening concept has proven helpful in many ways but we have to accept that if we knew exactly how a particular behavior was produced we would have no need for this term at all (Dawkins 1995). It is widely believed that neuromodulators relate to "motivational" changes in arousal, behavioral states, emotions, and mood by simultaneously altering the activity of many neural decision-making centers (Nader et al. 1997). Rather than produce behavior per se these substances may fine-tune ongoing activity and, in a given context, promote the occurrence of adaptive behaviors over contra-adaptive ones (Kravitz 1988). However, the very characteristics that make modulators so attractive for a distributed orchestration of behavior also make them inherently difficult to study. To examine their role it is essential that we combine studies of physiology, biochemistry, and molecular genetics with quantitative, behavioral analyses.

Biogenic amines occur in the nervous system of all animals and include several prime candidates for behaviorally active compounds. Serotonin, norepinephrine and dopamine are all strongly implicated in human neurological disorders, psychiatric disturbances, uncontrolled aggression, alcoholism and drug abuse (Eichelman 1990; Goodman et al. 1990; Jimerson et al. 1990; Mauri et al. 1996). Central neurons containing these substances often exhibit widespread projection patterns with release sites generally lacking specialized postsynaptic features. Thus, biogenic amines can spread across large regions of neuropil (i.e., "volume transmission"; Fuxe and Agnati 1990) or even throughout the general circulation and may thereby interact with many different sites concurrently. Well established in this context are the multiple and parallel effects of epinephrine which orchestrates a variety of physiological and biochemical

aspects during "fight/fright/flight" situations (Schmidt-Nielsen 1997). The specificity of the response arises from the fact that only a small subset of neurons contains receptors for a particular modulator. Amine receptors are commonly associated with G-proteins tied to second messenger systems and are in only few cases ligand-gated ion channels. Both the lack of anatomically-contained release in classical synapses, and its coupling to predominantly, "slow" signal transduction cascades are indicative of more dynamic, sustained, modulatory interactions with target neurons (Vaughan 1988) rather than rapid, direct, and primary effects.

Of the various substances in this group, serotonin (5-hydroxytryptamine, 5HT) has received a disproportionate amount of attention in recent years as evidence of its involvement in a broad spectrum of behavioral phenomena has mounted (Roth 1994; Weiger 1997). In particular, hypotheses suggesting a role in aggression in a wide range of taxa including man (Burrowes et al. 1988; Coccaro 1989, 1992), are supported by correlative evidence (Kruesi et al. 1990; Mehlman et al. 1994; Reisner et al. 1996), studies of family histories (Brunner et al. 1993; Coccaro et al. 1994; Virkkunen et al. 1995), amine depletion with neurotoxins or diet (Vergnes et al. 1988; Giammanco et al. 1990; Cleare and Bond 1995), receptor pharmacology (Bell and Hobson 1994; Mühlenkamp et al. 1995; Olivier et al. 1995), application of reuptake inhibitors (Olivier et al. 1989; Hilakivi-Clarke and Goldberg 1993; Fuller 1996), 5HT infusions (Puciowski et al. 1985; Maler and Ellis 1987; Raleigh et al. 1991), and genetic knock-out studies (Saudou et al. 1994; Cases et al. 1995; Hen 1996). In vertebrates, decreased effectiveness of serotonin is generally accompanied by increased levels of aggression, while in invertebrates the converse appears to be true (Weiger 1997).

Several lines of evidence suggest that in lobsters, crayfish, and other decapod crustaceans, an increase in 5HT neuron function is closely associated with aggressive or dominant behavior (Huber et al. 1997b). Direct injections of substantial amounts of serotonin (1–10 mg) produce a posture resembling "meral spread", a threat stance commonly seen in dominant animals, where the animal stands high on its walking legs and lifts up the claws (Livingstone et al. 1980). Within the central nervous system a general activation of contractor muscles in thorax and abdomen is elicited to produce this posture (Kravitz et al. 1985) and an increase in activity of serotonergic neurons enhances these motor systems (Ma et al. 1992). Neurons within local circuits controlling tail flip, a common behavior of retreat (Glanzman and Krasne 1983, 1986; Bustamante and Krasne 1991), exhibit reduced responsiveness in the presence of serotonin, and changes in their excitability (Krasne et al. 1997) and serotonin receptor subtype populations (Yeh et al. 1996) have been reported as a consequence of social status.

Once elicited, agonistic behavior of clawed decapod crustaceans proceeds according to prominent rules of conduct with success based largely on physical superiority (Glass and Huntingford 1988). Individual agonistic interactions escalate with a temporal sequence of characteristic behavior patterns and end when one of the combatants withdraws. In a typical scenario, fight intensity slowly increases with extensive threat displays upon first contact, then continues with phases of ritualized aggression and restrained use of the claws, and frequently ends in brief periods of unbridled combat (Huber and Kravitz 1995). These results are in close agreement with predictions of game theory (Parker and Rubenstein 1981; Enquist and Leimar 1983; Austad 1989), where animals acquire information about the opponents' strength and fighting abilities in a stepwise manner (i.e., assessment strategies) during fights. In this context, the timing of the decision to withdraw by either animal becomes the key factor in determining the duration and progress of resulting encounters (Maynard-Smith 1974; Bishop and Cannings 1978). Decisions may range from a brief meeting in many cases, to prolonged fighting when physical asymmetries are small (Scrivener 1971; Atema and Cobb 1980).

The presence of such a highly structured, quantifiable behavioral system, combined with the potential to bring the analysis to the level of individual neurons, thus offers us unique opportunities to explore the relationship between serotonin and aggression in this species. First we infused serotonin into freely moving crayfish and examined whether subsequent agonistic interactions differed in their temporal structure, eventual outcome or the occurrence of specific agonistic behavior patterns. Using multivariate statistical techniques we then identified the particular characteristics that were most altered as a result of amine treatment. Finally, pharmacological manipulations with infusions of serotonin in the presence and absence of the high-affinity serotonin re-uptake inhibitor fluoxetine (Prozac) were employed to further characterize the neural mechanisms underlying these behavioral processes.

Materials and methods

Experimental animals and general laboratory setup

Juvenile crayfish, *Astacus astacus*, were obtained from local, commercial suppliers and maintained under controlled environmental conditions in a holding facility at the Department of Zoology, University of Graz. Only healthy specimens with intact appendages were used. Body mass ranged from 12 g to 52 g corresponding to a carapace length of 2–6 cm and 2–3 years of age. Crayfish which had molted within a week or which showed signs of imminent molt (e.g., softened cephalothorax around gill chamber, new carapace visible within uropods), were not used. At least 5 days prior to the experiment, animals were moved to individual containers permitting visual and tactile but not chemical isolation. All containers and experimental observation chambers were supplied with water from a central holding tank (ca. 2000 l) where water was partially recirculated, filtered, aerated, and held at stable temperature (12 °C in winter; 18 °C in summer). Animals were fed twice a week ad libitum with pelleted fish food (Tagger, Graz, Austria).

The observation chamber (46 cm wide × 21 cm deep × 14 cm water level) was constructed from white, non-reflecting Plexiglass,

and a glass front pane. Tanks were filled with gravel to a depth of 1 cm and partitioned into two adjacent compartments of equal size by a removable plastic divider. Within each compartment, the back pane contained holes for water in-flow located towards the midline of the aquarium and out-flow close to the aquarium corners.

Delivering substances into freely moving animals

Fifty-three pairs of crayfish were chosen each with a size difference approximately 35–40% (descriptive statistics in Table 1). The smaller animal of each pair was fitted with a fused-silica fine-bore cannula connected to a syringe pump (Razel Scientific Instruments, Stamford, Conn., USA, Model A-99). Using a 26-gauge needle a small hole was drilled through the dorsal carapace within the caudal third of the pericard, a few millimeters to the right of the midline to avoid damaging the underlying heart. Ten centimeters of fused silica tubing (J & W Scientific, 160-2644) were cut, pre-rinsed with 125 mmol·l^{-1} NaCl, and fitted with a small piece of paper towel 3 mm from one end to restrict protrusion of the tube into the sinus. The tubing was then placed into the hole and secured with small drops of cyano-acrylate 'Superglue' and small pieces of paper towel (Fig. 1a). Deactivated needle material (J & W Scientific, 160-1010) was cut to 0.5 m length and both ends reinforced with 1 cm of 0.250 OD fused silica material (J & W Scientific, 160-2255). A small piece of 0.28 mm ID polyethylene tubing connected one end of this tubing to a blunt-tipped, hypodermic needle (26 ga $\times 1/2''$). The needle was attached to a 1-ml syringe filled with 125 mmol·l^{-1} NaCl and the assembly positioned in the infusion pump. After the entire length of the canula was pre-rinsed with NaCl it was connected with PE tubing material (see above) to the short piece of fused silica already attached to the animal. Serotonin (5HT creatinine sulfate complex; FW: 387.4; Sigma H-7752) was purchased from commercial sources. Fluoxetine hydrochloride (Prozac), a potent inhibitor of high-affinity serotonin re-uptake mechanisms, was supplied through the generosity of Mrs. M Niedenthal of the Lilly Research Laboratories and Dr. M. Angerer of Lannacher Heilmittel, Graz. Further biochemical particulars of its action in crustaceans have been presented elsewhere (Huber et al. 1997a).

The implanted animal was placed into a compartment of the observation tank with its larger opponent in the adjacent cell. Following an acclimation period of 10–15 min the divider was removed and all interactions were recorded on videotape (Hi-8, Sony CCD V6000E) along with a time display for 3 h. After 30 min (pre-injection period, Fig. 1b) the syringe pump was turned on to deliver 0.125 mol·l^{-1} NaCl into the smaller animal at a rate of 8 µl min^{-1} for another 30 min (control injection period). The infusion then switched to saline containing either serotonin, fluoxetine, or serotonin together with fluoxetine at a rate of 3 µg min^{-1} for each substance for 1 h (substance injection period). After the syringe pump was turned off, behavioral observation continued for another hour (post-injection period).

Table 1 Descriptive statistics for 53 pairs of crayfish, *Astacus astacus*, used in fighting experiments. The number of pairs in each experimental group (*n*) are listed with the average mass of the smaller, treated animal (mean ± standard deviation), the relative size advantage of the larger opponent (mean% ± standard deviation), and the sex composition (M – male, F – female) within these pairs

Experiments	N	Mass (g)	% Size Difference	Sex composition
5HT	20	23.12 ± 10.15	139.78 ± 9.02	18 MM, 1 MF, 1 FF
Fluoxetine	11	26.33 ± 9.24	135.03 ± 9.48	11 MM
5HT and fluoxetine	22	30.76 ± 10.47	139.25 ± 8.22	22 MM

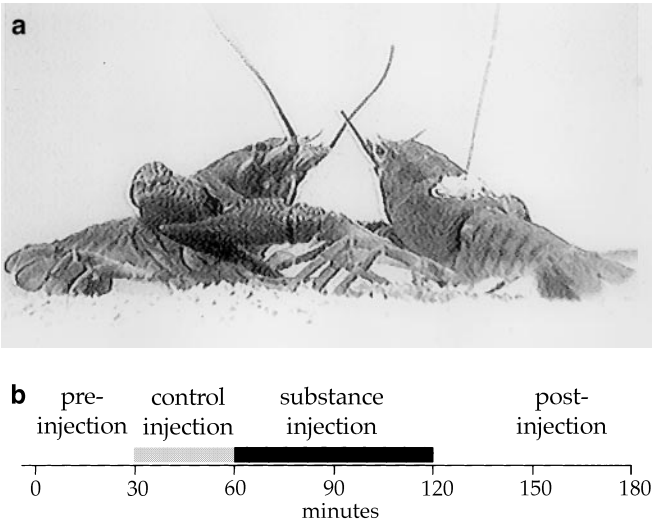


Fig. 1a, b Experimental setup for delivering substances into freely moving crayfish. **a** A fused-silica fine-bore cannula connected to a syringe pump was implanted into the pericardial sinus. The implanted animal was brought together with a 30% larger opponent. **b** After 30 min (pre-injection period) a syringe pump delivered 125 mmol·l^{-1} NaCl at a rate of 8 µl min^{-1} for 30 min (control-injection period). For 1 h, the infusion then switched to saline containing serotonin alone, fluoxetine alone, or serotonin and fluoxetine together at a rate of 3 µg min^{-1} (substance-injection period). After the syringe pump was turned off, behavioral observation continued for another hour (post-injection period)

Although we did not directly measure amine hemolymph concentrations at different stages of the experiment, we can infer its levels for particular hemolymph volumes when we combine the rate of infusion controlled by the pump and the speed at which the injected compound is eliminated from the hemolymph through metabolic degradation or uptake into surrounding tissues. In a series of accompanying experiments, the rate of removal was determined in another set of animals by injecting a single pulse (10–20 µg) of amine into the dorsal hemolymph space and measuring the amount remaining over time using high-performance liquid chromatography with electrochemical detection (HPLC-EC). For a period of 120 min after injection, a series of hemolymph samples were removed from the pericardial sinus at intervals ranging from 2 min – 50 min. Eighty microliters were immediately pipetted into a 1.5 ml microfuge tube containing 20 µl of 0.5 mol·l^{-1} perchloric acid, the insoluble residue consisting of denatured proteins and cell debris was pelleted in a table top centrifuge (15 min, 15 000 rpm, Beckman Microfuge), and the clear supernatant diluted 100–1000-fold with mobile phase. A 20 µl sample was applied directly onto a reverse-phase C_{18} column (Spherisorb ODS2, 3 µm, 100 mm × 4.6 mm) for isocratic separation (see Benton et al. 1997 for details). At hemolymph concentrations comparable to those during our infusion experiments, serotonin is characterized by a half-life of 10.1 min (Fig. 2a). Thus, when the infusion is switched on, hemolymph serotonin levels rise rapidly, stabilize at a concentration of approximately 10^{-5} mol·l^{-1} within 10 min (Fig. 2b), and return to base level within 13 min after the pump is again switched off.

Quantitative behavioral data

In a total 3425 agonistic encounters (Table 2) five behavioral measures were quantified, namely fight duration, two measures of intensity, and the identities of the initiating animal and the one which eventually retreated. The beginning of an interaction was defined as the time when two opponents advanced within one body length and overtly reacted to each others presence. The ap-

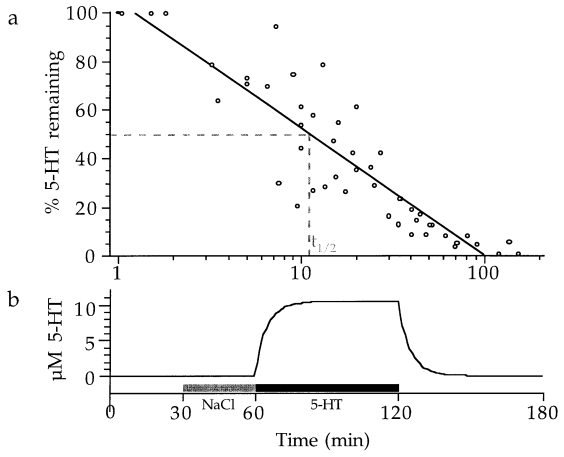


Fig. 2a, b Rates for experimental infusion and organismic removal of serotonin in *A. astacus* hemolymph. **a** To estimate how rapidly serotonin is removed from circulation, a single dose (10–20 µg) was injected into the pericardial sinus and the decrease in serotonin concentration over time (percentage substance remaining relative to first measure at 1–2 min) determined using high-performance liquid chromatography with electrochemical detection (HPLC-EC). A logarithmic function ($y = 104.61 + -52.187^{log(x)}$; $R^2 = 0.833$) was fitted to the data to estimate the time at which half the substance was removed from hemolymph. The *dotted line* indicates a serotonin "half-life" time $t_{1/2}$ of 10.1 min. **b** Knowledge of the rate at which serotonin is infused and removed by surrounding tissues allowed us to model the dynamic serotonin hemolymph concentrations at different stages of the experiment. The concentration of serotonin in an animal with a hemolymph volume of 8 ml (corresponding to a body weight of 30–35 g) rises rapidly when the infusion switches to serotonin (3 µg 5HT $min^{-1} \cdot l^{-1}$). Hemolymph levels plateau around 10 $\mu mol \cdot l^{-1}$ within 10 min, and rapidly decay back to below 100 $nmol \cdot l^{-1}$ within 13 min of the infusion pump being turned off

proaching animal was termed the initiator. As the interaction progressed, maximum intensity was determined according to the following criteria: (0) *no fighting*: neither animal attacked its opponent or one animal consistently retreated from the advances of the other; (1) *threat postures*: both animal contested the interaction using threat displays or ritualized fighting without resorting to the use of their claws; (2) *claw lock*: both animals contested the encounter and at least one animal used its claws to grab onto the opponent; (3) *strike and rip*: both animals contested the encounter and at least one animal made unrestrained use of the claws in an attempt to rip or tear off an opponent's appendages. If fights progressed to unrestrained combat, the number of such episodes was noted. The fight ended when one animal turned or walked away so that separation distance exceeded one body length, and the identity of the retreating animal was recorded.

Statistical evaluation of treatment effects

Univariate analyses

Based on the starting time, each interaction was assigned to one of six 30-min treatment periods (Fig. 1): pre-injection (I – between removal of divider and start of NaCl infusion), control injection (II – NaCl infusion), substance injection (III, IV – 1 h infusion of substances was split into two half-hour periods), and post-injection (V, VI – the time after the infusion pump was turned off was again split into two half-hour periods). Differences in behavior among these groups of interactions were compared by either ANOVA (Analysis of Variance) in the case of continuous data (e.g., fight duration) or Analysis of LogLikelihood for categorical and frequency data (i.e., which animal initiated or retreated). The latter analysis is comparable to ANOVA since the negative log likelihood for categorical data plays the same role as do sums of squares in interval data (JMP 2.0, SAS Institute 1994).

Multivariate analyses

A conceptual problem in assigning behavioral differences arises if the various fighting characteristics are related to some degree. Escalating fights will automatically reach higher levels of intensity the longer they last (Huber and Kravitz 1995). Moreover, since attacking crayfish have a higher chance of success (Rubenstein and Hazlett 1974), the progress a fight takes may depend on which animal initiates and which one retreats. If such dependencies exist, then variables need to be considered simultaneously within a multivariate design to include information about these relationships. In addition, the degree to which variables occur together provides valuable information about the underlying behavioral structure of fighting. Principal Components Analysis (PCA) is well suited to test for the existence of such correlations in behavioral data (Temoshok et al. 1988; Bouchard and Lynch 1989; Huber and Kravitz 1995; Rodgers and Johnson 1995). It identifies which behaviors tend to vary together as linked groups and thus identifies the number of independent, underlying behavioral dimensions present in the data (Sokal and Rohlf 1981; SPSS 1988). Discriminant Function Analysis (DFA) was used to test whether behavior differed among the half-hour experimental periods described above ("Specify Model", JMP 2.0, SAS Institute 1994), and if so, which behavioral characteristics best distinguished between them (Seal 1964).

Although all included behavioral variables should ideally be derived from multivariate normal distributions, PCA and DFA

Table 2 Summary statistics demonstrate that fighting was most pronounced during the later stages of 5HT infusion. Listed for each group are the number of 30-min observation periods (No. Obs.), the total (No. Int.) and average (Mean Int.) number of interactions in these periods, fight duration (mean ± standard error), and the mean percent of time spent interacting with the opponent

Period	Treatment group	No. Obs.	No. Int.	mean Int.	duration (s)	% Time
Pre-inj.	No injection	53	918	17.32	639.42 ± 46.0	35.52
Control inj.	NaCl	53	665	12.55	537.38 ± 50.5	29.85
Substance inj.	5HT (0–30)	19	233	12.26	616.11 ± 71.6	34.23
	5HT (30–60)	18	176	9.78	684.61 ± 116.6	38.03
	5HT/fluoxetine (0–30)	22	221	10.05	542.68 ± 82.3	31.37
	5HT/fluoxetine (30–60)	21	178	8.48	522.33 ± 110.8	29.02
	Fluoxetine (0–30)	11	156	14.18	564.36 ± 88.0	31.36
	Fluoxetine (30–60)	11	96	8.73	317.36 ± 70.2	17.63
Post-inj.	Post 5HT (0–30)	17	172	10.12	822.94 ± 138.2	45.72
	Post 5HT (30–60)	16	166	10.38	339.44 ± 67.03	18.86
	Post 5HT/fluoxetine (0–30)	21	161	7.67	419.91 ± 74.4	23.33
	Post 5HT/fluoxetine (30–60)	21	163	7.76	328.14 ± 65.5	18.23
	Post fluoxetine (0–30)	11	64	5.82	181.27 ± 66.1	10.88
	Post fluoxetine (30–60)	10	56	5.60	144.40 ± 51.5	8.02

should still perform reasonably well if these conditions are not met (Gilbert 1968; Moore 1973), particularly if sample sizes are large. As a precaution, a square root transformation was applied to three measures; fight duration, maximum intensity, and the frequency of intensity 3. Categorical variables were recoded as follows: "who initiated" was recoded to "interaction initiated by the larger animal" resulting in a positive value (+1) for each interaction where this condition was met and negative (−1) where not. An interaction initiated by both individuals simultaneously was awarded a value of zero (0). Similarly, "who retreated" was renamed to "retreat by the smaller animal" with a "+1" in all interactions where the smaller animal turned away from the fight, "0" when both retreated or turned away simultaneously, and "−1" when the larger animal fled. In both variables ("retreat" and "initiated") a constant (+2) was added to each value and a log transformation was performed to reduce heteroscedasticity (i.e., inequality of variances). Significance in Bartlett's Test of Sphericity ($X^2 = 1929.19$; $P \ll 0.001$) indicated that the variables correlate sufficiently well with each other (SPSS 1988) to justify the use of PCA. In addition, the Kaiser-Meyer-Olkin measure of sampling adequacy was in a usable range (Kaiser 1974) with a total matrix sampling adequacy of 0.674.

Results

Fighting behavior of crayfish

Crayfish which had been isolated for several days prior to the experiment rapidly engaged other individuals when placed together in an aquarium. Stereotyped behavior patterns and the structure of the ensuing fights closely resembled those reported for the American lobster (*Homarus americanus*), where fights escalate in a probabilistic manner until one of the opponents withdraws (Huber and Kravitz 1995). In our data set, fight duration significantly explained the maximum intensity reached during encounters ($R^2 = 0.166; F = 681.97; df = 1, 3424; P \ll 0.001$). PCA examined the characteristics of crayfish fights and delineated to what degree behavioral measures are linked (Pearce 1969). A strong association between duration and both measures of fight intensity confirmed that interactions become increasingly violent the longer they last (Fig. 3a). Moreover, which animal initiated or retreated had little influence on how the interaction escalated. This analysis was then performed separately for subsets of fights during: (1) pre- and control periods, (2) serotonin injection, (3) fluoxetine infusion, and (4) fluoxetine and serotonin injected together. The factor structure proved similar in all four instances, with the same number of important axes and virtually identical behavioral characteristics (Fig. 3b), although minor differences in the relative importance of these factors were observed. Thus, the set of "rules" according to which fighting among crayfish progresses was not affected by pharmacological treatment and interactions in all experimental groups tended to escalate in similar ways.

Univariate analyses of behavioral effects

In crayfish, a 30% advantage in body size constituted a strong predictor of dominance, and a few, brief agonistic interactions were sufficient to induce subordinate behavior in the smaller individual. A decrease in fighting corresponded to the formation of dominance as losers began to retreat from the advances of their winning opponent or avoided contact when they encountered it during explorative forays. The infusion of small amounts of NaCl into subordinates altered neither qualitative nor quantitative aspects of fighting. Significant differences in all measures of fighting resulted, however, when the infusion switched to serotonin (Fig. 4a). At that time, mean duration and the intensity

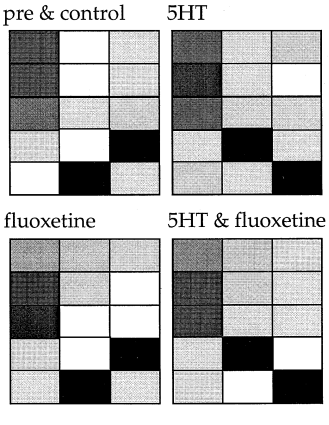
Fig. 3a, b Principal Components Analysis (PCA) identified the underlying behavioral structure and its heterogeneity for all interactions independent of treatment. **a** Three factors were extracted cumulatively accounting for 77.8% of total variation. The factor matrix lists loadings (Varimax rotated) for behavioral variables on each Principal Component (*PC*). Large loadings indicate variables that contribute strongly to a specific factor and are shown *highlighted*. Cases where several variables load highly together, signify the presence of a strong correlation between them, e.g., duration and intensity on factor 1. PC2 and PC3 accounted for similar amounts of variation, 20.0 and 19.3% respectively. **b** Separate analyses were then performed for subsets of interactions during (1) pre- and control injection, (2) 5HT injection, (3) fluoxetine injection, and (4) 5HT and fluoxetine injection and reveal high levels of homogeneity in these patterns. An equal number of significant factors was extracted within each treatment and the same behavioral variables contributed in similar ways to these factors. Slight differences in the importance of these factors were observed, reversing the order of PC2 and PC3 in 5HT and 5HT together with fluoxetine, relative to all other groups. Absolute values of correlation coefficients are depicted by the *darkness* of the corresponding box as indicated

a.

Variable	PC 1	PC 2	PC 3
Duration	0.779	0.035	-0.108
N Intensity 3	0.822	-0.073	0.026
Max. Intensity	0.765	0.133	-0.057
Initiate large	-0.048	-0.993	0.013
Retreat small	-0.073	-0.013	0.995
Eigenvalue	1.927	1.001	0.963
% explained	38.54	20.02	19.26

0.0 - 0.1
0.1 - 0.2
0.2 - 0.3
0.3 - 0.4
0.4 - 0.5
0.5 - 0.6
0.6 - 0.7
0.7 - 0.8
0.8 - 0.9
0.9 - 1.0

b.



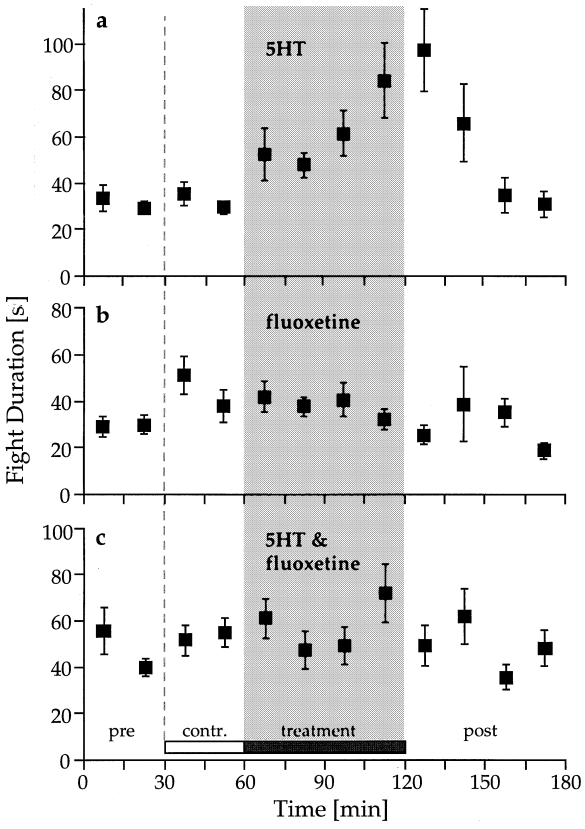


Fig. 4a–c Average duration of encounters summarized for 15-min periods of pre-injection, control injection, injection of substances (5HT, fluoxetine, or 5HT and fluoxetine), and post-injection. Dominance was established quickly as animals began to retreat from or avoid contact with the larger opponent. Infusion of small amounts of NaCl into subordinates did not alter qualitative or quantitative aspects of fighting behavior and dominance. Interactions escalated in the presence of serotonin (**a**) with fight duration returning back to pre-injection levels after the pump was turned off. Fluoxetine alone (**b**) did not result in significant changes in behavior, and presented together with serotonin (**c**), it prevented the behavioral changes associated with the amine

of fights increased steadily as the subordinate animal became less likely to retreat, but returned to pre-injection levels when the infusion pump was again turned off. Such increases in fighting were not observed with fluoxetine (Fig. 4b) or fluoxetine together with serotonin (Fig. 4c). When each behavioral variable associated with fighting was compared separately among treatment periods, significant differences were detected in duration, both measures of intensity, and the identity of the retreating animal (Table 3a). In contrast, the reuptake inhibitor fluoxetine injected together with 5HT (Table 3c) greatly reduced 5HT-associated changes in fighting. Fluoxetine infused alone resulted in few changes in behavior (Table 3b); only fight duration was slightly higher among controls.

Multivariate analyses of behavioral effects

Although the underlying structure of fighting proved similar in all pharmacological treatments, several univariate tests indicated distinct differences in intensity and duration of fights. A highly significant MANOVA confirmed these results (Table 4a), hence the main behavioral characteristics associated with each treatment were explored using canonical DFA. Three discriminant axes (DF1–DF3) together accounted for more than 90% of all differences (Table 4b), and DF1 proved by far the most important, explaining in excess of 59%. A high positive correlation with fight duration and a negative association with retreat were the main features of this axis. A second, less important axis (DF2) represented variation in intensity. Thus, differences among treatment groups primarily reflected changes in fight duration depending on how likely the smaller animal was to withdraw without escalation. In this context, serotonin commanded the most prominent behavioral effects (Fig. 5b) making retreat less likely and thereby increasing the probability of prolonged, escalated fighting. Once serotonin was turned off, behavioral effects quickly reversed and returned to pre- and control injection levels. When fluoxetine was infused alone no such changes in fighting were observed (Fig. 5c), and fluoxetine given together with serotonin effectively prevented the behavioral changes associated with the amine alone (Fig. 5d).

Discussion

Research in neuroethology is driven by a desire to understand the proximal causal mechanisms underlying behavior. Although the term "motivation" is commonly invoked as an intervening concept, its value is confined to situations where it effectively simplifies what we need to understand and predict behavior. If we are forced to postulate separate motivational biases for each behavior pattern we wish to explain, the use of motivations will fail to reduce the levels of conceptual complexity. Historically, motivations have been viewed as a collection of individual, unitary properties corresponding to broad behavioral categories such as eating, parental care, or fighting (Tinbergen 1951; Lorenz 1966). This model has now largely been superseded by a more complex, multi-dimensional view, where each particular behavioral response may arise from a close interaction of several independent physiological or stimulus axes (Immelmann and Beer 1989). Our data are in close agreement with the latter model, as occurrences of agonistic patterns in crayfish map onto several, independent axes and appear to be linked by a complex, underlying system of causal forces. Specifically, behavioral changes associated with serotonin infusion were largely a reduction of retreat with a concurrent increase in fight duration. In contrast, changes taking place when dominance becomes established differ mainly in the intensity of interactions. The success of a "dimensional" approach when applied to behavioral pharmacology (Apter et al. 1990; Tuinier and Verhoeven 1995; Huber et al. 1997b) provides strong support for a multivariate

Table 3 Univariate analyses tested whether individual behavioral variables differed among experimental periods. Duration, both measures of intensity, and the identity of the initiating and retreating animal were compared among six 30-min treatment periods (pre-injection, control injection, treatment injection 1 and 2, and post-injection 1 and 2) for 5HT (**a**), fluoxetine (**b**), and 5HT and fluoxetine (**c**) using univariate Analysis of Variance (continuous data) and Analysis of LogLikelihood (ordinal and nominal variables). Between group effects (ANOVA or -LLH) are listed over within group effects (*Error*) with associated degrees of freedom (*df*), sum of squares (*SS*), mean squares (*MS*), statistics (*F* or chi-squared) and levels of significance (*P*)

	Variable	Source	df	SS/-LogLH	MS	F/Chi^2	*P*
a. 5HT	Fight Duration	ANOVA	5	474413.0	94882.6	11.258	$\ll$0.001
		Error	1372	11562876.0	8427.8		***
	N. Intensity 3	ANOVA	5	6.256	1.25	3.806	0.002
		Error	1372	450.992	0.33		**
	Max. Intensity	-LLH	15	40.162		80.323	$\ll$0.001
		Error	1360	1541.763			***
	Larger Initiated	-LLH	10	6.238		12.476	0.255
		Error	1366	1197.616			ns
	Smaller retreated	-LLH	10	56.284		112.567	$\ll$0.001
		Error	1366	889.114			***
b. Fluoxetine	Fight Duration	ANOVA	5	31655.3	6331.1	2.419	0.035
		Error	742	1941896.4	2617.1		*
	N. Intensity 3	ANOVA	5	1.768	0.35	1.210	0.303
		Error	742	216.888	0.29		ns
	Max. Intensity	-LLH	15	6.103		12.206	0.663
		Error	730	897.134			ns
	Larger Initiated	-LLH	10	5.383		10.766	0.376
		Error	736	622.395			ns
	Smaller retreated	-LLH	10	5.012		10.024	0.438
		Error	736	565.309			ns
c. 5HT fluoxetine	Fight Duration	ANOVA	5	40188.0	8037.6	1.024	0.402
		Error	1293	10147701.0	7848.2		ns
	N. Intensity 3	ANOVA	5	4.560	0.91	1.560	0.168
		Error	1293	755.733	0.59		ns
	Max. Intensity	-LLH	15	6.81		13.620	0.555
		Error	1281	1552.54			ns
	Larger Initiated	-LLH	10	15.34		30.681	$<$0.001
		Error	1287	1026.05			***
	Smaller retreated	-LLH	10	7.09		14.178	0.165
		Error	1287	812.66			ns

Table 4 Results of a Multivariate Analysis of Variance (MANOVA) and Discriminant Function Analysis (DFA) demonstrated highly significant differences in behavior among 30-min treatment periods (pre-injection, control injection, treatment injection 1 and 2, and post-injection 1 and 2) for serotonin, fluoxetine, and serotonin together with fluoxetine. **a** For MANOVA similar statistics and identical levels of significance were obtained with Pillai's Trace, Hotelling-Lawley, and Roy's Max Root. Abbreviations are degrees of freedom (*df*), and level of significance (*P*). **b** Canonical DFA was used to identify those particular behavioral characteristics which best separated the treatment groups. Five discriminant axes were extracted and Eigenvalue (*EV*) and proportion of variance explained (%) are reported below correlation coefficients with duration, maximum intensity, number of intensity 3 (N Intensity 3), which animal initiated (*Init*) and which had retreated (*Retr*). Coefficients exceeding 0.5 or −0.5 are highlighted to improve readability

a. MANOVA	Value	df	Approx. F	*P*
Wilk's Lambda	0.932	65, 16105	3.745	$<$0.001
Pillai's Trace	0.070	65, 17055	3.719	$<$0.001
Hotelling-Lawley	0.072	65, 17027	3.769	$<$0.001
Roy's Max Root	0.043	13, 3411	11.288	$<$0.001

b. DFA	DF1	DF2	DF3	DF4	DF5
Duration		0.198	−0.437	0.359	0.270
Max. Intensity	0.426	−0.417	0.167	0.768	0.166
N. Intensity 3	0.290	−0.671	−0.420	0.036	0.536
Init.	0.139	0.187	0.635	−0.416	0.608
Retr.	−0.687	0.324	0.138	0.360	0.511
EV	0.043	0.014	0.008	0.004	0.003
%	59.80	19.19	11.21	5.11	4.68

view of motivations rather than the more traditional, univariate one. Thus, focusing on simple frequencies of behaviors and ignoring the relationships between them will necessarily fail to account for its underlying organization and will invariably reveal a less than complete subset of true effects.

In crayfish, serotonin greatly enhanced the likelihood of an individual to continue with escalated fighting. Game theory models provide a theoretical framework to assess advantages and disadvantages inherent to various individual behavioral strategies as a function of resource value, prior residency, or size asymmetry (Parker 1974; Enquist and Leimar 1987; Leimar et al. 1991). In this context, agonistic interactions are viewed in terms of individual decisions, such as whether and when an animal "chooses" to initiate encounters, escalate towards a higher level of intensity, or withdraw. Several of these decisions appear to be differentially constrained by sensory limitations or fight structure. In crayfish, initiating interactions (when an animal approaches to within one body length of another), is more closely tied to locomotory activity than to social status. "Limited war" strategies, where animals retaliate if opponents escalate, form an evolutionary stable strategy (ESS) (Maynard-Smith and Price 1973; Parker 1974), and it appears that in clawed decapods such decisions

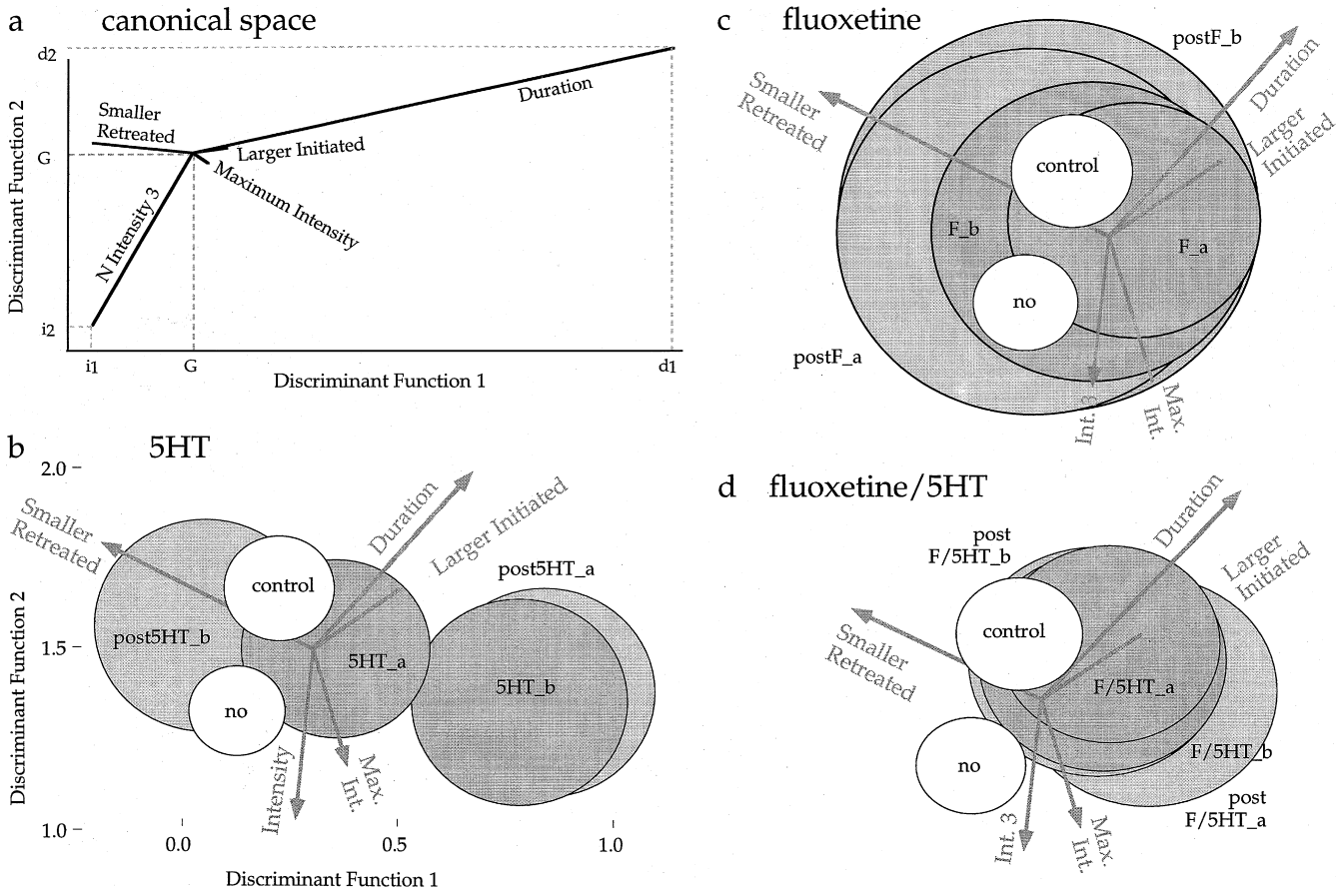


Fig. 5a–d Behavioral differences associated with pharmacological treatment are identified by Discriminant Function Analysis (DFA) and depicted as canonical centroid plots. In a two-step procedure, we first identified an optimized coordinate system which maximized separation among treatment groups while minimizing variation within them (**a**). We then plotted the fighting behavior of different treatment groups into this space. Data for 5HT (**b**), fluoxetine (**c**) and 5HT together with fluoxetine (**d**) are depicted separately to improve readability of the graphs. **a** The optimized coordinate system is defined by the first two discriminant axes (DF1, DF2). Original variables are represented as vectors originating from the overall (grand) mean (G) with the length of each vector indicating its ability to separate the groups and its direction aiding in the interpretation of these differences. Duration (d_1) proved several times more important than any other variable for variation in the horizontal dimension (DF1), while intensity (i_2) dominated others on DF2. **b–d** Fights of different experimental periods are represented as 95% confidence regions, and spatial separation of these circles indicates significant differences. Confidence regions are labelled with a combination of the following abbreviations: fluoxetine (F), serotonin (5HT), first half-hour (_a), and second half-hour (_b). Interactions during pre- and control injection were lumped irrespective of the treatment that followed. Thus, only one set of confidence regions was calculated for these two periods and the same circles appear in all three subgraphs. Significant differences in behavior were apparent for experiments involving serotonin (**b**) with fights during the second half-hour of infusion and the first half-hour post infusion moving towards the right on the graph. The behavioral characteristics associated with this change on DF1 are concurrent increases in fight duration and decreases in the probability of retreat (see Table 4). In contrast, interactions during and after acute infusion of fluoxetine (**c**) overlapped to a large extent, indicating that the behavioral characteristics of fighting were similar. The presence of fluoxetine prevented the behavioral effects associated with serotonin alone (**d**)

are strongly governed by innate rules (Huber and Kravitz 1995). Thus, the most sovereign decision a crayfish faces concerns picking the optimal time to retreat depending on the balance of perceived gains and losses (Enquist and Leimar 1990; DiMarco and Hanlon 1997; Pellis 1997). If the outcome of a fight is predictable it should pay both animals to settle the dispute quickly with minimal risks and costs (Maynard-Smith 1982). Under normal circumstances, differences in body size provide a powerful cue to predict eventual success (Glass and Huntingford 1988) and untreated crayfish were able to assess and respond to such asymmetries without excessive escalation. Serotonin-treated individuals, however, failed to withdraw from agonistic encounters even when they had little hope of winning. It appears that for a period of time this amine has taken away an animal's option to retreat and become subordinate. Resulting fights are particularly drawn out, contain several behavior patterns and levels of intensity, and often feature multiple episodes of unbridled aggression. Although this study did not gauge the specific consequences associated with individual behavioral strategies, we expect that continued fighting would prove disadvantageous in such situations, resulting in lost time and energy, or increased risk of injury and predation. Further experiments will explore whether serotonin impairs the ability to assess an opponents fighting potential, or whether the animal

arrives at an inappropriate central decision despite proper peripheral sensory cues.

Our ability to interfere with decision-making as a result of serotonin infusion indicates the fundamental importance of amine function in such processes. We believe that this may involve a balance between serotonin and some other substances (possibly octopamine) at certain key sites in the brain of these animals which we disturb with injections of serotonin. A potential role for re-uptake mechanisms was suggested by the slow onset and time-course of these changes, and by the fact that hemolymph concentrations with pronounced behavioral effects coincided with those where high-affinity serotonin re-uptake mechanism was maximal ($K_m = 0.7\ \mu mol \cdot l^{-1}$; Livingstone et al. 1981). By effectively reducing the fight-enhancing effects of serotonin, fluoxetine sheds light on the specifics of its neurobiological substrates and suggests that a functional high-affinity serotonin reuptake mechanism (Livingstone et al. 1981) is a prerequisite. We believe that increased amounts of amine are taken up into neurons when circulating levels are high, subsequently leading to elevated release and serotonin function in defined areas of neuropil involved in decision-making. However, reminders not to view this system too simplistically arise from a variety of sources. For instance, fluoxetine, which by itself ought to have similar short-term effects as serotonin infusion, did not result in similar increases in fighting. Although intriguing, the particulars of this association are not yet clear and await further clarification perhaps using pharmacological manipulation of specific 5HT receptor subtypes.

There are currently few limits to our molecular and biochemical ambitions when we attempt, for instance, to identify and describe new serotonin receptors. This will be of only limited value, however, unless we can eventually array alongside it a behavioral profile of functional significances in healthy and diseased brains. Screening based on convenient behavioral categories (pre-chosen based on the availability of a simple assay) is short on interpretive value and inherently unsuitable to characterize novel behavioral consequences. This is particularly true in the case of the complex behavioral effects produced by amine modulators and novel, highly specific pharmacological chemicals such as fluoxetine. In our view, the characterization of individual components underlying complex behaviors and the elucidation of effects within an empirically determined framework, provides a significantly better alternative.

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