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A Parasitoid Wasp Manipulates the Drive for Walking of Its Cockroach Prey

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Summary

The parasitoid wasp A. compressa hunts cockroaches as a live food supply for its offspring. The wasp selectively injects venom into the cerebral ganglia of the prey to induce long-term hypokinesia [1–5], during which the stung cockroach, although not paralyzed, does not initiate spontaneous walking and fails to escape aversive stimuli. This allows the wasp to grab the cockroach by the antenna and walk it to a nest much like a dog on a leash. There, the wasp lays an egg on the prey, seals the nest, and leaves. The stung cockroach, however, does not fight to escape its tomb but rather awaits its fate, being consumed alive by the hatching larva over several days. We investigated whether the venom-induced hypokinesia is a result of an overall decrease in arousal or, alternatively, a specific decrease in the drive to initiate or maintain walking. We found that the venom specifically affects both the threshold for the initiation and the maintenance of walking-related behaviors. Nevertheless, the walking pattern generator itself appears to be intact. We thus report that the venom, rather than decreasing overall arousal, manipulates neuronal centers within the cerebral ganglia that are specifically involved in the initiation and maintenance of walking.

Results and Discussion

Wasp Venom Decreases Spontaneous and Evoked Walking

Stung cockroaches are hypokinetic and rarely, if ever, initiate walking under natural conditions [1]. We first confirmed these results (see Figure S1 available online) and found that although >90% of the control group explored a novel area (n = 14) and effectively escaped aversive stimuli (n = 26), stung cockroaches showed no exploratory behavior (n = 14) and in only <15% of the trials (n = 33) escaped different types of stimuli, although each stimulus recruits different sensory and central premotor pathways [6, 7]. Nevertheless, stung cockroaches were not completely motionless; antennae occasionally moved in an exploratory manner. In 8% of the stung cockroaches, a tactile stimulus directed at the abdomen evoked a forward jump immediately followed by one or two steps backward, i.e., in the direction of the stimulus (data not shown). Such behavior was never observed in the control group. None of the observed behavioral deficits could be explained by direct sensory or motor deficits (Figure S1 and see also [2]).

Wasp Venom Elevates the Threshold for Walking Initiation

Are stung cockroaches unable to initiate walking, or is their behavioral threshold for walking elevated? To answer this question, we applied either controlled noxious or repetitive stimuli (Figure 1). For noxious stimuli, we used a modified Shuttle Box [9] in which we administered escapable foot shocks to the cockroach’s legs, without previous training. The box comprised a shocking chamber and a neutral chamber. We recorded the threshold voltage required to elicit (1) leg contractions without subsequent locomotion (i.e., the “nociceptive” threshold) and (2) walking to the neutral chamber, a distance of 5 cm (based on [8, 9]). We recorded these two values before (time point 0 in Figure 1A) and at different time points after the sting (n = 33) or, in control individuals (n = 15), after stressing the cockroaches by handling. For each individual cockroach, we considered the arithmetic difference between the nociceptive and walking threshold values to be the “escape threshold” of the individual (Figure 1A). Prior to the sting, the average escape threshold was 3 ± 2 V, demonstrating that cockroaches escaped the shock soon after it became noxious. In marked contrast, after the sting but not after the control treatment, none of the cockroaches walked to the neutral chamber in response to noxious stimuli of initial-level intensities. By increasing the applied voltage in 3 V increments at 30 s intervals, we found that the escape threshold in stung cockroaches increased gradually after the sting, reaching peak levels at 2–4 hr, staying constant for at least 20 hr, and returning to baseline 72 hr after the sting (Figure 1A). In fact, at the peak of the venom’s effect, stung cockroaches endured voltages 2- to 3-fold stronger than their nociceptive threshold without moving to the neutral chamber, although leg contractions appeared normal. Cockroaches of the control group showed no significant change in the escape threshold voltage throughout the study.

Next, we tested whether repetitive stimuli of identical intensities could evoke walking in stung cockroaches (Figures 1B and 1C). This was achieved by applying trains of 1–5 brief calibrated tactile stimuli (<0.5 s, spaced 1.5 s apart) to the abdomen of tethered cockroaches standing on a slippery surface. In control individuals, a single stimulus was sufficient to evoke a stereotypical escape response (28 ± 16 steps; n = 5; data not shown). In stung cockroaches, by contrast, trains of one or two stimuli rarely initiated a synchronized walking response (one stimulus, 1 ± 1 steps; two stimuli, 5 ± 5 steps; n = 6; Figure 1B). Trains of three or more stimuli, however, typically evoked walking that outlasted the length of the actual stimulus train (roughly 15 steps). Increasing the number of stimuli in a train more reliably evoked walking.

Taken together, our results demonstrate that stung cockroaches have an elevated behavioral threshold for the initiation of walking. The venom is injected into the cerebral ganglia and
does not affect action potential propagation [10], so it seems that stung cockroaches have a deficit in “reaching the decision” to walk. This deficit could, however, result from (1) a change in the drive for walking initiation or (2) a change in the drive for walking maintenance, despite successful initiation [11].

To discriminate between these two alternatives, we tested whether the maintenance of walking, once initiated by a suprathreshold stimulus, is impaired in stung cockroaches.

**Wasp Venom Affects Walking Maintenance**

Walking episodes initiated by repetitive tactile stimuli (Figure 1C) were significantly slower in stung as compared with control individuals (2 ± 1 and 6 ± 1 steps/s, respectively; p < 0.05). To further analyze walking maintenance, we adopted a behavioral paradigm commonly used in the study of motivation in mammals, namely the forced swimming test [12]. For cockroaches, which are terrestrial insects, water immersion for periods longer than several minutes can prove fatal. Water immersion thus provides a reliable and continuous stressful stimulus that typically produces strong, stereotypic aversion responses [13, 14]. We can thus investigate whether stung cockroaches initiate and maintain normal aversion responses when exposed to continuous stress. Stung and control individuals (n = 20 and n = 34, respectively) were immersed in a water-filled cylinder and the duration of spontaneous active swimming within a 1 min trial period was recorded. All control individuals initiated swimming upon immersion, quickly reaching the wall and continuing to swim rigorously close to the rim, occasionally attempting to climb out (Figure 2A). Overall, control subjects spent 53 ± 1 s of the 1 min trial period actively swimming (data not shown). Swimming behavior typically ceased toward the end of the trial period, although the cockroaches continued to move their antennae in an exploratory manner while floating passively on the water surface. In this state, applying wind or tactile stimuli reliably reinitiated swimming.

Like their control counterparts, 93% of the stung cockroaches initiated rigorous wall-oriented swimming immediately upon immersion (Figure 2A). However, in marked contrast to members of the control group, swimming in stung individuals ceased soon afterwards although antennal movements, as in controls, persisted. The mean duration of active swimming in the stung group was 10 ± 8 s, significantly shorter than observed with the control group (p < 0.001). Three observations indicated that the reduction in swimming duration was not due to direct motor deficits or muscle fatigue. First, as in the control group, external stimuli applied during immobile periods tended to re-evoked swimming, although the resulting swimming episodes were shorter in stung cockroaches. Second, in rare cases (such as presented in Figure 2A), short bursts of active swimming were spontaneously initiated after long periods of immobility. Finally, after removal from the water cylinder, cockroaches were placed on their backs and their ability to right themselves, i.e., to turn over using their legs and stand upright, was tested. All stung cockroaches showed vigorous leg movements during righting attempts and successfully turned themselves over, similar to members of the control group.
of the control group. Thus, to summarize, it appears that venom injection into the cerebral ganglia affects the drive for both initiation and maintenance of walking.

**Wasp Venom Does Not Affect Pattern Generator Properties**

The motor pattern observed during active swimming in stung cockroaches was comparable to that presented by control individuals, as revealed by EMG electrodes implanted in the coxal depressor muscles of the legs. First, stung cockroaches (n = 4) presented a normal gait during active swimming (Figure 2B), namely the “alternating-tripod” gait that is also observed in untreated cockroaches during walking [13, 14]. Furthermore, and similar to control individuals, fast coxal depressor potentials (Df) were sometimes recruited during active swimming bursts in stung cockroaches (data not shown). This phenomenon was never observed in stung cockroaches standing on a solid surface. Thus, under potentially fatal environmental conditions, stung cockroaches are able to express a normal walking-like motor pattern for short periods.

Next, to characterize the intrinsic properties and activity of the thoracic pattern generator in control and stung cockroaches, we focused on the occurrence of Ds potentials in the metathoracic leg (Figure 4). The motor neurons producing these potentials are active during the retraction (stance) phase of swimming or walking to provide forward propulsion. An intrinsic property of the walking pattern generator in *Periplaneta* is that with increasing cycle period, the average Ds discharge rate decreases while the stance duration increases [15–17]. We began our investigation by validating that in untreated cockroaches (n = 5), swimming and walking are similar at the level of the pattern generator output, with correlation between cycle period, average Ds discharge rate, and duration of the stance phase all being comparable for swimming and walking (p < 0.001; Figure S2). These results support previous findings [13, 14] suggesting that walking and swimming in *Periplaneta* are highly similar behaviors and that swimming may be considered as “walking on water.” Thus, investigating changes in swimming patterns can provide insight into the walking deficits of stung cockroaches.

The characteristic correlation between cycle period, Ds discharge rate, and stance duration persisted in stung individuals during active swimming and was similar to the correlation noted with control individuals (n = 4 and n = 5, respectively; p < 0.001) (Figure 3). However, the average Ds discharge rates within the stance phase were significantly lower, and the stance duration significantly longer throughout the range of cycle periods in stung insects, as compared with the control group (p < 0.001) (Figure 3). These differences imply a decrease in the excitation level of the pattern generator responsible for swimming. Because swimming and walking appear to be governed by the same pattern generator, it is reasonable to assume that walking deficits in stung cockroaches are also the result of decreased excitability in thoracic circuits. Such a decrease in excitability could be due to a decrease in the activity of thoracic dorsal unpaired median (DUM) neurons [18], a population of excitatory octopamine-releasing neurons known to affect the walking [19] and escape pattern generators [20].

**Wasp Venom Has Little Effect on Other Locomotory Behaviors**

Finally, we considered whether the wasp’s venom specifically affects walking, or motor behavior in general. We assumed that if the venom specifically affects the drive to walk, rather than the overall arousal state, then the execution and maintenance of behavioral patterns other than walking should not be significantly affected. We investigated this claim by testing two distinct motor behaviors: righting, which involves motor structures similar to walking, and flying, which involves different motor structures (Figure 4). Stung cockroaches did not show any deficiency in righting behavior, with all cockroaches being able to turn themselves upright in less than 3 s (n = 15; data not shown). By placing cockroaches on their dorsal sides on a smooth surface, we were able to record ongoing muscle potentials from the mesothoracic leg during righting attempts. Here again, and similarly to what was observed in control
individuals, stung cockroaches showed rhythmic as well as sporadic Ds bursts, occasionally accompanied by Df potentials (Figure 4A).

In a different experiment, we removed the legs of cockroaches to eliminate leg-ground contact and then applied calibrated wind stimuli to wind-sensitive hairs on the cerci. This setup allowed us to investigate fictive flying episodes in tethered cockroaches via EMG electrodes implanted in the depressor muscles of three wings, simultaneously. Overall, stung cockroaches (n = 11) showed no deficits in initiating flying behavior or in the flight motor pattern, as compared with control individuals (n = 9). First, the distribution of the threshold wind velocity required to elicit flying overlapped in the two groups of cockroaches (p = 0.415; data not shown). Second, wingbeat frequency was not significantly different between the control and stung groups (24.4 ± 1.5 Hz and 26.1 ± 1.6 Hz, respectively; p = 0.159). Third, EMG recordings and successive phase analysis (Figure 4B) showed that contralateral wings on the same body segment moved synchronously and that the forewing burst preceded the hindwing burst by approximately 7 ms. Both parameters are characteristic of cockroach flying [14]. Taken together, our results show that the wasp venom appears to specifically affect walking, rather than motor behavior in general.

In conclusion, we provide here evidence that the venom injected by *A. compressa* into the head of its cockroach prey specifically manipulates the drive to initiate and maintain walking, rather than the general arousal state of the prey. The sum of intrinsic forces promoting defined sets of behaviors in response to relevant stimuli defines the “motivational state” of an animal to initiate or maintain a specific behavior [21–23]. We therefore suggest that the venom manipulates a precursor form of motivation in the cockroach prey. This could be achieved, for instance, if the venom targets neurotransmission in the cerebral ganglia. Monoaminergic systems, and in particular the octopaminergic and dopaminergic systems, are known to profoundly affect motivation and locomotion in insects [24–27] and are thus possible targets of the venom. Accordingly, recent evidence suggests that the monoamine pair dopamine/octopamine plays an important role in cerebral circuits in the induction of hypokinesia in the wasp’s cockroach prey [28, 29]. Such manipulation would have to affect specific pathways converging, directly or indirectly (for example, via thoracic DUM neurons [18]), onto thoracic pattern-generating circuits to specifically reduce the propensity of walking-related behaviors. Further investigation of these pathways, which represent the link between decisions made in the cerebral ganglia and their execution in the thoracic ganglia, might lead to further understanding of the neuronal underpinnings of motivation and goal-directed actions in insects.

Experimental Procedures

Animals
Parasitoid wasps, *Ampulex compressa* Fabricius (Hymenoptera: Ampulicidae), were taken 2–6 weeks after metamorphosis. Male *Periplaneta americana* cockroaches were taken 2–4 months after adult molt. All animals were reared under laboratory conditions [29]. A wasp was allowed to complete a full stinging sequence and the cockroach was immediately removed to prevent further manipulation by the wasp.
Behavioral Assays
A detailed description of some of the assays performed in the present work can be found in [14]. In brief, assays were performed on freely moving cockroaches in an open-field arena (radius = 30 cm) or, for more detailed behavioral analyses, on tethered individuals walking on an oiled surface. Exploratory behavior was measured as the total duration of exploration and climbing attempts in an open-field arena during a 10 min trial. Positive exploratory behavior was defined as walking >1 min in the novel environment. Escape responses were measured as the distance traveled after receiving a tactile stimulus to the antenna or abdomen or a wind stimulus to the cerci. A positive escape response was defined by walking >5 cm. Each stimulus was given three times for each cockroach with 1 min intervals. Escapable foot shocks were administered in a custom-built Perspex box (35 × 7 × 15 cm), divided into a shocking chamber and a neutral chamber. The shocking chamber was lined with a 6-mm-thick antistatic foam sheet (SJM Eurostat, Cheshire, UK), through which electric shocks (10 s pulse trains, pulse duration = 1 s, pulse period = 1.5 s, amplitude range = 30–100 V) were applied to the tarsi of standing cockroaches. Repetitive stimuli of identical intensity were applied to the abdomen of tethered cockroaches via a glass rod connected to a step motor. A digitized sequencer (Spike2, CED, Cambridge, UK) supplied a train of 1–5 brief (<0.5 s) stimuli, spaced 1.5 s apart. Different trains, consisting of a different number of stimuli, were applied at random order, and each cockroach received at least three trains of each type. In some instances, the same cockroach was tested before and after the sting. A photosensor was placed directly beneath the mesothoracic leg that was illuminated from above, allowing us to record voltage deflections resulting from leg movements evoked by the tactile stimuli. Swimming in a modified forced swimming test [12] was induced by placing a cockroach in an opaque pool (25 cm in diameter) filled with water to a height of 10 cm, maintained at 25°C. The duration of swimming during a 1 min period was measured, and swimming behavior was recorded with a camera (Cohu, San Diego, CA) for later analysis and motion tracking. Flying was tested by exciting the legs and applying a wind puff to the cerci, as described previously [14]. Righting was tested by placing a cockroach ventral side up on coarse sandpaper and measuring the duration it took for the cockroach to right itself, i.e., to turn over and stand upright. A righting attempt was considered successful if the cockroach turned over in less than 3 s.

Electrophysiology
Cockroaches used for electrophysiological studies were not pooled with cockroaches used for behavioral studies. Behavioral parameters measured in cockroaches used for electrophysiology, however, were not significantly different from data obtained in behavioral studies (data not shown). EMG electrodes used were 50 μm steel wires insulated to the tip, implanted into the coxal depressor muscles of the legs or the depressor muscles of the wings, and held in place with dental wax (for further details, see [14]).

Figure 4. Stung Cockroaches Show Righting and Flying Behaviors Comparable to Those of Normal Cockroaches
(A) EMG recordings from the coxal depressor muscles of the metathoracic leg during righting attempts are similar in control (left) and stung (right) individuals. Slow (Ds) and fast (Df) potentials occur regularly during righting attempts in control as well as in stung cockroaches. The scale bar applies to both traces.
(B) Simultaneous EMG recordings from depressor muscles of the right forewing (R1) and the right and left hindwings (R2 and L2, respectively) during a flying episode in a stung cockroach. A phase histogram (right) of wing depressor potentials demonstrates a normal flying pattern, in which the burst of the forewings slightly precedes the synchronous burst of the hindwings. Dashed lines represent the average phases, quantified by the values above.

We focused on swimming and flying because these are two rhythmic behaviors that could be elicited in stung cockroaches. For swimming, we analyzed, in detail, >800 retraction cycles of active swimming, based on [14, 16]. For flying, we analyzed, in detail, >4000 wingbeat cycles of active flight, based on [14]. All electrophysiological recordings were digitized and processed with Spike2 data acquisition software (CED, Cambridge, UK). Low-frequency movement artifacts were filtered out digitally.

Statistical Analysis
We used Student’s t test for analysis of normally distributed data or the Mann-Whitney Rank Sum Test for testing non-normally distributed data. Correlations were tested with the Pearson Product-Moment Correlation test. Data in this work are presented as means ± standard deviation (SD), with n representing the number of animals tested.

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