Direct Injection of Venom by a Predatory Wasp into Cockroach Brain

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ABSTRACT: In this article, we provide direct evidence for injection of venom by a wasp into the central nervous system of its cockroach prey. Venomous predators use neurotoxins that generally act at the neuromuscular junction, resulting in different types of prey paralysis. The sting of the parasitoid wasp *Ampulex compressa* is unusual, as it induces grooming behavior, followed by a long-term lethargic state of its insect prey, thus ultimately providing a living meal for the newborn wasp larvae. These behavioral modifications are induced only when a sting is inflicted into the head. These unique effects of the wasp venom on prey behavior suggest that the venom targets the insect's central nervous system. The mechanism by which behavior modifying compounds in the venom transverse the blood-brain barrier to induce these central and long-lasting effects has been the subject of debate. In this article, we demonstrate that the wasp stings directly into the target ganglia in the head of its prey. To prove this assertion, we produced "hot" wasps by injecting them with ^{14}C radiolabeled amino acids and used a combination of liquid scintillation and light microscopy autoradiography to trace radiolabeled venom in the prey. To our knowledge, this is the first direct evidence documenting targeted delivery of venom by a predator into the brain of its prey.
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Keywords: **neurotoxins; *Ampulex compressa*; mushroom bodies; central complex; subesophageal ganglion; stinging behavior**

INTRODUCTION

Animals as diverse as snakes, scorpions, spiders, insects, and snails manufacture venoms to capture their prey (Adams and Olivera, 1994; Rappuoli and Montecucco, 1997). Venoms contain various toxins, which affect mostly the ability of the prey's nervous system to generate muscle contractions resulting in an immobilization of the prey. Within the large group of venomous wasps, a few species do not paralyze but manipulate the behavior of their victims in the most interesting ways (Rathmayer, 1978; Steiner, 1986; Piek, 1990). The parasitoid solitary wasp *Ampulex compressa* hunts cockroaches (*Periplaneta americana*) and attacks them by stinging them first in the thorax and then in the head (Williams, 1942; Piek et al., 1984; Fouad et al., 1994) [Fig. 1(A)]. Unlike most venomous predators (Piek, 1990; Adams, 1996), the wasp does not induce paralysis in its prey. Instead, it applies the unique strategy of behavioral modulation of the cockroach (Fouad et al., 1994, 1996; Piek et al., 1984; Weisel-Eichler et al., 1999; Williams, 1942). The stung prey first grooms extensively (Weisel-Eichler et al., 1999), after which it becomes sluggish and is not responsive to various stimuli (Fouad et al., 1994, 1996; Libersat et al., 1999). The wasp grabs one of the antennae of the cockroach, which follows docilely to a suitable oviposition location,

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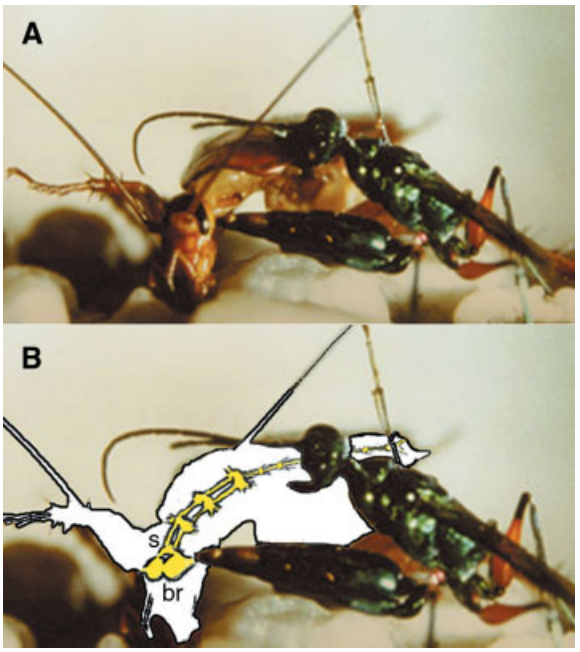

Figure 1 *Ampulex compressa* stinging behavior. (A) The wasp stings a cockroach in the head. (B) The ventral nerve cord is schematically illustrated to indicate the position of the head ganglia relative to the wasp sting (br, brain; s, subesophageal ganglion). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

where it will serve a few days later as an immobilized fresh food source for the wasp's offspring (Williams, 1942; Piek et al., 1984; Fouad et al., 1994). We have shown that these behavioral modifications are evoked by the sting in the head rather than by the sting in the thorax (Fouad et al., 1994). In addition, we have shown that the venom has no effect on the cockroach's neuromuscular junction (Fouad et al., 1996) and that specific motor behaviors are modulated while others, such as flight, are little affected (Fouad et al., 1994; Libersat et al., 1999; Weisel-Eichler and Libersat, 2002). Thus, the venom appears to target the central nervous system (CNS) in the head to modulate specific behaviors.

For more than a century, there has been a controversy over whether or not some parasitoid wasps such as *A. compressa* deliver their venom by stinging directly into the nervous system (Fabre, 1879; Ferton, 1902; Roubaud, 1917; Steiner, 1986). To resolve this issue, we have radiolabeled wasp peptides *in vivo* by injection of radioactive amino acids, allowed the wasps to sting prey, and then measured radioactivity levels in specific tissues of the head of stung cockroaches. Using this approach, we have explored the possibility that *A. compressa* delivers its venom by stinging directly into the head ganglia of its prey [Fig. 1(B)].

METHODS

Animals

The wasps, *A. compressa* Fabricius (*Hymenoptera*: *Sphecidae*), were raised at 30°C on a 12L:12D cycle in Perspex cages and provided with abundant water and honey. Adult cockroaches (*P. americana*) were raised at 26°C in plastic cages and provided with abundant water and cat food pellets.

Radiolabeling of Venom

The venom glands of five wasps were first depleted by milking. The wasps were then injected with a mixture of ^{14}C radiolabeled amino acids (10 μCi per wasp; Sigma). Two days later, the venom of these wasps was milked using the following procedure. Wasps were immobilized with CO_2 and confined to a small, conical, plastic tube open at both ends. A modified syringe plunger was fitted to one end of the tube and used to provoke the wasp to sting a small piece of Parafilm (American National Can) held in front of the other end. Venom droplets were collected into about 10 μL/wasp of 10 m*M* HEPES buffer (pH 7.4 with NaOH) containing 0.1 m*M* PMSF, a serine protease inhibitor (Sigma). The peptides were separated on SDS-PAGE, stained with Coomassie blue, and then exposed for 5 weeks to X-ray film. The milked venom contained more than 10 peptides, all of which were labeled to various extents. Five more wasps, radiolabeled in the same manner, were allowed to freely sting several cockroaches 2 days after the injection of radiolabeled amino acids. To reduce the diffusion of labeled venom, the stung cockroaches were collected immediately after the sting and put on ice for no more than 1 min before the dissection of the head tissues. To check the alternative possibility of diffusion of labeled venom from the head cavity into the CNS, we injected cockroaches with labeled amino acid mixture outside of the CNS and followed the distribution of the radioactive signal.

Liquid Scintillation

The amount of radioactivity in the tissues of cockroaches stung by the radiolabeled wasps or injected with radioactive amino acids was assessed using liquid scintillation (Ultima Gold LSC cocktail and Liquid Scintillation Analyzer 2100TR; Packard). We examined the brain, subesophageal ganglion (SEG), and the surrounding non-neuronal head tissues.

Light Microscope Autoradiography

To determine the precise location of injection, cockroaches stung by radiolabeled wasps were collected in fixative (4% paraformaldehyde, 0.16% glutaraldehyde, 0.03% picric acid in 0.2 *M* phosphate buffer, pH 7.2), their head ganglia were dissected and embedded in plastic resin (JB-4Plus; Polysciences, Inc.), and serially sectioned at 10 μm. Sections were mounted on slides, dipped in autoradiography emulsion (LM-1; Amersham-Pharmacia), and exposed for 6 weeks. After developing the slides, images were acquired through a microscope equipped with a digital camera (Dage MTI: CCD 300T RC).

RESULTS

We labeled venom peptides *in vivo* by injecting radioactive amino acids into wasps. We then allowed these radiolabeled wasps to sting cockroaches and measured radioactivity levels in specific tissues of the stung cockroaches. When measuring the levels of radioactivity in the various tissues (Fig. 2), we found that these were significantly higher ($p < 0.01$) in the head ganglia (brain, 80 ± 2%; SEG, 18 ± 2%) than in the surrounding head tissue (2 ± 0%). It is worth noticing that the radioactivity measured in the brain was always higher (2- to 10-fold, $p < 0.01$) than that measured in the SEG. To check the alternative possibility of diffusion of the venom into the head ganglia, we injected radiolabeled amino acids into the head cavity, that is, the space around the brain. We then measured the radioactive level in the same tissues as in the stung cockroaches. In the injected cockroaches, most of the radioactive signal was found in the surrounding head tissue (76 ± 2%) while significantly less ($p < 0.01$) was found in the head ganglia (brain, 14 ± 1%; SEG, 10 ± 1%). Thus, we conclude that the high levels of radioactivity detected in the head ganglia of cockroaches stung by radiolabeled wasps are not due to diffusion from the surrounding tissues. Rather, they are due to a direct sting and delivery of the venom into the CNS of the cockroach.

To determine the precise location of injection, we used autoradiography emulsion to visualize radioactivity in the head ganglia of cockroaches stung by radiolabeled wasps. Radioactivity was observed in the central part of defined structures of the brain (Chiang et al., 2001), posterior to the central complex and around the mushroom bodies [Fig. 3(C)]. This pattern of staining was observed in a total of five stung animals. Radioactivity was observed also around the ganglion midline of the SEG [Fig. 3(B)]. When looking through all the serial sections of each of the brain-SEG preparations ($n = 5$), we found no trace of radiolabeled signal in the circumoesophageal connectives [Fig. 3(C)].

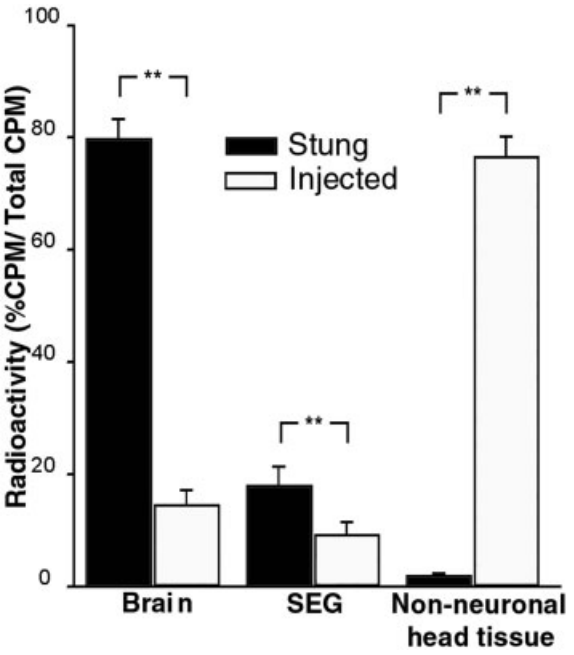

Figure 2 Localization of radiolabeled venom by liquid scintillation. In stung cockroaches (solid bars, $n = 16$; mean of percentile + standard error), the levels of radiolabeled venom were significantly higher ($p < 0.01$) in the head ganglia (brain and SEG) than in non-neuronal head tissue. When radioactive amino acids were manually injected into the head cavity of other cockroaches (open bars, $n = 15$), the levels of radiolabeled venom were significantly higher ($p < 0.01$) in non-neuronal head tissue than in the head ganglia. Furthermore, significantly different ($^{**}p < 0.01$) levels of radioactivity were measured in stung and injected cockroaches in each of the sampled tissues. The measurements are represented as the percentile fraction of the total CPM (counts per minute) of a specimen. All probability values are of Fisher LSD test following a two-way (stung or injected; tissue) ANOVA on the log transformed percentile values. The two-way ANOVA probability values were lower than 0.0001 for both factors and the interaction between them.

DISCUSSION

We have demonstrated unequivocally that the wasp *Ampulex compressa* stings into specific regions of the head ganglia of the cockroach. In a similar system, Gnatzy and Otto (1996) have provided indirect evidence that the wasp *Liris nigra* stings its cricket prey in the thoracic and subesophageal ganglion. However, in that study, visual observation of the penetration of the sting into the nervous system was obtained in

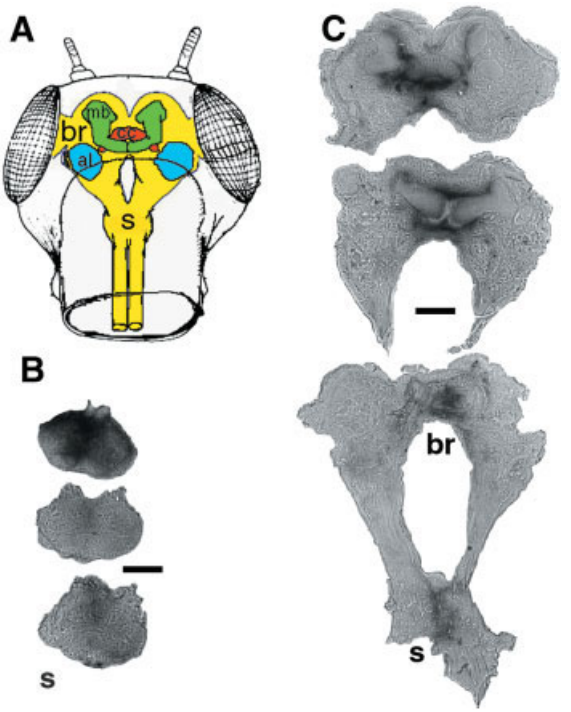

Figure 3 Neuroanatomical localization of radiolabeled venom. (A) Schematic representation of a dorsal view of a cockroach head shows the relative positions of the head ganglia in the head capsule. The brain (br) and subesophageal ganglion (s) are shown. The major structures of the brain include the central complex (cc), the mushroom bodies (mb), and the antennal lobes (al). (B) Three sections of a representative subesophageal ganglion (SEG) preparation of a cockroach, stung by a radiolabeled wasp. Radiolabeled venom, indicated as black stain, was located around the ganglion midline. (C) Three sections of a representative head ganglia (brain and SEG) preparation of a cockroach, stung by a radiolabeled wasp. Radiolabeled venom was located posterior to the central complex and around the mushroom bodies of the brain (br) and around the center of the SEG (s). Scale bars = 0.25 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

restrained conditions with a tethered wasp forced to sting a tethered prey. The first sting of *A. compressa* into the first thoracic segment induces 2 to 3 min of transient flaccid paralysis of the front legs. In a recent study, we demonstrated that the wasp injects its venom directly into the cockroach's first thoracic ganglion (Haspel and Libersat, 2003). In the same study, we showed that the transient paralytic effect of the thoracic sting can be mainly accounted for by the presence of a venom active component that induces a postsynaptic block of central cholinergic synaptic transmission (Haspel and Libersat, 2003). This focal injection causes a local and specific modulation of motor control of the front legs through a modulation of central synapses. We suggest that *A. compressa* stings the cockroach directly into the first thoracic ganglion to flaccidly paralyze the front legs, thereby facilitating the more difficult and precise head sting into the brain. This is consistent with the present study in which we show that *A. compressa* stings not only into the SEG, which lies directly underneath the site of the sting in the neck, but also further into the more distant brain. To insure that the sting is long enough to reach the brain, the stingers of a few wasps ($n = 5$) were dissected out and imaged under the microscope. We found that the protruding part of the stinger is long enough (2.5 ± 0.2 mm; $n = 5$) to reach the brain, which lies 1 to 2 mm deep in the head capsule (Fig. 4). To our knowledge, this is the first direct evidence documenting targeted delivery of venom into the CNS of a prey organism. Examination of the tip of the sting (ovipositor) in various families of social wasps and bees shows the presence of numerous receptors (Van Marle and Piek, 1986). However, such information is not available regarding the solitary wasps. It would be interesting to categorize the receptors found on the stinger of *Ampulex* and examine their possible role in distinguishing nervous tissue from non-nervous tissue. Although locomotory patterns are generated in the thoracic ganglia in insects, it has been suggested that the SEG and the brain are implicated in controlling the expression of motor actions (Kien and Altman, 1992; Martin et al., 1998). The SEG has been found to be involved in selection, organization, initiation, and maintenance of various behaviors in insects, such as walking (Altman and Kien, 1987; Kien, 1983), stridulation (Lins and Elsner, 1995), and flight (Ramirez, 1988). Thus, it appears that the SEG functions to control the excitability of motor networks in

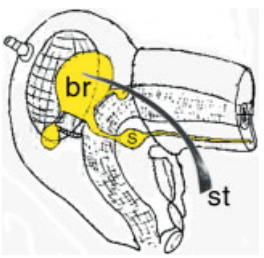

Figure 4 The wasp stinger can reach the brain. A micrograph of the stinger (st) is shown over a schematic lateral view of a cockroach head, scaled by measuring dissected stingers and cockroach heads, to demonstrate their relative proportions. The protruding part of the stinger is long enough (2.5 ± 0.2 mm; $n = 5$) to reach the brain (br), which lies 1 to 2 mm deep in the head capsule. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the thorax (Altman and Kien, 1987; Johnston et al., 1999). For the brain, the radioactive signal was concentrated in the central part of the brain, around the mushroom bodies and posterior to the central complex (Chiang et al., 2001). The mushroom bodies appear to conditionally relay information about sensory stimuli and their context to higher brain centers and to play a central role in olfactory and place learning and memory (Strausfeld et al., 1998). They also appear to be important in the control of the expression of motor actions (Martin et al., 1998). In addition, focal chemical stimulation of the central complex affects the threshold of initiation of a specific motor act, suggesting a role for this brain area in arousal (Heinrich et al., 2001). It is known that the long-lasting lethargic state is induced only when a sting is inflicted into the head (Fouad et al., 1994). Given these facts, the labeled venom detected in the SEG and around the central complex and mushroom bodies in the brain provides further support for the role of these neuronal structures in the control of insect locomotion and arousal. For *A. compressa*, it has been shown that the head sting affects neither the neuromuscular system nor the activation of thoracic interneurons by primary sensory interneurons (Fouad et al., 1994, 1996; Libersat et al., 1999). Therefore, the thoracic sites that are indirectly modulated by the venom injected into the head ganglia are likely to be the synaptic connections between thoracic interneurons and thoracic motoneurons for locomotion (Libersat et al., 1999). Because the wasp injects venom into the SEG and brain, it is possible that specific venom components directly affect neurons in the brain or SEG that modulate synapses in the thorax. Furthermore, we have shown that injection of a dopamine agonist directly into the SEG is alone sufficient to induce excessive grooming and that dopamine is present in the venom (Weisel-Eichler et al., 1999). Altogether, we propose that the dopamine in the venom is injected by the wasp directly into the SEG to induce prolonged grooming by stimulating dopamine receptors in the cockroach's SEG.

The specificity and effectiveness of neurotoxins lie in their exquisite selectivity for their molecular targets and are the outcome of evolutionary selection on one animal's strategy to incapacitate another (Adams and Oliviera, 1994). Here, we highlight the selection of an unusual behavioral strategy by a venomous predator for the delivery of these neurotoxins into the brain of its prey to cause specific and effective behavioral modifications. The precise anatomical targeting through the body wall and ganglionic sheath is akin to the most advanced stereotaxic delivery of drugs.

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