

## RESEARCH ARTICLE

# The role of the cerebral ganglia in the venom-induced behavioral manipulation of cockroaches stung by the parasitoid jewel wasp

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## ABSTRACT

The jewel wasp stings cockroaches and injects venom into their cerebral ganglia, namely the subesophageal ganglion (SOG) and supraesophageal ganglion (SupOG). The venom induces a long-term hypokinetic state, during which the stung cockroach shows little or no spontaneous walking. It was shown that venom injection to the SOG reduces neuronal activity, thereby suggesting a similar effect of venom injection in the SupOG. Paradoxically, SupOG-ablated cockroaches show increased spontaneous walking in comparison with control. Yet most of the venom in the SupOG of cockroaches is primarily concentrated in and around the central complex (CX). Thus the venom could chiefly decrease activity in the CX to contribute to the hypokinetic state. Our first aim was to resolve this discrepancy by using a combination of behavioral and neuropharmacological tools. Our results show that the CX is necessary for the initiation of spontaneous walking, and that focal injection of procaine to the CX is sufficient to induce the decrease in spontaneous walking. Furthermore, it was shown that artificial venom injection to the SOG decreases walking. Hence our second aim was to test the interactions between the SupOG and SOG in the venom-induced behavioral manipulation. We show that, in the absence of the inhibitory control of the SupOG on walking initiation, injection of venom in the SOG alone by the wasp is sufficient to induce the hypokinetic state. To summarize, we show that venom injection to either the SOG or the CX of the SupOG is, by itself, sufficient to decrease walking.

**KEY WORDS:** Cockroach, Wasp, Parasitoid, Central complex, Subesophageal ganglion, Supraesophageal ganglion, Behavioral manipulation, Locomotion

## INTRODUCTION

The parasitoid jewel wasp (*Ampulex compressa* (Fabricius 1781)) hunts and stings cockroaches (*Periplaneta americana* (Linnaeus 1758)) to use them as a live fresh food supply for its offspring. In contrast to other parasitoid wasps, the jewel wasp venom does not paralyze the cockroach. Instead, the venom causes specific changes in the cockroach behavior (Fouad et al., 1994). After the cockroach is stung, it grooms continuously for 30 min and then it enters a long-term hypokinetic state, during which it does not voluntarily engage in spontaneous locomotion and escapes neither wind nor tactile stimuli. Such a behavioral manipulation allows the wasp to walk the cockroach to a burrow, where it affixes its egg. The larva that hatches from the egg feeds on the cockroach hemolymph at first, and then enters the cockroach abdomen to devour the cockroach's internal organs (Haspel et al., 2005).

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Behavioral and electrophysiological studies have demonstrated that the venom effect on locomotion is limited to walking and escape behaviors (Libersat, 2003). Other behaviors, such as flying or righting, remain unaffected and the walking pattern generator, which locally controls the spatiotemporal motor pattern of leg movements during walking, remains functional in stung cockroaches. More specifically, the wasp's sting affects the spontaneous initiation and the maintenance of walking (Libersat, 2003; Gal and Libersat, 2008). It has been shown that the wasp injects its venom directly into the cockroach cerebral ganglia, namely the subesophageal ganglion (SOG) and the supraesophageal ganglion (SupOG) (Haspel et al., 2003). The SOG and SupOG, which are connected to each other by the circumesophageal connectives (CirC), are considered to be 'higher-order' neuronal centers that modulate different aspects of locomotion (Kien and Altman, 1992; Strauss and Heisenberg, 1993; Strausfeld, 1999; Schaefer and Ritzmann, 2001). As the venom is injected directly into the cerebral ganglia, the behavioral change observed in stung cockroaches must be the result of a pharmacological manipulation of neuronal circuits in these ganglia. However, the contribution of venom injection into each of those ganglia to the observed behavioral manipulation is still unclear.

Based on experiments performed on the SOG, venom injected into the SupOG almost certainly decreases neuronal activity there (Gal and Libersat, 2010). Paradoxically, SupOG-ablated cockroaches show increased spontaneous walking in comparison with control cockroaches (Kien, 1983; Ridgel and Ritzmann, 2005; Gal and Libersat, 2006), indicating that the SupOG plays a general inhibitory role in the control of walking. While the entire SupOG has a general inhibitory role, it is still possible that the wasp targets subregions in the SupOG.

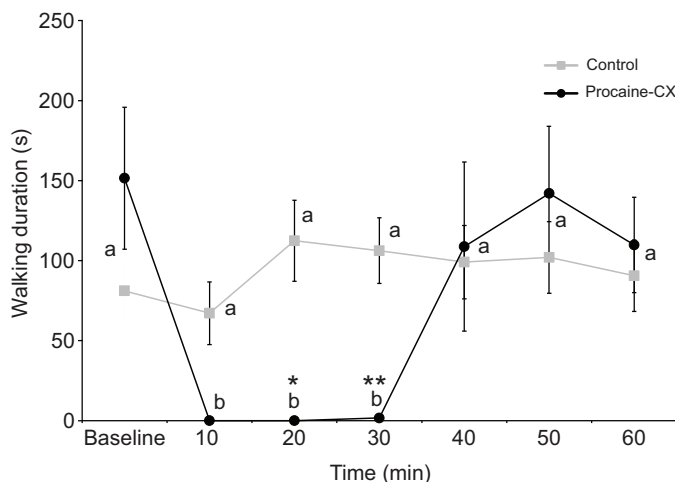
Most of the venom injected by the wasp into the SupOG is predominantly concentrated in its central part (Haspel et al., 2003), especially in and around the central complex (CX; also known previously as CC; Ito et al., 2014). Thus the venom could affect primarily this region to contribute to the hypokinetic state, leaving other regions unaffected. In fact, the CX is a target of choice for the wasp's venom, as many studies suggest that the CX is important for the initiation and maintenance of walking (Huber, 1960; Martin et al., 1999; Strausfeld, 1999; Winther et al., 2006; Bender et al., 2010; Kahsai et al., 2010). In addition, studies carried out on the fruit fly suggest that the CX plays a permissive role in the control of walking, whereas the mushroom bodies (MBs), a bilateral region in the SupOG, have a suppressive effect on walking (Martin et al., 1998). We suggest that the venom injected into the SupOG could affect mainly the CX to decrease spontaneous walking. To test this hypothesis and resolve the aforementioned paradox, we first performed a series of experiments in which we injected compounds into discrete regions of the SupOG and evaluated the resulting behavioral deficits.

**List of abbreviations**

CirC	circumesophageal connectives
CX	central complex
MBs	mushroom bodies
SOG	subesophageal ganglion
SupOG	supraesophageal ganglion

The SOG, anatomically, is the second cerebral ganglion, located below and connected to the SupOG through the CirC. A previous study has shown that injection of procaine (a reversible voltage-dependent sodium and potassium channel blocker) into the honey bee brain (Devaud et al., 2007), or into the SOG of cockroaches (Gal and Libersat, 2010) reduces neuronal activity in these ganglia. In the latter, such an injection results in a decrease in spontaneous walking. Likewise, venom injections to the SOG also decrease neuronal activity and, consequently, decrease spontaneous locomotion (Gal and Libersat, 2010). Thus this study and others show that the SOG exerts a net tonic permissive effect in the control of initiation and maintenance of walking (Kien, 1983; Johnston et al., 1999; Schaefer and Ritzmann, 2001). Hence the second aim of the present study was to examine the contribution of each cerebral ganglion to the manipulation of walking. We therefore performed a series of experiments involving lesions and natural venom injection by wasps into the cerebral ganglia.

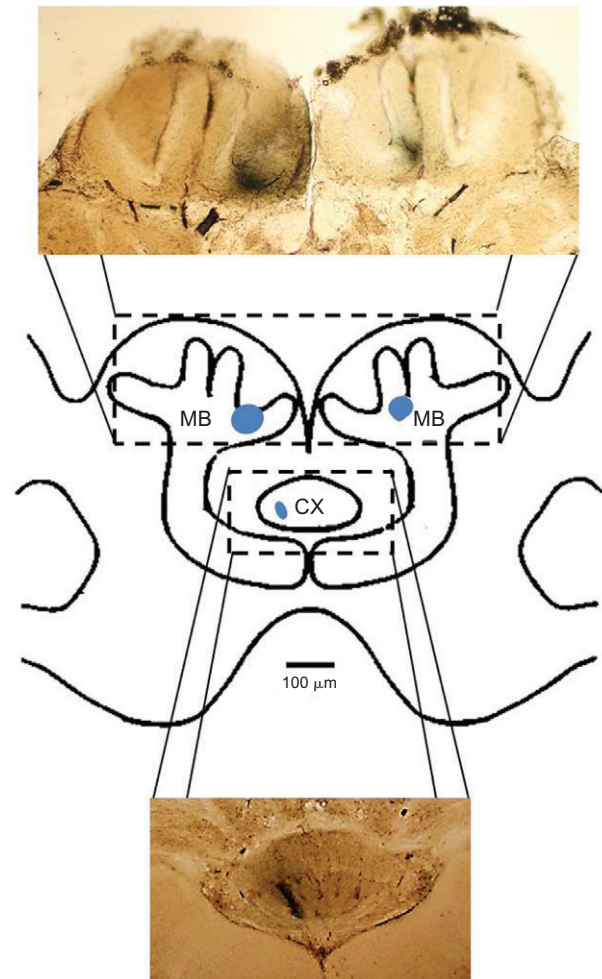
Our results show that the CX is predominantly permissive for the initiation of spontaneous walking and that its role in the control of spontaneous walking is antagonistic to that of the MBs. We show that a focal injection of venom or procaine into the CX is sufficient to induce the decrease in spontaneous walking. However, we also show that when the wasp stings a cockroach with a disabled SupOG but a normally functioning SOG, the cockroach shows decreased spontaneous locomotion. Thus venom in each of the two cerebral ganglia is sufficient to induce a decrease in spontaneous walking, as seen in stung hypokinetic cockroaches.



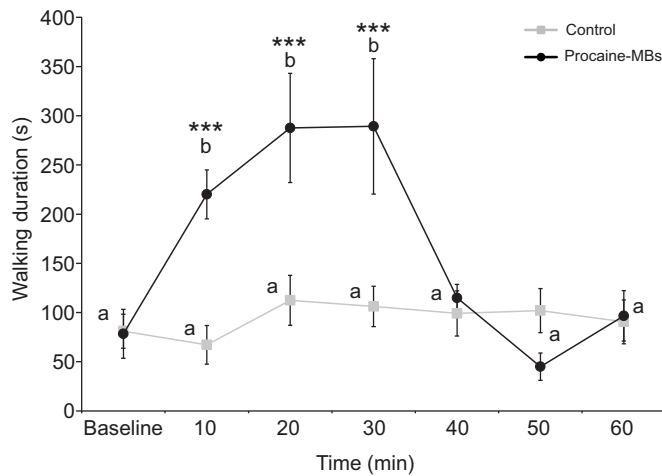
**Fig. 1. A procaine injection into the CX decreases spontaneous walking in cockroaches.** Spontaneous walking duration is measured in seconds, in 10 min bins, for baseline walking and for a continuous hour after nano-injection treatments. Data points represent means  $\pm$  s.e.m. Data points with the same letter are not significantly different, whereas data points with different letters are significantly different ( $P < 0.05$ ). Asterisks represent significant difference between treated and control groups (\* $P < 0.05$ ; \*\* $P < 0.01$ ).  $N = 22$  and  $N = 8$  cockroaches in control and 'Procaine-CX' groups, respectively.

**RESULTS****A procaine injection to the CX decreases spontaneous walking**

First, to determine the role that the CX plays in the control of spontaneous walking, we focally injected procaine to the CX to reversibly decrease neuronal activity specifically in this region (Devaud et al., 2007; Gal and Libersat, 2010; Kathman et al., 2014). Compared with the baseline walking duration, cockroaches that were injected with procaine to the CX walked significantly less, if at all, for 30 min after the injections ( $P < 0.05$ ; Fig. 1). After this time period, the walking duration returned to baseline, as expected given the reversible nature of procaine. In addition, compared with control cockroaches, the walking duration was significantly lower in the 'Procaine-CX' cockroaches, especially 20 and 30 min after injections ( $P = 0.013$  and  $P = 0.009$ , respectively; Fig. 1). Post-mortem verification of the injection site confirmed that the injections were focused in the central body of the CX (Fig. 2). These results show that procaine injections to the CX have an inhibitory effect on walking.



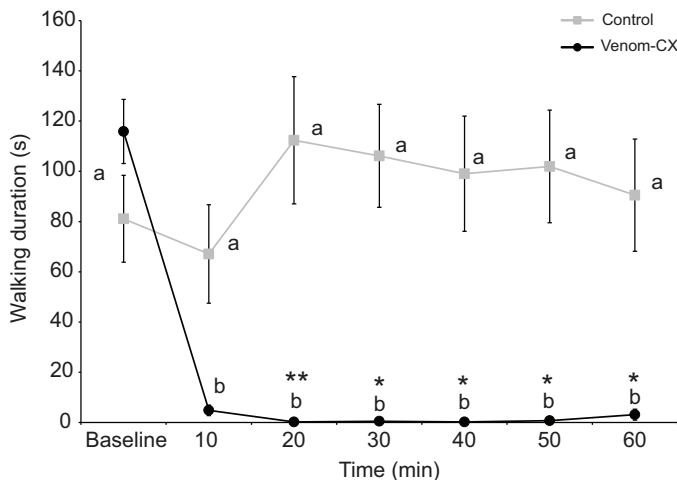
**Fig. 2. Representative images for post-mortem verification of injection site.** In the center, a schematic drawing shows the general anatomy of the SupOG (Mizunami et al., 1998). Darker spots in the drawing indicate the injection sites as shown in the representative images (bottom and top). The bottom photograph shows the injection site in the central body of the central complex (CX), and the top photograph shows the injection sites of the mushroom bodies (MBs).



**Fig. 3. A procaine injection into the MBs increases spontaneous walking.** Spontaneous walking duration is measured in seconds, in 10 min bins, for baseline walking and for a continuous hour after nano-injection treatments. Data points represent means $\pm$ s.e.m. Data points with the same letter are not significantly different, whereas data points with different letters are significantly different ( $P<0.05$ ). Asterisks represent significant difference between treated and control groups ( $***P<0.001$ ).  $N=22$  and  $N=8$  cockroaches in control and 'Procaine-MBs' groups, respectively.

#### A procaine injection to the MBs increases spontaneous walking

We then tested the effect of a bilateral procaine injection into the MBs, which are also known to be involved in walking. Compared with baseline walking duration, cockroaches that were injected with procaine to the MBs walked significantly more for 30 min after the injections ( $P<0.005$ ; Fig. 3). Likewise, compared with control cockroaches, the walking duration was significantly higher in the 'Procaine-MBs' cockroaches for 30 min after the injection ( $P<0.001$ ; Fig. 3). As expected, the walking duration returned to baseline 30 min following the injections.



**Fig. 4. An injection of jewel wasp venom into the CX decreases spontaneous walking.** Spontaneous walking duration is measured in seconds, in 10 min bins, for baseline walking and for a continuous hour after nano-injection treatments. Data points represent means $\pm$ s.e.m. Data points with the same letter are not significantly different, whereas data points with different letters are significantly different ( $P<0.05$ ). Asterisks represent significant differences between treated and control groups ( $*P<0.05$ ,  $**P<0.01$ ).  $N=22$  and  $N=8$  cockroaches in control and 'Venom-CX' groups, respectively.

Post-mortem verification of the injection site confirmed the location of the injections to the MBs (calyces region, Fig. 2). These results show that the procaine injections to MBs have an opposite effect to procaine injections to CX, and that these two regions in the SupOG appear to have antagonistic roles in the control of initiation of walking.

#### A venom injection to the CX decreases spontaneous walking

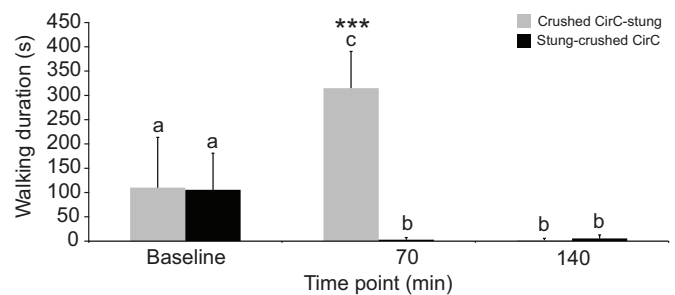
Most of the venom injected to the SupOG is concentrated in or around the CX. This means that the wasp could target the CX specifically to decrease spontaneous walking in the cockroach. If true, then venom injection to the CX should have a comparable effect to procaine injections to the CX, and should decrease spontaneous walking.

To test our hypothesis, we focally injected freshly milked venom to CX. Compared with the baseline walking duration, cockroaches injected with venom to the CX walked significantly less for the entire duration of the behavioral test ( $P<0.05$ ; Fig. 4). Likewise, compared with control cockroaches, the walking duration was lower in the 'Venom-CX' injected cockroaches throughout the entire duration of behavioral testing ( $P<0.05$ , 20–60 min; Fig. 4). Post-mortem verification of the injection site confirmed the location of the injections to the central body in the CX (not shown). These results show that venom injection to the CX alone is sufficient to decrease spontaneous walking.

#### SupOG-ablated stung cockroaches show decreased spontaneous walking

To examine the contribution of each cerebral ganglion to the drastic effect of the sting on walking, we performed another series of experiments. Although it has been shown that venom injection to the SOG is, in itself, sufficient to decrease spontaneous walking, it is not known if a natural direct injection by the wasp to cockroaches with only SOG will be sufficient to decrease spontaneous walking. If this was the case, the effect of venom in the SOG will need to overcome the increased walking observed previously in SupOG-ablated cockroaches (Gal and Libersat, 2006).

To investigate this possibility, we measured spontaneous walking in two groups: 'Crushed CirC-stung' cockroaches and 'Stung-crushed CirC' cockroaches. In the first group, the CirC were crushed before the cockroach was stung whereas in the second group, the



**Fig. 5. SupOG-ablated stung cockroaches show decreased spontaneous walking.** Spontaneous walking duration is measured in seconds, in 10 min bins, at three time points: baseline (before treatment), 70 min (an hour after the first treatment) and 140 min (an hour after second treatment). For 'Crushed CirC-stung' cockroaches, the first treatment was crushing the CirC and the second one was stinging by a wasp. For 'Stung-crushed CirC' cockroaches, the first treatment was stinging by a wasp and the second one was crushing the CirC. Data points represent means $\pm$ s.e.m. Data points with the same letter are not significantly different, whereas data points with different letters are significantly different ( $P<0.05$ ). Asterisks represents significant difference between treatments ( $***P<0.001$ ).  $N=8$  cockroaches in each treatment.

CirC were crushed after the cockroach was stung. By crushing the CirC we removed descending inhibitory control of the SupOG on walking (Gal and Libersat, 2006). We expected that, if venom in the SOG is sufficient to decrease spontaneous walking, then crushing the CirC before or after the sting would not change the effect of the sting and the cockroach will be hypokinetic in either one of the treatments.

As expected, for ‘Crushed CirC-stung’, there was a significant increase in walking duration after surgery (70 min,  $P < 0.001$ ; Fig. 5) compared with baseline. Following the sting, the walking duration dramatically decreased (140 min,  $P < 0.001$ ; Fig. 5), similar to when a wasp stung a normal cockroach (‘Stung-crushed CirC’, 70 min,  $P < 0.001$  compared with baseline; Fig. 5).

Crushing the CirC after the sting (‘Stung-crushed CirC’ group) did not affect the walking duration of stung cockroaches as they remained hypokinetic (140 min; Fig. 5).

These results show that venom injection to the SOG alone is sufficient to induce a decrease in spontaneous walking and to overcome the increased walking of SupOG-ablated cockroaches.

## DISCUSSION

The parasitoid jewel wasp stings cockroaches and injects venom directly into their cerebral ganglia, namely into the SOG and the SupOG (Haspel et al., 2003). While venom or procaine injection into the SOG alone decreases spontaneous walking, venom or procaine injection into the entire SupOG paradoxically increases spontaneous walking (Gal and Libersat, 2010). Yet the wasp does not inject venom into the entire SupOG but, rather, mostly into a discrete region of the SupOG known as the CX (Haspel et al., 2003). Hence the goal of the present study was to resolve this paradox by using behavioral, surgical and neuropharmacological methods.

We show here that procaine focally injected into the CX dramatically decreased spontaneous walking in cockroaches; in fact, most cockroaches did not show any spontaneous walking (Fig. 1). This clearly indicates that the CX is permissive for the initiation of spontaneous walking. Our result is consistent with work carried out on *Drosophila melanogaster*, which shows that flies with genetically manipulated neurons or structural mutations of the CX demonstrate shorter activity duration, lower walking speed, decreased levels of locomotor activity and changes in walking time intervals (Strauss et al., 1992; Strauss and Heisenberg, 1993; Martin et al., 1999; Strauss, 2002; Poock et al., 2008; Kahsai et al., 2010; Pfeiffer and Homberg, 2014). It is also in good agreement with experiments showing that current injection into the CX can elicit walking in crickets (Huber, 1960) and cockroaches (Bender et al., 2010). While most CX units show an increase in their firing rate preceding the initiation of locomotion, some show a decrease indicating that the CX is predominantly permissive in walking (Guo and Ritzmann, 2013). In a recently published study, injection of procaine into the CX of *Blaberus discoidalis* was performed, to investigate its role in optomotor behavior (Kathman et al., 2014); the authors found deficits in optomotor behavior after procaine injection but no significant change in general activity (i.e. walking). We can only speculate as to what could account for such a difference. First, it is possible that size matters, as *Blaberus* CX is much larger than *Periplaneta* CX. A more convincing argument would be a simple concentration effect. In the present study, we used a high concentration solution of procaine (50% versus 20% and 10% in Kathman’s study) and a larger volume of diluted procaine (10 nl in the present study versus 2 nl in Kathman’s study). Hence we expect that in the *Blaberus* study, the effect is limited to a

smaller portion of the CX, whereas in the present study the effect results from a more complete inhibition of the entire CX.

While the CX is at least necessary for spontaneous walking, the entire SupOG in general has a suppressive role in the control of walking (Gal and Libersat, 2006). The cockroach’s head ganglia play a major role in initiating and maintaining the walking pattern generated in the thoracic ganglia (Kien and Altman, 1992; Gal and Libersat, 2006). We have shown previously that the SupOG inhibits walking, whereas the SOG exerts the opposite effect (Gal and Libersat, 2006; Gal and Libersat, 2010). This suggests the existence of other regions within the SupOG that exert inhibitory control on walking initiation. As already suggested by studies on *Drosophila* (Martin et al., 1998), we hypothesized that this observed suppressive effect of SupOG removal is a result of the inhibitory influence of the MBs on walking. Our results showed that procaine injections into the MBs have the opposite effect on walking to that of injections into the CX. A procaine injection into the MBs reversibly increases spontaneous walking. Our results are in agreement with other studies that suggest that these two major ‘higher’ brain centers of the SupOG have an antagonistic role in the control of spontaneous walking (Huber, 1960; Strauss and Heisenberg, 1993; Martin et al., 1998; Helfrich-Förster et al., 2002; Svidersky and Plotnikova, 2002).

As it was shown before that the venom decreases neuronal activity similarly to procaine (Gal and Libersat, 2010), we focally injected venom into the CX to mimic the wasp’s venom injection in this discrete region. We show that an accurate venom injection into CX alone is sufficient to decrease spontaneous walking. Thus venom injection by the jewel wasp into SupOG must be fairly accurate in order to have an impact only on the CX, without affecting the MBs, to achieve the desired behavioral manipulation.

Finally, in the present study, we also addressed the role of the venom in each ganglion in the venom-induced hypokinesia. As it is not possible to coerce the wasp to sting in the SOG alone and prevent it from stinging in the SupOG, we used the following approaches. We surgically crushed the CirC of normal cockroaches or of cockroaches that were already stung by a wasp, thereby removing the inhibitory input from the SupOG. Those ‘Crushed CirC’ cockroaches tend to be more active due to the removal of inhibition. When these cockroaches were stung by the wasp, their spontaneous walking significantly decreased, comparably to the effect of the sting in both ganglia of normal cockroaches. Crushing the CirC after the sting did not change the spontaneous walking of the cockroaches as they remained hypokinetic. These experiments show that the venom injection into the SOG is sufficient to decrease spontaneous walking, and that the effect of the venom in the SOG is sufficient to ‘override’ the effect of the ablation of the SupOG. Thus the jewel wasp injects venom to both cerebral ganglia, while venom injection to either one would be sufficient to cause a decrease in spontaneous walking in the cockroach. It is only possible to speculate about the adaptive significance of such a double venom injection, where one alone would be sufficient to induce hypokinesia. It is possible that the venom injection to both ganglia is a kind of ‘insurance policy’, in which the wasp maximizes the odds that the cockroach will turn and remains hypokinetic. Indeed, if the wasp were to fail to sting properly in the SupOG, it would still achieve its goal. Similar ‘insurance’ mechanisms can be found in other venomous organisms, such as the cone snail that produces different types of conotoxins, each of which should, in principle, be sufficient for blocking the neuromuscular junction, ensuring paralysis of the

prey (Olivera and Cruz, 2001). Another, yet not exclusive, alternative is that the injection into the central portion of the SupOG is involved in other aspects of the manipulation. For instance, an important aspect of the manipulation is the modification of the cockroach's metabolism (Haspel et al., 2005). Venom in the SupOG could slow down the metabolism in the stung cockroach by affecting, directly or indirectly, neuroptidergic modulation in the CX (Zornik et al., 1999; Nässel, 2002). Another possibility is that the venom in the SupOG is involved in the stereotypic grooming behavior of stung cockroaches. Indeed, injection of dopamine, which is present in the venom (Weisel-Eichler et al., 1999), of dopamine agonists (Libersat and Gal, 2014), or of venom (data not shown) into the central portion of the SupOG induces intense grooming. One caveat to this possibility is that the benefit of the grooming behavior in this parasitic manipulation remains unclear (Weisel-Eichler et al., 1999).

To summarize, in the present study, we were able to demonstrate the role of different regions in the SupOG in the control of spontaneous walking by using the wasp–cockroach association as a model. We have shown that venom injection into each of the cerebral ganglia is, by itself, sufficient to decrease spontaneous walking, as observed in stung cockroaches. This presumably insures the wasp with an effective and lasting hypokinetic state of its offspring's host. Our work also provides further insights into the interplay between the SupOG and the SOG of insects and their role in the processes of motivation and decision to engage in a behavioral act.

## MATERIALS AND METHODS

### Animals

*Ampulex compressa* (Hymenoptera: Ampulicidae) wasps and *Periplaneta americana* cockroaches were reared in crowded colonies under laboratory conditions of 40–60% humidity, 30°C and a 12 h:12 h light:dark cycle. All animals were supplied with water and food (cat chow for cockroaches and honey for wasps) *ad libitum*.

To obtain stung cockroaches, a single cockroach was introduced to a wasp and the stinging duration was measured to ensure normal stinging behavior (Fouad et al., 1994).

### Venom milking

Wasps were immobilized by chilling on ice for 5 min and were confined in a small, conical, plastic tube open at both ends. A modified syringe plunger was fitted to one end of the tube and was used to provoke the wasp to sting a small piece of Parafilm held in front of the other end. Venom droplets were collected from the distal side of the Parafilm with a nanovolumetric injector (NVI-570A/V, Medical Systems, Greenvale, NY, USA). From each wasp, approximately 10 nl of crude venom was collected to a glass micropipette containing 10 nl saline.

### Pharmacology

Procaine was freshly prepared and dissolved to a concentration of 500 mg ml<sup>-1</sup> in vehicle containing cockroach saline and 0.1% Janus Green dye. Venom was freshly milked and dissolved 1:1 in cockroach saline containing 0.1% Janus Green dye.

### Injections and surgical procedures

#### General

Prior to the procedures, cockroaches were anesthetized with carbon dioxide and immobilized dorsal side up with modeling clay on a wax platform. A staple-shaped pin was softly pressed against the neck to regulate hemolymph flow to the head during the procedure. A U-shaped incision was then performed to open a small flap between the ocelli. Then, we either injected various compounds in specific regions of the SupOG or performed a surgical crush of the CirC. After the procedure, the cuticular incisions were sealed with wax.

### Procedures

#### Micro-injection

A nanovolumetric injector was used to deliver solutions directly into the CX or MB. Using physical landmarks, a glass needle was inserted into the specific location and the solution was injected. For two groups of cockroaches, we used procaine, a reversible voltage-dependent sodium and potassium channel blocker, which eliminates neuronal activity in the injected area as shown by Devaud et al. (Devaud et al., 2007) and by Gal and Libersat (Gal and Libersat, 2010). 'Procaine-CX' cockroaches were injected with 10 nl of procaine into the CX; 'Procaine-MB' cockroaches were injected with 20 nl of procaine (bilateral injection) into the MB; and 'Venom-CX' cockroaches were injected with 20 nl of venom diluted in saline into the CX. Control cockroaches were injected with saline containing 0.1% Janus Green dye (10–20 nl) into the CX or MBs. The effect of procaine injection on neuronal activity is known to be restricted to the injection site (Kathman et al., 2014).

#### Surgical crush of the CirC

Two groups of cockroaches were prepared as follows. For the first group, the CirC were gently crushed at first (carefully, to not cut the connectives and to avoid damage to surrounding tissue) and afterwards the cockroach was introduced to a wasp for a sting ('Crushed CirC-stung'). For the second group, the CirC were crushed after the cockroach was stung by a wasp ('Stung-crushed CirC'). The experimental timeline is explained in 'Behavioral assays' below.

### Behavioral assays

#### Micro-injections

Behavioral assays were performed on freely moving cockroaches in an open-field arena (radius, 30 cm). Spontaneous walking was quantified with a stopwatch in continuous 10 min bins. Baseline walking duration was measured prior to the nano-injections procedure. After the procedure, the cockroach was given 10 min to recover. Then spontaneous walking was again recorded continuously for 1 h.

#### Surgical crush of the CirC

Freely moving 'SupOG-ablated' cockroaches in an open-field arena have a tendency to collide with the arena wall and often get turned on their back. This could affect the spontaneous walking measurements. Since 'Crushed CirC' cockroaches were expected to behave similarly to 'SupOG-ablated' cockroaches, behavioral assays were performed on tethered cockroaches walking on an oiled glass plate. In such a fixed position, cockroaches are able to move their legs in short bouts of stationary walking or running. The walking and escape movements are similar to those of free-ranging animals (Camhi and Levy, 1988). Spontaneous walking was measured in 10 min bins at three time points. For both groups, the cockroaches were first tethered and left to acclimate for 20 min. Afterwards, baseline walking duration was measured. Following the baseline measurement, the protocol differed for both groups: for 'Stung-crushed CirC' cockroaches, the second time point (70 min) was an hour after the cockroach was stung by a wasp. After the second time point, the CirC were crushed (see 'Injections and surgical procedures' above) and the third time point (140 min) was an hour afterwards. For 'Crushed CirC-stung' cockroaches, the second time point (70 min) was an hour after the CirC were crushed. After the second time point, the cockroach was stung by a wasp and the third time point (140 min) was an hour afterwards.

#### Statistical analysis

For the different nano-injection treatments, we used a two-way repeated measures ANOVA, with Treatment as the between-subject factor and Time as the within-subject factor, to compare spontaneous walking between different treatment groups ( $N=22$ , 8, 8 and 8 cockroaches in the control, 'Procaine-CX', 'Procaine-MB' and 'Venom-CX' groups, respectively).

For 'Crushed CirC-stung' and 'Stung-crushed CirC' cockroaches, we used a two-way ANOVA with a Student–Newman–Keuls *post hoc* test to identify differences in time and treatment between the groups ( $N=8$  for each group).

### Post-mortem verification of the injection site

After the behavioral assay, the cockroach head was removed and fixed overnight in formalin. Then the SupOG was removed from the head, embedded in 6% agarose and sliced (60  $\mu$ m) with a vibratome. The location of the Janus Green tracer was used to verify the exact injection site.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

M.K. contributed to the study conception, design, execution, interpretation of the findings and writing. F.L. contributed to the study conception, design, interpretation of the findings and writing.

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