The Fight and Flight Responses of Crickets Depleted of Biogenic Amines

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Received 20 September 1999; accepted 19 January 2000

ABSTRACT: Aggressive and escape behaviors were analysed in crickets (Orthoptera) treated with either reserpine, a nonspecific depleter of biogenic amines, or the synthesis inhibitors α-methyltryptophan (AMTP) and α-methyl-*p*-tyrosine (AMT) to specifically deplete serotonin, respectively dopamine and octopamine. Standard immunocytochemical techniques were used to verify depletion from central nervous tissue, and determine the effective dosages. Reserpinized crickets became exceedingly lethargic and had severely depressed escape responses. However, they were still able to express all the major elements of the escalating sequences of stereotype motor performances that typifies normal aggressive behavior in the cricket. AMT and AMTP treatment had opposing influences on escape behavior, being enhanced by serotonin depletion, but depressed by dopamine/octopamine depletion. AMTP-induced serotonin depletion had no influence on aggressive or submissive behaviors. AMT-treated crickets could normally only be brought to fight by coaxing. Though capable of expressing aggressive behavior per se, agonistic encounters between AMT-treated crickets were shorter, and rarely involved actual physical interactions. Hence, although amines seem to have similar actions on escape behavior in insects and crustaceans, the aminergic control of aggression seems to be fundamentally different in these arthropods groups. We conclude that amines are not in principle required for the initiation and operation of the motor circuits underlying aggression in the cricket. However, octopamine and/or dopamine seem necessary for establishing a level of excitability sufficient for aggressive behavior to become overt in response to appropriate natural releasing stimuli. J Neurobiol 43: 107–120, 2000

Keywords: **aggression; escape behavior; octopamine; dopamine; serotonin**

Aggressive interactions between conspecifics are a common phenomenon throughout the animal kingdom, having been described in the lowest metazoans (Brace and Purvey, 1978) and of course in humans (Eibl-Eibelsfeld, 1974; Albert et al., 1993). As a complex social interaction with multivariate intrinsic and extrinsic determinants, aggressive behavior is predispositioned for neurochemical biasing via biogenic amines (see Eichelman, 1990). Despite the intricacies involved, several general principles have emerged. In vertebrates at least, hormonally released adrenaline and noradrenaline are considered instrumental in preparing metabolic body functions for the prospective fight, whereas the central nervous noradrenergic system appears required for the start, maintenance, and situation-dependent fine tuning of aggression (review: Haller et al., 1998). On the other hand, manipulations that raise brain serotonin levels are said to lower aggression, whereas serotonin depletion increases it (Vergnes et al., 1988; Sijbesma et al., 1990; for review, see Olivier and Moss, 1990), possibly by interacting with the aggression-promoting effects of the neuropeptide vasopressin (Deville et al., 1996; Ferris et al., 1997).

Although in vertebrates a decreased effectiveness of the serotonergic system is generally accompanied by increased levels of aggression, the converse is

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Contract grant sponsor: Deutsche Forschungsgemeinschaft; contract grant number: Schi 379-1/1.

believed to hold for invertebrates (reviews, see Kravitz, 1988; Kravitz and Edwards, 1997; Wieger, 1997). Thus, lobsters injected with serotonin adopt the aggressive posture typical of dominant animals, whereas the subordinate posture is assumed after injections with octopamine (Livingstone et al., 1980; Antonsen and Paul, 1997), the invertebrate *pendant* of adrenaline/noradrenaline (cf. Evans, 1980; David and Coulon, 1985). These postural effects result from both peripheral modulation of muscular contraction and central activation of specific motor pathways (Harris-Warrick and Kravitz, 1984; Harris-Warrick, 1985). Serotonin is also believed to renew the willingness of subordinates to engage in further agonistic encounters by interfering with the decision to withdraw, and thereby increases fight duration in both lobsters and crayfish (review: Huber et al., 1997b; Huber and Delago, 1998; Huber et al., 1997a).

In this article, we investigate the role of amines in aggressive encounters between isolated mature adult crickets. Apart from an early report that serotonin increases mutual aggressiveness in ants (Kostowoski and Tarchalska, 1972) and the finding that hemolymph octopamine levels increase in crickets after aggressive interactions as in various other behaviors (Adamo et al., 1995), little is known of amine function in insect aggression. Several features of cricket aggressive behavior (Alexander, 1961; Adamo and Hoy, 1995; Hofmann, 1996, 1997; Hofmann and Stevenson, 2000) attracted our attention as potentially modifiable by amines. First, cricket fights comprise a highly stereotyped, stepwise escalation of several motor performances. Second, the outcome of a fight establishes a clear winner and loser, each exhibiting characteristic behaviors. Third, although losers will not normally reengage an opponent for several hours after a defeat, they are "reset" to an aggressive state immediately after flying (Hofmann et al., 1996; Hofmann and Stevenson, 2000), a behavior known to involve the release of numerous hormones including octopamine (cf. Orchard et al., 1993; Adamo et al., 1995). Since insects possess a formidable blood–brain barrier (Treherne and Schofield, 1979) to the passage of amines (cf. Stevenson and Kutsch, 1987), we opted to use established amine-depleting agents for insects: reserpine, which nonspecifically removes serotonin, octopamine and dopamine from the central nervous system (Sloley and Owen, 1982), and the more specific competitive synthesis inhibitors α-methyltryptophan (AMTP) to deplete serotonin and α-methyl-*p*-tyrosine (AMT) to deplete dopamine and octopamine (cf. Sloley and Orikasa, 1988). To evaluate the effectiveness of depletion and its influence on aggression, we also examined evoked escape-running behavior (cf. Gras and Hörner, 1992), for which the functionally antagonistic actions of serotonin and octopamine have already been established in insects (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992).

Our results demonstrate that amines are not in principle required for the initiation and operation of the motor circuits underlying aggression in the cricket. However, octopamine and/or dopamine seem necessary for establishing a level of excitability sufficient for aggressive behavior to become overt in response to appropriate natural releasing stimuli. In this respect, the aggressive system of insects appears to be more similar to that of vertebrates than crustaceans. A preliminary account of earlier findings has appeared in abstract form (Hofmann et al., 1996).

MATERIALS AND METHODS

Experimental Animals

Mature adult (10–20 days after the final moult) male crickets, *Gryllus bimaculatus* were taken from colonies maintained at Leipzig University under standard conditions (cf. Staudacher and Schildberger, 1998). These were then kept individually in glass jars under the same conditions for at least 2 days prior to experimentation.

Amine Depletion

Three different reagents were used to deplete amines from the cricket nervous system. Reserpine (Sigma, Deisenhofen, Germany), a nonspecific depleter for catecholamines and serotonin, was dissolved in dimethylsulfoxide (DMSO; Sigma) to give a concentration of 50 mg/mL. Competitive blockers of dopamine synthesis (AMT; Sigma) and serotonin synthesis (AMTP, Sigma) were each dissolved in distilled water to give concentrations of 75 and 25 mg/mL, respectively. Solutions were injected into the thoracic cavity via the abdomen using a microsyringe (Hamilton, Bonaduz, Switzerland). Controls received DMSO or insect ringer. The effective dosages for achieving depletion were determined postmortem by immunocytochemistry (see below and Results): reserpine: 24–48 h after a single injection of 200 μg in 4 μL DMSO. AMT: 48 h after the last of 2 successive injections of 1.5 mg in 20 μL *A. dest.* administered at 48-h intervals. AMTP: 48 h after the last of 3 successive injections of 1.0 mg in 40 μL *A. dest.* administered at 48-h intervals.

Immunocytochemistry

Amine depletion was checked by immunocytochemistry using established polyclonal antisera raised in rabbits against the amines octopamine, dopamine, and serotonin.

The same basic procedure was used throughout, with variations appropriate for each antiserum.

The animals were cooled, and the head capsule was opened to reveal the cerebral ganglion, which was flushed with fixative as specifically required for each of the primary antisera used (see below), excised, and transferred to fresh fixative for 2 h. Brains were subsequently passed through an ascending ethanol series (50, 70, and 90%, two times at 100%, each for 5 min), xylene (one time, 2 min), and then molten paraffin wax (1 h, 58°C). Horizontal sections (14 μm, relative to embryonic axis) were cut with a microtome (Jung-Biocut; Leica, Wetzlar, Germany), passed through xylene (two times for 5 min), rehydrated in a descending alcohol series (two times at 100% and 90, 70, and 50% ethanol; each for 5 min), and bathed in buffer (Tris-HCl, 0.1 *M*, pH 7.6) containing 1% hydrogen peroxide to block endogenous peroxidase activity. After washing (two times for 10 min) in buffer containing 0.1% Triton-X100 ("Tris-Tx"), sections were incubated in normal goat serum (10% Sigma) and then overnight at room temperature in primary antiserum diluted in Tris-Tx containing 1% normal goat serum (see below). After washing (Tris-Tx, 3 times 10 min), bound antiserum was detected by the avidin/biotin technique with a commercial kit (Vector Laboratories, Burlingame, CA) with adherence to the recommended dilutions and incubation times, and using diaminobenzidine (2 mg/mL buffer; Polyscience, St. Goar, Germany) as chromogen. After dehydration in an ascending alcohol series and clearing in xylene, sections were mounted in Entellan (Merck, Darmstadt, Germany) under coverslips.

The octopamine antiserum originated from Dr. M. Eckert (Jena, Germany). It detects glutaraldehyde conjugate of octopamine, but not of noradrenaline, dopamine, or serotonin (Eckert et al., 1992) and has been used extensively in insects (cf. Stevenson and Spörhase-Eichmann, 1995). We used this serum diluted 1:1000 on tissues fixed in 6% glutaraldehyde and 0.5% glacial acetic acid in saturated aqueous picric acid (2 h). It was essential to treat sections prior to incubation in normal goat serum with sodium borohydride (0.5% in Tris-HCl, 30 min) to saturate double bonds and subsequently rinse the sections thoroughly (twice 15 min in Tris-HCl, 15 min in Tris-HCl-Tx).

The dopamine antiserum originated from Dr. H. W. M. Steinbusch (Maastricht, The Netherlands) and was raised against dopamine coupled to thyroglobulin with glutaraldehyde. Its specificity has been well established *in vitro* and in tissue (Steinbusch and Tilders, 1987; Steinbusch et al., 1991). We used this serum diluted 1:1000 in tissues fixed for 2 h in 6% glutaraldehyde, 0.5% glacial acetic acid with 1% sodium metabisulphide in saturated aqueous picric acid. It was essential to add sodium metabisulphide (1%) to the aqueous phases of the fixative to all alcohol dilutions and to all buffer solutions used prior to incubation in primary antiserum.

The serotonin antiserum was obtained from Eugene Tech (Ridgefield Park, NJ). It recognises insect serotonergic neurons, producing a cleaner signal than most other available serotonin antisera (Spörhase-Eichmann, personal communication). We used this serum diluted 1:1500 on tissues fixed for 2 h in 2.5% paraformaldehyde and 0.5% glacial acetic acid in saturated aqueous picric acid.

Preparations were viewed with a compound microscope (Leitz DMR; Leica) using phase interference contrast. Optical images were obtained with a mounted CCD camera (SensiCam; PCO Computer Optics, Kelheim, Germany) using automatic exposure and color/brightness compensation. Images were scaled, trimmed, and converted to 300 dpi 8-bit greyscale using standard software (Canvas 5.3; Deneba, Florida) running on a Power Macintosh computer (Apple Computers, Cupertino, CA). Beyond this, no further image processing was undertaken.

Analysis of Escape Behavior

Escape responses were evaluated as a measure of general excitability and for determining the effectiveness of amine depletion on an insect behavioral system where amines have established influences (e.g., Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992) and which functions antagonistically to aggression. For this purpose, the animals were positioned by means of a holder fastened to the prothoracic shield so that they could run on a hollow, air-cushioned Styrofoam ball. Intended running sequences were evaluated from the optically recorded translational and rotational movements of the Styrofoam ball [see Fig. 2(A)] and Staudacher and Schildberger (1998) for operational details)]. Escape responses were elicited by air pulses (20 ms, 2–3 m/s) directed at the wind-sensitive cerci from a 10-cm-distant glass tube (inner diameter, 5 mm) connected to a compressed air supply via an electrically controlled two-way magnetic valve (Lee, Westbrook, CT). Recorded activity was stored and evaluated on a Power Macintosh computer using a MacLab (AD Instruments, Mountain View, CA 94043) interface and software.

Analysis of Aggressive Behavior

To evaluate aggressive behavior, two crickets were placed at opposite ends of a small Perspex glass arena (16 $\times$ 9 cm) covered with sand and allowed to contact each other after removing a door that divided the arena. If the animals appeared reluctant to move, they were coaxed by stroking the cerci and or antennae with a fine-hair brush. On contacting each other a fight generally ensued. Because these interactions follow a strict, sequentially escalating series of stereotyped motor performances (Adamo and Hoy, 1995; Hofmann and Stevenson, 2000), the intensity of a fight can be awarded a score on a scale of 0–6, denoting the level to which a fight escalates before the winner is established by the retreat of his opponent (cf. Fig. 4 and text). Fight duration, from first contact until establishment, was measured to the nearest second with a stop watch, whereby the duration of any pauses that occasionally occurred when the animals lost contact in level 6 fights were deducted. All fights investigated were between crickets that received the same treatment.

Since cricket aggressiveness appears to fluctuate with the seasons, during the course of a day and under various meteorological conditions, we took the precaution of performing test and control experiments in parallel in the summer months, avoiding times when aggression tends to be depressed (just after midday, late at night, and on generally dreary days).

Flight motor activity, which increases the aggressiveness of crickets after losing a fight (Hofmann et al., 1996; Hofmann and Stevenson, 2000), was evoked by suspending the animals from a holder fastened to the pronotum in the warmed air stream of a household-grade hair dryer.

Statistical Analysis of Data

The mean and S.D. was calculated for normally distributed metric data (Excel 5.0; Microsoft, Redmond, WA); Student's *t* test for paired and unpaired data sets was applied where appropriate for calculating the significance of differences between means (GB-STAT; Dynamic Microsystems, MD). The median with the lower and upper quartiles was calculated for ordinal and parametric data sets (Excel 5.0); the significance of differences in the distributions was tested by the Mann-Whitney *U* test for unpaired data, and by the Wilcoxon test for paired data sets (GB-STAT). The chi-square test was used for comparing relative frequencies (GB-STAT).

RESULTS

Immunocytochemistry of Amine-Depleted Tissue

The effectiveness of amine-depleting agents was checked post mortem by immunocytochemistry using established antisera directed against biogenic amines (Fig. 1).

In comparison to DMSO-injected controls [4 μL, Fig. 1(A)], thoracic injections of reserpine in DMSO (200 μg/4 μL) resulted in a significant reduction of octopamine-, dopamine- and serotonin-immunoreactive material in the cricket brain [Fig. 1(B)] when examined 24–48 h later. Brains of all examined reserpinized crickets ($n = 5$) were completely void of octopamine-immunoreactive stained tissue, although very faintly labeled dopamine- and serotonin-immunoreactive somata and dendritic processes were still visible in some regions. At this dosage, mortality was under 30%. At higher reserpine dosages (300 μg/6 μL DMSO), mortality exceeded 50%, and deleterious effects also became apparent in DMSO-treated (6 μL) controls. At the lower dosage (100 μg reserpine/2μL DMSO) reported to reduce the levels of electrochemically detected amines by more than 90% in cockroaches (Sloley and Owen, 1982), we still observed significant dopamine- and particularly serotonin-immuoreactive labeling in cricket brains.

The selective depletion of immunoreactivity for octopamine and dopamine by AMT and of serotonin by AMTP only became significant in the cricket brain after successive injections of the dosages reported to be effective in cockroaches after a single administration (cf. Sloley and Orikasa, 1988). Some 48 h after the last of two thoracic injections of AMT (1.5 mg/20 μL *A. dest*.) spaced at 48-h intervals, octopamine-immunoreactive processes were barely visible, and only very faintly labeled dopamine-immunoreactive somata and some axon processes were apparent, whereas serotonin-immunoreactive staining was unchanged [Fig. 1(C)]. Mortality was under 25%. Contrasting this, octopamine- and dopamine-immunoreactive staining in the brain appeared unchanged 48 h after the last of three thoracic injections of AMTP (1.0 mg/40 μL *A. dest*.) spaced at 48-h intervals, whereas significant, albeit incomplete, depletion of serotonin-immunoreactive material occurred [Fig. (1D)]. No deaths appeared to result from AMTP treatment.

Influences on General Excitability and Escape Running

Reserpine, AMT, and AMTP each produced clear changes in the behavior of crickets when administered at the dosages found to produce maximal depletion of amine-immunoreactive material. All examined reserpine-treated (octopamine/dopamine/serotonin-depleted) and AMT-treated (octopamine/ dopamine-depleted) crickets were extremely lethargic and generally unresponsive to stimuli of modalities that generally tend to startle the animals (e.g., wind puffs and touching the cerci or antennae). AMTP-treated (serotonin-depleted) crickets, on the other hand, appeared to be hyperresponsive to stimuli of modalities that generally tend to startle the animals. These apparent influences on excitability were evaluated by analyzing the escape responses of tethered crickets to a single wind puff directed at the cerci.

Figure 2 shows the escape responses of three representative untreated crickets and crickets tested 48 h after injections of either DMSO (4 μL) or reserpine (200 μg/4 μL DMSO). Untreated animals produced relatively uniform responses to the wind stimulus [Fig. 2(B)], characterized by a brief (< 250 ms) bout of intended-running having a mean maximal translation velocity of 14.5 m/s [S.D. 3.4, $n = 10$ animals, 3 trials averaged for each; Fig. 2(E)] that resulted in a mean translation distance of 3.3 cm [S.D. 1.0; Fig. 2(F)] covered within 2 s from stimulus begin. Escape response appeared unaffected by DMSO [4 μL; Fig.

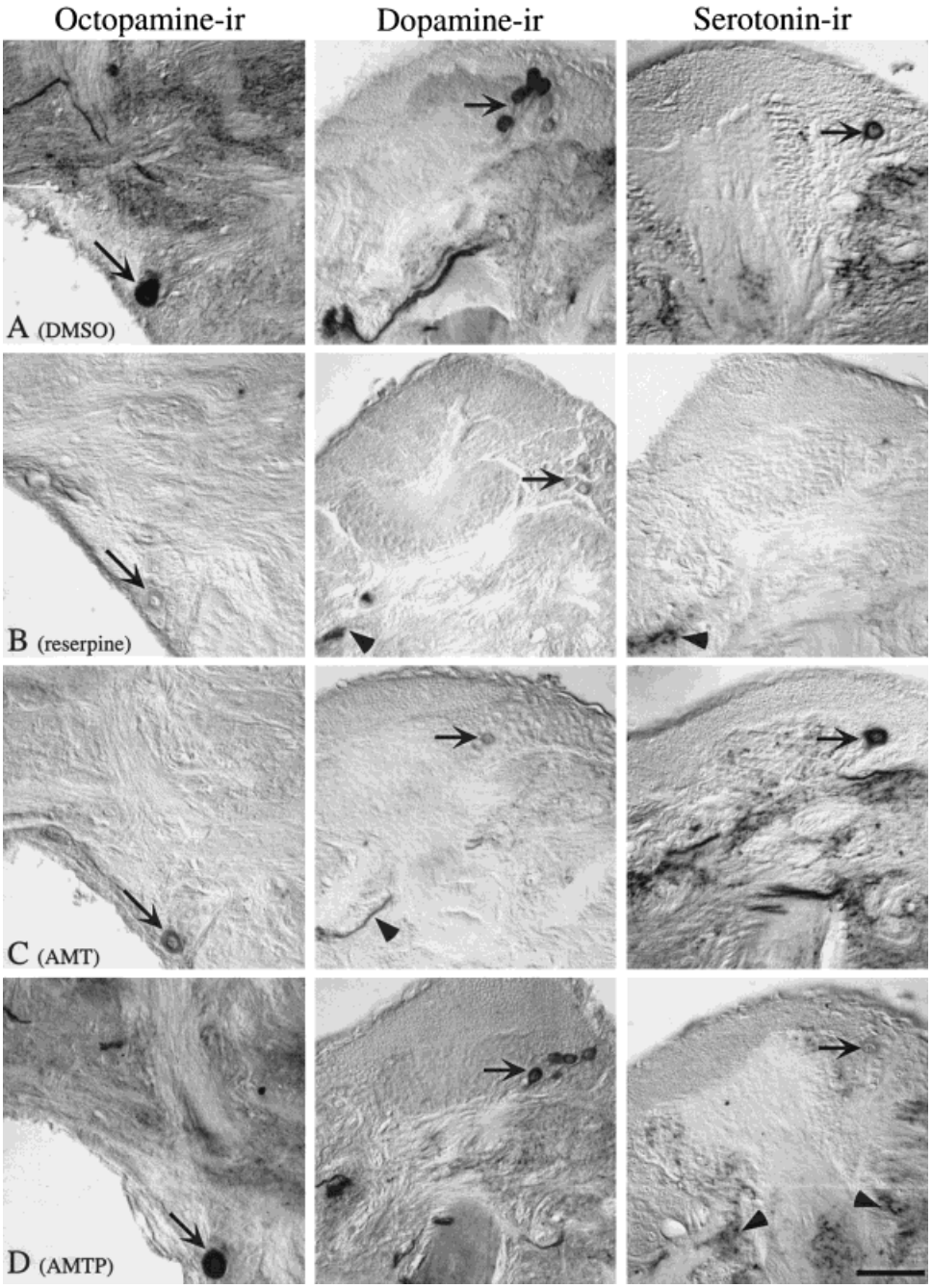

Figure 1 Photomicrographs of horizontal brain sections showing from left to right octopamine-, dopamine- and serotonin-immunoreactive (-ir) labeling following injections of (A) DMSO (4 μL), (B) reserpine (200 μg in 4 μL DMSO), (C) AMT (two times, 1.5 mg/20 μL *A. dest.*), and (D) AMTP (three times,3 $\times$ 1.0 mg/40 μL *A. dest.*). The same brain regions are shown for all treatments: right side deutocerebrum for octopamine-immunocytochemistry (left column) and right side protocerebrum for dopamine- (middle column), and serotonin-immunocytochemistry. Arrows: position of somata prominently labeled in (A). Arrowheads: areas with labeled nerve cell processes. Scale bar = 100 μm

2(C)], neither the maximal translational escape velocity (mean 11.7 m/s, S.D. 5.1, $n = 10$), nor the translational distance covered in 2 s (mean 2.6 cm, S.D. 1.4) was significantly different from untreated controls [Fig. 2(E,F)]. However, the escape response of reserpine-treated animals was severely impaired in all

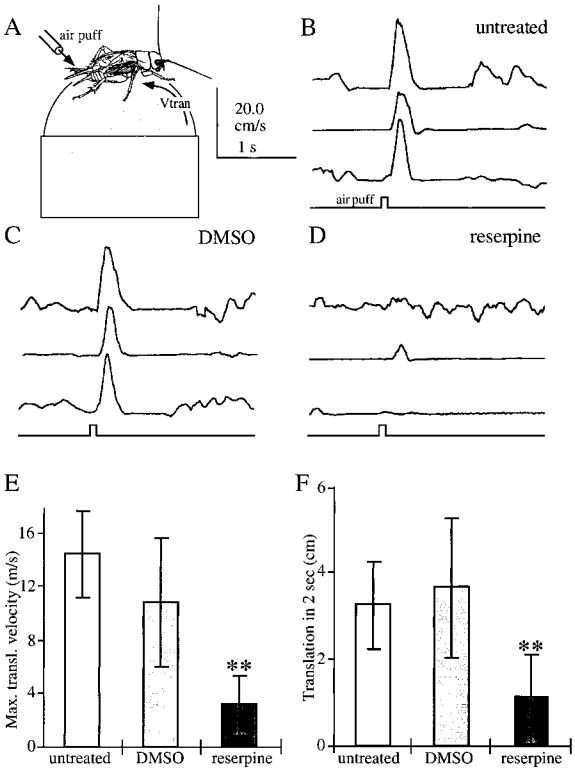

Figure 2 Wind-stimulus–evoked escape responses of untreated compared to DMSO and reserpine-injected crickets. (A) Pictogram of the experimental set up. (B) Escape traces of three different untreated crickets showing the translational velocity following a wind stimulus directed to the cerci (lower trace). (C) As in (B) for three DMSO-treated animals. (D) As in (B) for three reserpine-treated animals. (E) Bar chart showing the maximal translation velocity of untreated, DMSO-injected, and reserpine-injected crickets (mean ± S.D. calculated from the average of 3 successive trials of 10 animals in each group). (F) Bar chart showing the translation distances covered in 2 s (mean ± S.D.) for the data set shown in (E). The mean values for reserpine-treated crickets are significantly different from means of untreated and DMSO-treated crickets (*t* test: $^{**}p < .01$).

animals and trials. Some crickets remained stationary and produced no visible response, others exhibited bouts of trembling movements that were not influenced by the wind stimulus, and a few animals made a short (< 250 ms) intended-translational movement of maximally 6 m/s [Fig. 2(D)]. Both the mean maximal velocity [3.2 m/s, S.D. 2.4; Fig. 2(E)] and the mean translational distance covered in 2 s [1.1 cm, S.D. 1.1: Fig. 2(F)] was significantly reduced compared to controls (*t* test, $p < .01$).

Figure 3 compares the wind-induced escape responses of AMT, AMTP, and vehicle-injected (*A. dest.*, three times, 40 μL) crickets. AMT treatment resulted in significantly reduced escape responses in all crickets evaluated ($n = 11$); escape traces of three representative individuals are shown in Figure 3(A). The mean maximum translational velocity [4.3 m/s, Fig. 3(C)] and mean translational distance covered in 2 s [1.4 cm, S.D. 2.2; Fig. 3(D)] were both significantly reduced compared to vehicle-injected controls [Fig. 3(C,D); *t* test: $p < .01$, $p < .05$, respectively], but not statistically different from the corresponding means of reserpine-treated animals [Figs. 2(E, F)]. Contrasting this, the escape responses of AMTP-treated crickets ($n = 13$) were clearly enhanced. They usually consisted of a short, rapid forward movement (<250 ms), followed by a prolonged running sequence lasting a few seconds [Fig. 3(B)]. Compared to vehicle-injected controls, both the mean maximum velocity [14.2 ms, S.D. 3.9; Fig. 3(C)] and the mean translational distance covered in 2 s was significantly greater [6.7 m/s, S.D. 2.7, *t* tests: $p < .05$, $p < .01$, respectively; Fig. 3(D)].

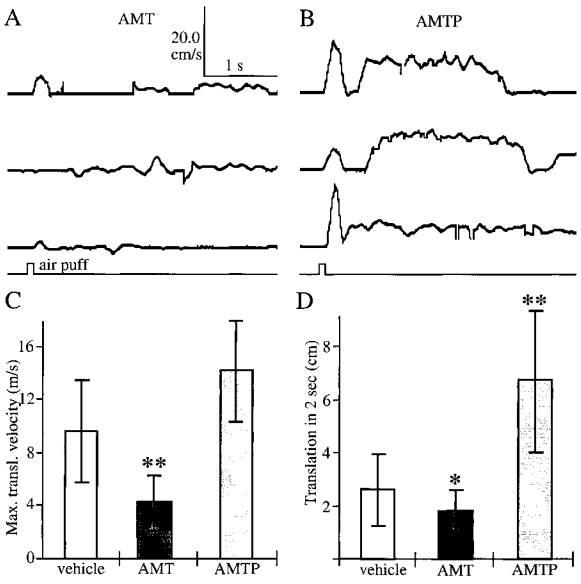

Figure 3 Wind-stimulus–evoked escape responses of AMT- and AMTP-injected crickets. (A) A Escape traces showing the translational velocity over time for 3 AMT-treated animals. (B) As in (A) for 3 AMTP-injected animals. (C) Bar chart of the maximal translation velocity (mean ± standard deviation) of crickets injected with vehicle (*A. dest.*; 11 animals), AMT (10 animals) and AMTP (13 animals) calculated from the average of three successive trials for each animal in each group. (D) Bar chart of the translational distances covered in 2 s (mean ± S.D.) for the same data set shown in (C). The mean values for AMT-treated crickets are significantly lower than the means of vehicle-injected crickets; the mean distance covered in 2 s is significantly higher in AMTP-injected crickets (*t* test: $^{*}p < .05$, $^{**}p < .01$).

Influences on Aggressive Behavior

When two previously isolated adult male crickets meet they exhibit a highly stereotyped, escalating sequence of motor acts that culminates in combat (Alexander 1961; Hofmann, 1997). We distinguish between several stages that denote the level of aggression to which the two opponents escalate before the winner is established (cf. Hofmann and Stevenson, 2000; Fig. 4). Level 0 applies to interactions where both crickets avoid each other and show no indication of aggressive behavior. Level 1 describes a situation of clearly preestablished dominance, in which one animal retreats from an approaching potential aggressor. At level 2, the antennae of the two contestants make contact and lash each other in a fashion only expressed during agonistic encounters (Alexander, 1961). This is followed by level 3, in which one of the crickets faces its opponent with broadly spread mandibles and then level 4 where both crickets display their spread mandibles. Mandible spreading is only exhibited during agonistic encounters between conspecifics (Alexander, 1961). At level 5, the spread mandibles of the two combatants engage, each animal pushing forward by stemming both hind legs in the ground. At level 6 the animals enter a stage of all-out combat, during which the contestants mandibles may disengage and reengage several times, with intermittent sallies by each cricket to attack and bite the other. A fight can be concluded at any level by one animal retreating, and this establishes a clear winner and a clear loser, each showing characteristic behavioral traits.

Untreated Crickets. Fights between the nontreated crickets examined in the present study generally escalated to level 5 (median, interquartile range 4–6, $n = 28$ pairs; Fig. 5A, "initial fight") and lasted 10 s [median, interquartile range 10–18; Fig. 5(B)]. In these encounters, 90% of all crickets exhibited mandible spreading, 60% engaged mandibles, and 40% of the flights lasted longer than the median duration [Fig. 6A, open bars). The winners, once established by the opponent retreating (Fig. 4), typically performed a series of body jerking movements (64% of winners), and they produced the aggressive rival song by stridulating with the forewings [57% of winners, Fig. 6(A); cf. Huber et al., 1989]. Just after fighting, each loser immediately retreated on confronting the potentially aggressive winner (level 1; see Fig. 5, "reengagement $<$ 3 min").

After fighting the losers normally continue to be nonaggressive for one (Adamo and Hoy, 1995) to several hours (Hofmann, 1997). Nevertheless, we

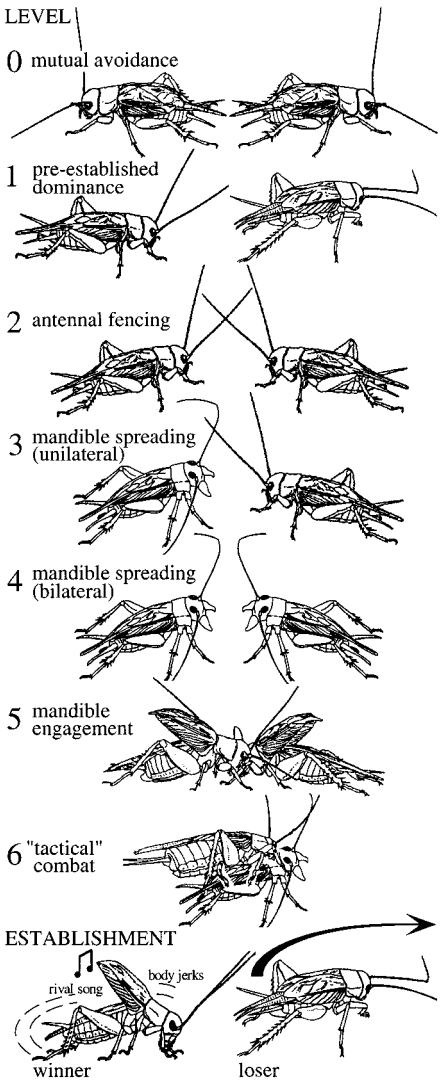

Figure 4 Pictogram illustrating the stereotyped escalating sequence of motor performances (level 0–6) characteristic for aggressive encounters between male crickets (modified from Alexander, 1961). Level 0 *mutual avoidance*: no aggressive interaction. Level 1 *preestablished dominance*: one cricket attacks, the other retreats. Level 2 *antennal fencing*: the two crickets lash with their antennae. Level 3 *mandible spreading* (*unilateral*): one cricket displays broadly spread mandibles. Level 4 *mandible spreading* (*bilateral*): both crickets displays their spread mandibles. Level 5 *mandible engagement*: the mandibles interlock and the animals push against each other. Level 6 *"tactical" combat*: an all-out fight where the animals may repeatedly disengage, struggle for position, bite other body parts, and reengage mandibles to push the opponent. Establishment: the fight can be concluded at any of the levels 2–6 by one opponent, the loser, retreating, upon which the established winner typically produces the rival song together with characteristic body-jerking movements. (Sketches of crickets modified from Huber et al., 1989)

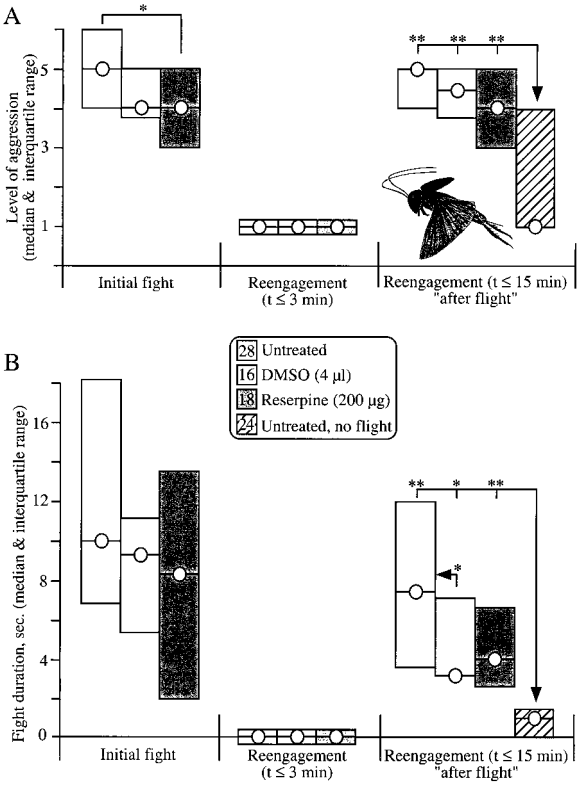

Figure 5 Bar graphs showing in (A) the level of aggression (median ± interquartile range; cf. Fig. 4) and in (B) the duration (median ± interquartile range) of aggressive encounters between previously isolated, weight matched pairs of male crickets that were either untreated (open bars, $n = 28$ pairs), injected with DMSO (light grey bars, $n = 16$ pairs), or injected with reserpine (dark grey bars, $n = 18$ pairs). The same pairs of crickets were tested three times in succession: Initial fight: the first encounter of the crickets. Reengagement ($t \leq 3$ min): less than 3 min after the winner of the initial fight had been established. Reengagement ($t \leq 15$ min, after flight): some 15 min after establishment and subsequent to a 1-min bout of induced tethered flight (data for nonflown untreated control are shown for comparison: hatched bars, $n = 24$ pairs). Statistically significant differences between data sets are indicated (Mann–Whitney U test: $*p < .05$, $**p < .01$).

have found that their behavioral status can be "reset" by inducing flight motor activity (Hofmann and Stevenson, 2000). As shown in Figure 5, losers tested after an induced 1-min flight in a warmed air stream will reengage to fight with the previous winner (median level and duration not significantly different from initial fight; Wilcoxon test, $p < .01$). Of these flown losers, 82% exhibited mandible spreading and 54% mandible engagement, 39% of the fights exceeded the median fight duration of naive animals, and 37% actually defeated the previous winner [Fig. 6(B); all values significantly different from nonflown losers, but not significantly different from initial fight, chi-square, $p < .01$]. Contrasting this, no change in the losers' aggressiveness was observed in response to wind stimulation when the animals were provided with tarsal contact so that they did not fly (data not shown).

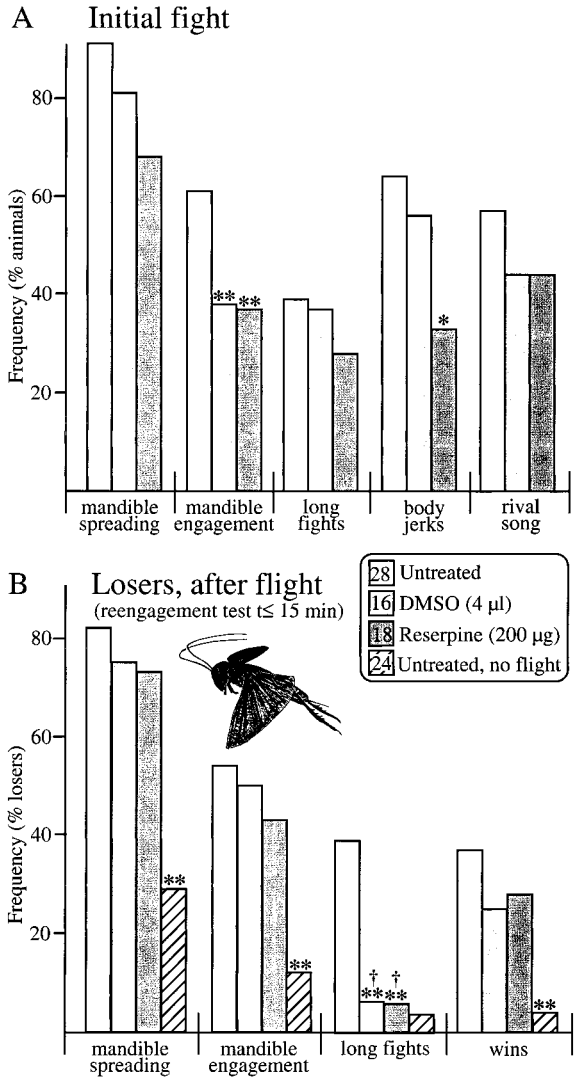

Figure 6 Bar graphs showing the relative frequencies (%) of key elements of aggressive behavior (cf. Fig. 4) for untreated crickets (open bars, $n = 28$ pairs) compared to crickets injected with DMSO (light grey bars, $n = 16$ pairs) and reserpine (dark grey bars, $n = 18$ pairs). "Long fights" lasted longer than the median initial fight of untreated crickets. (A) Data for the initial fight. (B) Data for losers of the initial fight tested approximately 15 min later and subsequent to a 1-min bout of flying. *,**Relative frequencies significantly different from untreated crickets (chi-square, $p < .05$, $p < .01$, respectively). †Relative frequencies of losers that are *not* significantly different from nonflown, untreated losers.

DMSO-Treated Crickets. The vehicle used for injecting reserpine appeared to have no major effect on the examined aspects of cricket aggressive behavior (Figs. 5, 6). The only statistically significant difference from untreated controls was that fights between DMSO-injected crickets (4 μL, $n = 16$ pairs) less frequently escalated above level 4, evidenced in Figure 6(A) by the lower mandible engagement frequency (chi-square, $p < .01$).

Reserpine-Treated Crickets. Reserpinized crickets generally seemed reluctant to interact with each other. Some pairs would lay motionless, next to each other for minutes, without sign of responding to the conspecific. However, considering their exceedingly lethargic condition, it was surprising to find that if brought to face each other and coaxed by stroking their antennae, such crickets would frequently engage each other, as if awakened, and proceed through the stereotyped sequence of motor performances that characterize cricket fights, although the movements of the appendages generally appeared somewhat slower.

Regarding the level of aggression to which these encounters escalated and their total duration, there were no significant group differences between DMSO and reserpine-treated crickets (Figs. 5, 6). In all, 68% of reserpinized crickets exhibited mandible spreading and 37% mandible engagement, and 28% of the interactions lasted longer than the median fight duration of nontreated controls [Fig. 6(A), no significant differences from DMSO group frequencies]. These aggressive encounters established clear winners, of which 33% exhibited body jerks (significantly less frequent than DMSO controls) and 44% produced the rival song [not significantly different from DMSO controls, Fig. 6(A)], along with losers, which avoided the winners when confronted.

When tethered in a wind stream, reserpinized crickets generally produced short (2–7 s) bouts of wing fluttering, but rarely fully opened their wings to produce the excursions typical of normal tethered flight. Nevertheless, this behavior proved to be as effective as normal flight for resetting the aggressive status of subordinate crickets. Some 15 min after losing, and subsequent to a 1-min period of repeatedly induced bouts of wing fluttering, 81% of reserpinized losers exhibited mandible spreading on meeting the previous winner (not significantly different from initial fight of untreated crickets). With regards to the median level and duration, these aggressive interactions were not different from fights between DMSO-injected losers after flying, but were significantly different from nonflown untreated losers (Fig. 5; Mann–Whitney U test, $p < .01$). However, long fights

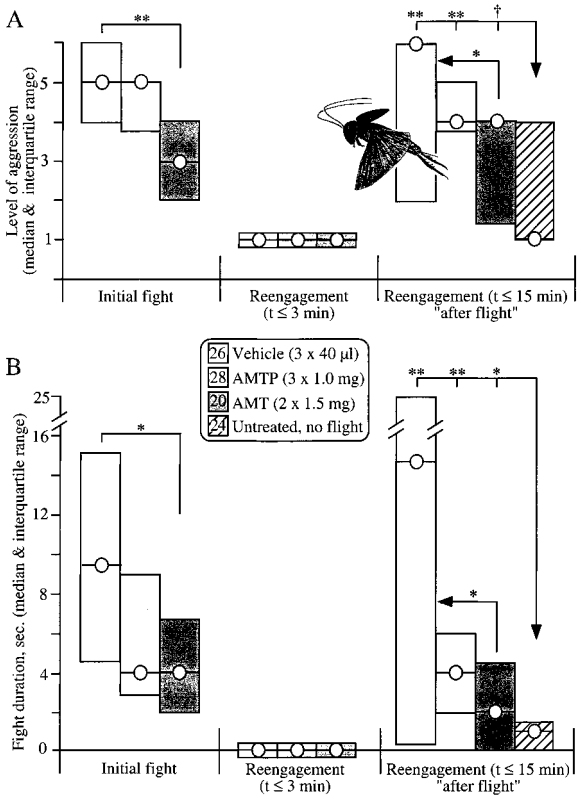

Figure 7 Bar graphs showing in (A) the median level of aggression (± interquartile range; cf. Fig. 4) and in (B) the median duration (± interquartile range) of aggressive encounters between previously isolated, weight matched pairs of male crickets that were injected with either vehicle (*A. dest.*, open bars, $n = 26$ pairs), AMTP (light grey bars, $n = 28$ pairs), or AMT (dark-grey bars, $n = 20$ pairs). As in Figure 5, the same pairs of crickets were tested three times in succession: Initial fight: the first encounter of the crickets. Reengagement ($t \leq 3$ min): less than 3 min after the winner of the initial fight had been established. Reengagement ($t \leq 15$ min, after flight): some 15 min after establishment and subsequent to a 1-min bout of induced tethered flight (data for nonflown untreated control is shown for comparison: hatched bars, $n = 24$ pairs). Statistically significant differences between data sets are indicated (U test: *$p < .05$, **$p < .01$, †not significantly different).

(>median initial fight for untreated crickets) were significantly less frequent [Fig. 6(B)]. Nevertheless, the flown reserpinized losers managed to win against the previous winner in 28% of cases [no difference from controls, Fig. 6(B)].

AMTP-treated crickets. Despite their clearly hyperactive condition (Fig. 3), aggressive encounters between crickets depleted of serotonin by AMTP treatment appeared normal in all major respects (Figs. 7, 8). In comparison to vehicle-injected (*A. dest.*) con-

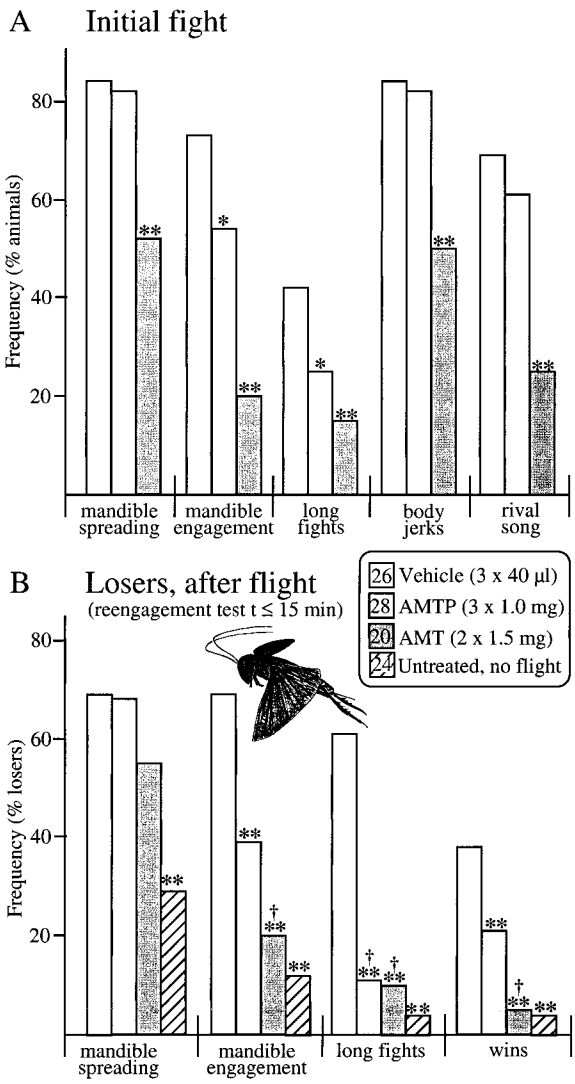

Figure 8 Bar graphs showing the relative frequencies (%) of key elements of aggressive behavior (cf. Fig. 3) for vehicle-injected crickets (open bars, $n = 26$ pairs) compared to crickets injected with AMTP (light grey bars, $n = 28$ pairs) and AMT (dark-grey bars, $n = 20$ pairs). "Long fights" lasted longer than the median initial fight of untreated crickets. (A) Data for the initial fight. (B) Data for losers of the initial fight tested approximately 15 min later and subsequent to a 1-min bout of flying. *, **Relative frequencies significantly different from vehicle-injected crickets (chi-square: $p < .05$, $p < .01$, respectively). †Relative frequencies of losers that are *not* significantly different from nonflown, untreated control.

trols there were no significant differences in the median level and duration of the initial fights [Fig. 7(A)]. However, comparatively fewer AMTP-treated crickets escalated above level 4, and their fights were less often longer than the median duration of untreated cricket fights (Fig. 6, mandible engagement and long-fight frequency both significantly different from vehicle-injected controls, chi-square, $p < .05$).

Again, these interactions produced clear winners and losers, and the aggressiveness of losers could be restored by flying [Figs. 7, 8(B); e.g., 77% mandible spreading, no difference from vehicle control]. However, as for the initial fights, these aggressive interactions between the flown loser and the previous winner escalated less frequently to level 5 and were less frequently longer than the median duration of fights between naive crickets [Fig. 8(B): mandible engagement and long fight frequencies significantly different from vehicle control, chi-square, $p < .01$.].

AMT-Treated Crickets. Crickets depleted of octopamine and dopamine by AMT appeared even more reluctant than reserpinized animals to exhibit aggressive behavior. Both the level of aggression [Fig. 7(A)] and duration [Fig. 7(B)] of fights between AMT-treated crickets was significantly reduced compared to vehicle-injected controls (Mann–Whitney U test, $p < .01$, $p < .5$, respectively). Only 52% of the treated crickets exhibited mandible spreading and mandible engagement; long fights, body jerks, and rival song all occurred significantly less frequently than in controls [chi-square, $p < .01$ for all parameters, Fig. 8(A)]. Furthermore, AMT-treated crickets were clearly less aggressive than AMTP-treated crickets [Fig 7(A), level of aggression for initial and reengagement fight both significantly lower, U test, $p < .05$; Fig. 8(A), all values for frequency of occurrence of mandible spreading, mandible engagement, body jerks and rival song production significantly lower, chi-square, $p < .05$)].

When placed in a wind stream, the losers of these encounters only produced short bouts of wing fluttering as observed for reserpinized crickets. Subsequent to this treatment, the recorded level of aggression towards the previous winner was not significantly different from nonflown, untreated loser crickets [Fig. 7(A)]. Despite this, we observed that 58% of the losers spread their mandibles on confronting an opponent after inducing flight-like activity [chi-square, not significantly different from vehicle-injected flown losers; Fig. 8(B)]. However, the mandible engagement frequency and long-fight frequency of flown, AMT-treated losers were not significantly different from untreated, nonflown losers.

DISCUSSION

Contrary to what one might have expected from knowledge of the control of aggression by amines in mammals (e.g., Olivier and Moss, 1990; Haller et al.,

1998) and arthropods (Antonsen and Paul 1997; Huber et al., 1997a,b; Kravitz and Edwards, 1997; Huber and Delago, 1998; Kravitz, 1988), we found that crickets with nervous systems depleted of dopamine, octopamine, and serotonin with reserpine were still able to express the major components of normal aggressive behavior (Figs. 3, 5, 6). Despite their exceedingly lethargic condition, evidenced here by severely depressed escape behavior (Fig. 2) and general reluctance to interact, more than two thirds of all reserpinized crickets exhibited mandible spreading (Fig. 6), a motor response and clearly aggressive display not known outside of agonistic encounters between conspecifics (cf. Alexander, 1961; Adamo and Hoy, 1995). Once initiated, aggressive interactions between reserpinized crickets lasted as long and escalated to the same level as fights between control crickets (Fig. 5). Furthermore, the contests established clear winners and losers, which each exhibited behaviors characteristic for dominant and subordinate crickets.

These findings with reserpine can hardly be discounted on the grounds that depletion was insufficient. The almost complete loss of octopamine-, dopamine- and serotonin-immunoreactive material from the cricket brain was verified using highly sensitive antisera [Fig. 1(B)]. The reserpine doses that we administered corresponded to twice that required to deplete amines below levels detectable by electrochemical techniques in cockroaches (cf. Sloley and Owen, 1982), which are somewhat larger in size. Thus, although only qualitative, amine immunocytochemistry appears to be far more sensitive than electrochemical detection. Furthermore, it appears unlikely that the effects of long-term depletion on aggression had been counteracted by compensatory mechanisms (e.g., increased receptor sensitivity), since this would probably also have been accompanied by some recovery of the animals' responsiveness to escape inducing stimuli. Our data thus indicate that biogenic amines are not in principle essential for the initiation, generation, and coordination of the escalating sequences of stereotype motor performances underlying aggressive behavior in the male cricket.

This result is, however, perhaps not so surprising. First, although amines can activate various central pattern generators, such as those for the crustacean gastric mill (Flamm and Harris-Warrick, 1986), leech swimming (Nusbaum and Kristan, 1986), locust flight (Stevenson and Kutsch, 1987), and snail feeding (cf. Yeoman et al., 1994), they probably operate in parallel with more rapidly acting commands (cf. Pearson and Ramirez, 1992; Angstadt and Friesen, 1993; Yeoman, et al., 1995, 1996). Second, in systems where details are known, it appears that the pivotal neuronal components of motor pattern generating circuits do not utilize amine transmitters themselves (e.g., Harris-Warrick et al., 1992).

Amines are best known for their neuromodulatory actions, which serve to fine tune neural circuits and bias motor output and behavior (Kravitz, 1988; Bicker and Menzel, 1989; Harris-Warrick et al., 1992). In crayfish and lobsters, serotonin can induce a renewed willingness of subordinates to engage the dominant in further agonistic encounters, apparently by altering the decision to retreat. It has thus been suggested that serotonin influences the animals "aggressive motivation" (Huber et al., 1997a,b; Huber and Delago, 1998). In crickets, we accordingly predicted that the renewed willingness of subordinate crickets to fight after flying (Hofmann, 1996, 1997; Hofmann and Stevenson, 2000) may also be directly due to amines, for example, octopamine that is released from the nervous system into the haemolymph during flight (Adamo et al., 1995). However, flight motor activity effectively restored the aggressiveness of reserpinized subordinate crickets after a defeat in 73% of all cases [Fig. 6(B)]. At present we have no conclusive explanation to account for this "behavioral modulation" of aggressive motivation by flying. Ganglion-connective ablation studies (Hofmann and Stevenson, 2000) suggest the involvement of interneurons that ascend from the thoracic motor centers to the brain and are activated during flight, such as those described by Homberg (1994). Irrespective of this, our present findings indicate that the underlying mechanism can operate without the intervention of amines.

In many systems, the behavioral actions of dopamine, noradrenaline, and octopamine are often functionally antagonistic to those of the indolamine serotonin (see Kravitz, 1988; Erber, et al., 1993; Menzel et al., 1994). Hence, the aminergic control of aggression may depend on the relative balance of these two amine classes, which of course need not be perturbed by nonspecific depletion with reserpine.

With respect to escape responses, we could show that AMT and AMTP can be used to differentially bias cricket behavior in the directions expected from the effects of the amines they specifically deplete. In general terms, escape behavior is depressed by serotonin, but enhanced by octopamine in both cockroaches (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992) and crayfish (Glanzman and Krasne, 1983), whereas serotonin neurotoxins lower the threshold in the escape system of the crayfish (Glanzman and Krasne, 1986). These observations clearly conform to our findings in crickets that serotonin depletion results in enhanced escape responses, whereas the depletion of octopamine and dopamine

depressed the escape responses (Fig. 3). The similarities between the escape responses of reserpine- and AMT-treated animals may reflect the more complete depletion of octopamine relative to serotonin in reserpinized crickets [Fig. 1(B)].

Despite the similarities in the ways biogenic amines appear to function in the insect and crustacean escape systems, our experiments with amine depletors suggest that the aminergic control of aggression may be fundamentally different in these two arthropod groups. First, although serotonin is strongly implicated in inducing aggressive behavior in crustaceans (e.g., Kravitz, 1988; Antonsen and Paul, 1997), we observed no appreciable detrimental effects of AMTP-mediated serotonin depletion on the fighting ability of crickets (Figs. 7, 8). Second, although serotonin can renew the willingness of subordinate lobsters and crayfish to engage in further agonistic encounters [Huber et al., 1997a(review),b; Huber and Delago, 1998], serotonin depletion was without consequence for the analogous paradigm of restoring aggressiveness by inducing flight behavior in subordinate crickets [Figs. 7, 8(B)]. Third, although octopamine injections appear to bias the aggressiveness of crustaceans toward submissive behavior (Kravitz, 1988; Antonsen and Paul, 1997), the converse may be true for insects. In crickets at least, the selective depletion of octopamine and dopamine with AMT led to a significant reduction in the group level and group duration of aggressive encounters (Fig. 7) as well as the frequency of actual physical interactions (mandible engagement, Fig. 8).

Although, more than half the AMT-treated crickets could be coaxed to exhibit aggressive behavior per se [mandible spreading; Fig. 8(A)], and the aggressiveness of subordinates could still be restored by flying in many cases [54%, Fig. 8(B)], they appeared even more reluctant to fight than crickets depleted of octopamine, dopamine, and serotonin by means of reserpine. Indeed, if left to their own devices the majority showed no interactions whatsoever. This implies that the presence of serotonin may subdue the expression of aggression and oppose the action of octopamine and dopamine. The fact that amine depletion resulting form AMT and AMTP treatment was not as complete as with reserpine favors this notion [Fig. 1(C,D)]. Clearly, we cannot know the consequences of complete selective depletion, although our current results do allow us to make a reasonable prediction. If anything, the total and selective loss of octopamine and dopamine might result in a condition in which it is practically impossible to evoke aggression; conversely more pronounced selective depletion of serotonin might enhance some aspects of aggression. Hence, the aminergic control of aggression in insects may have some analogies with the situation in vertebrates (reviews: Olivier and Moss, 1990; Haller et al., 1998), where aggression is reduced by catecholamine depletion (Ross and Ogren, 1976; see also Serova and Naumenko, 1996), but enhanced by serotonin depletion (Vergnes et al., 1988). Current investigations with the octopamine agonist chlordimeform support this notion (Stevenson et al., 1999; Stevenson et al., submitted).

In conclusion, our results demonstrate that amines are not in principle required for the initiation and operation of the motor circuits underlying aggression in the cricket. Nevertheless, this need not mean that amines have no effect on insect aggression. At the very least, octopamine and/or dopamine seem necessary for establishing a level of excitability sufficient for aggressive behavior to become overt in response to appropriate natural releasing stimuli.

We thank Varya Dyakonova, Moscow, Stephan Michel, Leipzig, and Werner Loher, Berkeley, for fruitful discussions on this manuscript.

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