Insect Biochemistry and Molecular Biology 106 (2019) 64–78

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Molecular cross-talk in a unique parasitoid manipulation strategy

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ARTICLE INFO

Keywords:
Parasitoid wasp
Behaviour
Cockroach
Venom
Proteomics
Synapses

ABSTRACT

Envenomation of cockroach cerebral ganglia by the parasitoid Jewel wasp, *Ampulex compressa*, induces specific, long-lasting behavioural changes. We hypothesized that this prolonged action results from venom-induced changes in brain neurochemistry. Here, we address this issue by first identifying molecular targets of the venom, i.e., proteins to which venom components bind and interact with to mediate altered behaviour. Our results show that venom components bind to synaptic proteins and likely interfere with both pre- and postsynaptic processes. Since behavioural changes induced by the sting are long-lasting and reversible, we hypothesized further that long-term effects of the venom must be mediated by up or down regulation of cerebral ganglia proteins. We therefore characterize changes in cerebral ganglia protein abundance of stung cockroaches at different time points after the sting by quantitative mass spectrometry. Our findings indicate that numerous proteins are differentially expressed in cerebral ganglia of stung cockroaches, many of which are involved in signal transduction, such as the Rho GTPase pathway, which is implicated in synaptic plasticity. Altogether, our data suggest that the Jewel wasp commandeers cockroach behaviour through molecular cross-talk between venom components and molecular targets in the cockroach central nervous system, leading to broad-based alteration of synaptic efficacy and behavioural changes that promote successful development of wasp progeny.

1. Introduction

The parasitoid Jewel wasp (*Ampulex compressa*) hunts and stings cockroaches (*Periplaneta americana*) to use them as a live fresh food supply for its offspring. The wasp directs its first sting into the cockroach thorax, inducing a transient paralysis of the front legs (2–3 min). This allows the wasp to execute a second accurate sting into the head ganglia, causing instead of paralysis, specific long-term changes in behaviour (Fouad et al., 1994). The stung cockroach grooms continuously for ~30 min, then descends into a long-term hypokinetic state characterized by failure to initiate spontaneous locomotion or to respond to wind or tactile stimuli. Such behavioural manipulation allows the wasp to lead the cockroach into its burrow, as one would pull a domestic animal with a leash. Inside the burrow, the wasp deposits a single egg onto the cockroach leg before sealing the entrance. Upon hatching, the wasp larva feeds on cockroach hemolymph and then enters the cockroach abdomen to devour its internal organs (Haspel et al., 2003, 2005). Eventually, a fully-grown wasp emerges from the remaining shell of the consumed cockroach.

The venom seems to modulate descending signals from cerebral ganglia, resulting in suppression of host escape behaviour and decreased spontaneous walking, without affecting other behaviours. More specifically, the wasp sting suppresses spontaneous initiation and the maintenance of walking (Gal and Libersat, 2008, 2010; Libersat, 2003).

The wasp injects its venom directly into the cockroach cerebral ganglia, namely, the subeosophageal ganglion (SEG) and the supraeosophageal ganglion (brain) (Haspel et al., 2003). The SEG and brain are considered to be 'higher-order' neuronal centers, which modulate different aspects of locomotion (Kien and Altman, 1992; Schaefer and Ritzmann, 2001; Strausfeld, 1999; Strauss and Heisenberg, 1993). Since

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Abbreviations: SEG, Sub-esophageal ganglion; CX, Central complex; CNS, Central nervous system; MS, Mass spectrometry; OBCAM, Opioid-binding protein/cell adhesion molecule; Ig, Immunoglobulin; CAM, Cell adhesion molecules; NHS, N-hydroxysuccinimide; BLAST, Basic Local Alignment Search Tool; GO, Gene Ontology; DAVID, The Database for Annotation Visualization and Integrated Discovery; GEF, Guanine nucleotide exchange factor; GAP, GTPase-activating protein; PICK1, Protein interacting with C kinase 1

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https://doi.org/10.1016/j.ibmb.2018.11.009
Received 12 September 2018; Received in revised form 18 November 2018; Accepted 29 November 2018
Available online 30 November 2018

the venom is injected directly into the cerebral ganglia, the behavioural change observed in stung cockroaches must result from modification of neuronal circuits in these ganglia. The venom molecular components involved in the short-term effects of the venom (transient paralysis of the front legs and the grooming phase) have been identified (Moore et al., 2006; Weisel-Eichler et al., 1999).

Neuroparasitology is an emerging branch of science dealing with parasites that induce changes in host behaviour to benefit their progeny. Studies of behavioural manipulations are shedding light on various strategies, which have evolved in parasites, to achieve a desired altered behaviour (Hughes and Libersat, 2018; van Houte et al., 2013). Some studies have focused on changes in biogenic amines in the central nervous system (CNS) of the host (Adamo et al., 2017; Hughes, 2015; Hughes and Libersat, 2018; Miles and Booker, 2000). For example, octopamine concentration increases in cerebral ganglia of *Manduca sexta* parasitized by the wasp *Cotesia congregate* (Adamo et al., 1997). However, no changes in biogenic amines were observed in the cerebral ganglia of stung cockroaches (Banks and Adams, 2012). In parasitized *M. sexta*, distinct changes in biogenic amines were found to be accompanied by an accumulation of neuropeptides in the host cerebral neurosecretory system (Zitnan et al., 1995). Proteomics approaches have been applied to studying parasite-host interactions more recently (Barrett et al., 2000; Biron et al., 2005b; Lefevre et al., 2007). For example, methods such as two-dimensional gel electrophoresis and mass spectrometry (MS) were used to identify proteins from the grasshopper that are associated with the behavioural manipulation by the nematomorph hairworm *Spinochordodes tellinii* (Biron et al., 2005a). Similar studies found differentially expressed proteins in two gammarid species infected with two behaviour-altering parasites: the trematode, *Microphallus papillorobustus* and the acanthocephalan, *Polymorphus minutes* (Ponton et al., 2006). Those studies used 2D gels for comparing proteomes between infected and not-infected host. However, advances in quantitative MS methods now allow direct comparison of protein levels between groups. Analysis of protein abundance provides better insight into differential expression compared to the more frequently used RNA expression analyses, since RNA abundance does not necessarily predict protein levels (Greenbaum et al., 2003; Maier et al., 2009). Although most quantitative MS methods make use of labelled proteins, label-free methods are less time and resource consuming and still provide relatively reliable and accurate data with high coverage and dynamic range (Bantscheff et al., 2007; Cox et al., 2014; Megger et al., 2013; Patel et al., 2009; Zhu et al., 2010).

In order to understand molecular mechanisms underlying venom-induced behavioural manipulation, an essential step is to identify which cerebral ganglion proteins mediate the effect of the venom, either by direct interaction with venom components or as part of dysregulated signal transduction pathways contributing to hypokinesia. The wasp venom is a rich mixture of peptides and proteins, many of them only recently characterized (Arvidson et al., 2018). Envenomation by the jewel wasp is unusual, since it is injected directly into the CNS and contains many proteins predicted to act inside the cell. Although most venom components target membrane proteins such as receptors, ions channels and pumps, some have intracellular targets such as proteins kinases/phosphatases, small GTP binding proteins, nucleic acids, ribosomal proteins and others (Lahiani et al., 2017). Since the venom contains at least 264 proteins and peptides, identifying each molecular target in the cerebral ganglia is a staggering task (Arvidson et al., 2018).

Therefore, in order to identify at least some of the venom targets, we constructed a venom affinity column using a N-hydroxysuccinimide activated Sepharose (NHS), which has been used previously to purify antibodies directed against crude snake venom (Jorge et al., 2015; Liu et al., 2017). Our results show that venom components bind to synaptic proteins and therefore likely interfere with synaptic processes. Furthermore, we show that numerous proteins are differentially expressed in cerebral ganglia of stung cockroaches, many of which are involved in signal transduction pathways such as the Rho GTPase pathway implicated in synaptic plasticity. Altogether, our findings suggest that wasp venom components engage in molecular cross-talk with CNS proteins involved in many endogenous signalling pathways, causing changes in synaptic efficacy that induce behavioural changes in the host that benefit developing wasp progeny.

2. Methods

2.1. Animals

Ampulex compressa Fabricius (*Hymenoptera: Ampulicidae*) wasps and *Periplaneta americana* cockroaches were reared in crowded colonies under laboratory conditions of 40–60% humidity, 30 °C and a 12 L:12D 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 behaviour *(*Fouad et al., 1994*)*.

2.2. Venom collection

Milking procedure: Wasps were immobilized by chilling on ice for 5 min and subsequently confined in a small, conical, plastic tube open at both ends. A modified syringe plunger, fitted to one end of the tube, was used to provoke the wasp to sting through a small piece of Parafilm held close to the stinger. Venom droplets were collected from the distal side of the Parafilm with a capillary and kept directly in coupling solution (see below) on ice and then stored at −20 °C (Haspel and Libersat, 2003).

Extraction of venom from venom sac: Venom sacs were dissected from wasps and immersed in high potassium saline (Arvidson et al., 2018). After venom sac contraction, saline containing expelled venom was collected and kept in −20 °C. Before use, the saline was exchanged with coupling buffer (see below), using Amicon Ultra-4 Centrifugal Filter Units (Millipore Sigma, Darmstadt, Germany).

2.3. Venom affinity column preparation

In order to identify venom targets in cockroach cerebral ganglia, we used HiTrap NHS-activated HP (GE Healthcare, Product No. 17-0716-01) to create a venom affinity column. Venom samples collected from milking and venom sac were pooled in 1 ml of standard coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) using a combination of milking and extraction from the venom sac (total of approximately 100 milking procedures and 20 venom sac extracts). The venom was then covalently bound to Sepharose beads to construct a HiTrap NHS-activated affinity column by activation with ice cold 1 mM HCl using a syringe as recommended by the product manual. The column was then washed and blocked by alternating washes, each of 6 ml, of pH 4 (sodium acetate) and pH 8.3 (ethanolamine) and stored at 4 °C as recommended by the product instructions. The binding efficiency was calculated by the measuring absorbance at 280 nm before and after coupling to the NHS column. Two venom columns were prepared and the average binding efficacy was 90%. For control, a column was prepared with the same procedure as the venom column, only without any venom. No peaks were observed at the elution (for at least 10 ml of elution with high salt).

2.4. Purification of venom molecular targets

For purification of venom targets, we conducted two experiments, each utilizing 12 cockroach cerebral ganglia (SEG or brain) collected in binding buffer (10 mM HEPES, pH 7.3–7.4, containing 0.25% CHAPS). Ganglia were homogenized and centrifuged (60 min, 12000 G, 4 °C), after which supernatants were loaded onto the column using an AKTA chromatography system. Proteins were eluted with high salt solution (0.5 M NaCl, 10 mM HEPES, pH 7.3–7.4, 0.25% CHAPS). Fractions of 1 ml were collected and separated on a sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) for protein detection. This procedure was repeated 4 times for brain samples and 3 times for SEG samples. Fractions with visible proteins in the SDS-PAGE were sent for identification by MS at the Technion, Technion City, Haifa, Israel. The mass spectrometry data was analysed using the MaxQuant software 1.5.2.8 (Mathias Mann's group) vs. the a custom cerebral ganglia transcriptome database (Bio-project number PRJNA356979, www.ncbi.nlm.nih.gov/nuccore/1172798147). For the elaborated mass spectrometry protocol please see Supplementary: Elaborated mass spectrometry methodology.

2.5. Label Free Quantitative mass spectrometry analysis

Differentially expressed proteins were identified at two different time points, corresponding to different phases of behavioural change induced by the sting: 1) 10 min after the sting, prior to onset of hypokinesia, and 2) 24 h after the sting, when hypokinesia is fully expressed. For each time point, brain and SEG were surgically removed (after behavioural assay as described below), analysed separately and compared to age-matched untreated cockroaches. In addition, since most of the venom was found in the region of the central complex (CX), we surgically removed the brain from 3 control and 3 stung cockroaches (24 h after the sting) and isolated the central region of the brain to enrich the contribution of the CX. All samples were analysed at the Smoler Proteomic Center. In order to identify changes in protein expression in cockroach cerebral ganglia after the sting, we used quantitative mass spectrometry (Label Free Quantitative MS). Samples were analysed by LC-MS/MS on a Q Exactive plus mass spectrometer (Thermo). Mass spectrometry data from all biological repeats were analysed using MaxQuant software 1.5.2.8 (Laboratory of Mathias Mann, Max-Planck Institute (Cox and Mann, 2008) vs. a custom cerebral ganglia transcriptome database with 1% FDR. Data were quantified by label free analysis using the same software, based on extracted ion currents (XICs) of peptides enabling quantitation from each LC/MS run for each peptide identified in any of the experiments. Statistical analysis of the identification and quantization results was performed using Perseus 1.5.2.4 software (Mathias Mann's group) (Cox and Mann, 2008). To identify differentially expressed proteins, Student's t-test was used for comparing each protein in a group to its age-matched untreated control. A protein group is defined when a set of identified peptides is found to be equal to or completely contained in multiple proteins. Comparison was made between proteins that were identified with at least 3 Razor + unique peptides. Razor peptides are shared among multiple proteins, while being most parsimoniously associated with the group with the highest number of identified peptides, but stay in all groups where they occur. The use of unique and razor peptides is a compromise between unequivocal peptide assignment and most accurate quantification (Cox and Mann, 2008; Tyanova et al., 2016). The relative protein quantification across all samples is represented by the LFQ intensities, normalized intensity profile (Cox et al., 2014). Differential expression was defined if the difference between the averaged LFQ of the two groups was of at least 2-fold and if the Student's t-test P-value was less than 0.05. For the elaborated mass spectrometry protocol please see Supplementary Information: Elaborated mass spectrometry methodology.

Heat maps for the differentially expressed protein were created using Morpheus, https://software.broadinstitute.org/morpheus.

2.6. Protein annotation

Proteins were annotated using BLAST (Basic Local Alignment Search Tool) with the Blast2GO software (Gotz et al., 2008) (Blastp, E-value threshold: 10^{-3}) against the SWISS-PROT database (Manually reviewed, high quality protein sequences and functional annotations produced by UniProt), the Non-redundant (nr) database (All non-redundant GenBank CDS translations, PDB, SWISS-PROT, PIR, PRF excluding environmental samples from WGS projects) and Refseq (NCBI Protein Reference Sequences). In addition, proteins were annotated using the HMMER web server (phmmer) against the SWISS-PROT database with restriction to *Drosophila melanogaster* proteins only.

2.7. Functional enrichment analysis

The annotated list of the *D. melanogaster* homolog proteins was analysed by DAVID (The Database for Annotation, Visualization and Integrated Discovery v6.8), against the *D. melanogaster,* the closest well-annotated genome. This on-line service (https://david.ncifcrf.gov/home.jsp) is used for ranking functional categories (gene ontology terms) based on co-occurrence with sets of genes in a gene list (Ashburner et al., 2000; Huang da et al., 2009; Huang et al., 2009). This can aid in unravelling new biological processes associated with cellular functions and pathways. The threshold of the EASE Score, a modified Fisher Exact P-Value, for gene-enrichment analysis, was set to include only results with values smaller than 0.05 and the threshold for minimum gene counts belonging to an annotation term was set to at least 2.

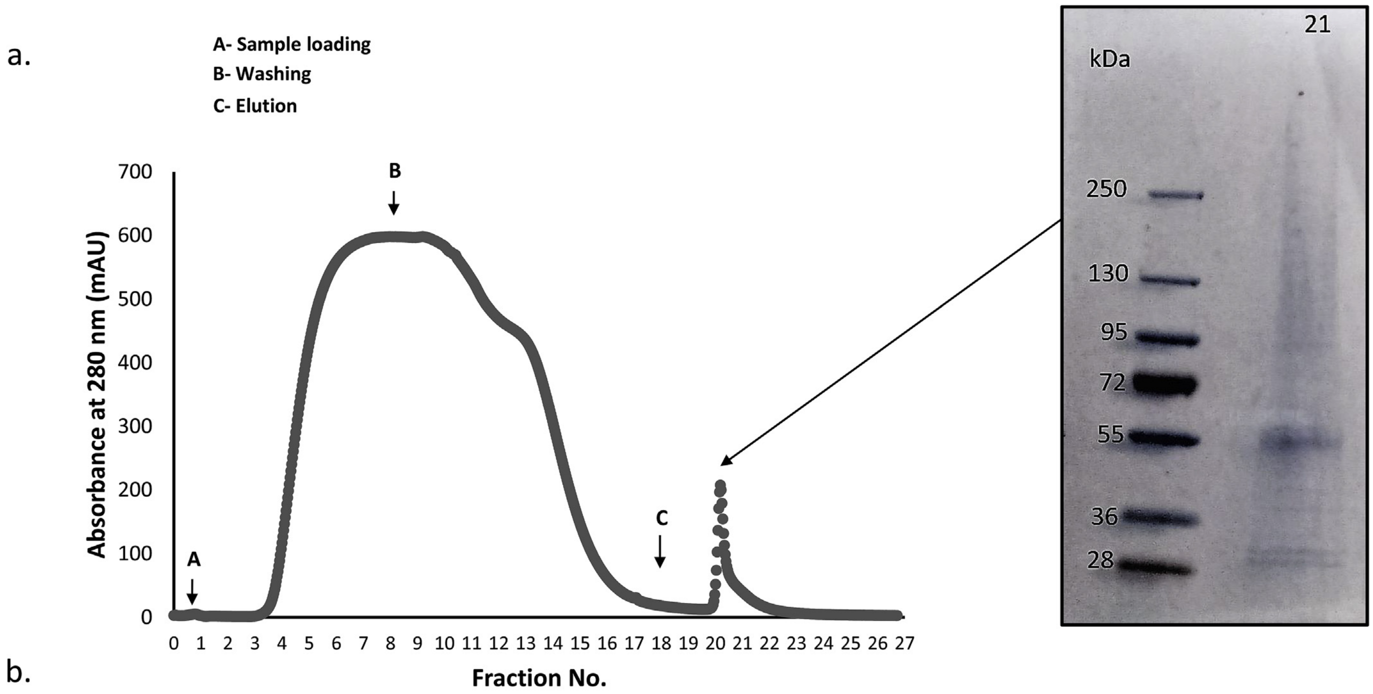
2.8. Behavioural assays

Behavioural assays for quantitative MS analysis were performed as described in detail in (Gal and Libersat, 2006; Kaiser and Libersat, 2015). Briefly, behavioural assays were performed on freely-moving cockroaches in an open-field arena (radius = 30 cm). Spontaneous walking duration was quantified with a stopwatch for 10 min, while escape response distance was measured in response to a tactile stimulus after the spontaneous walking observations. Cockroaches were placed at the center of the arena and were given a tactile stimulus by gentle touch with a paint brush. Prior to the wasp sting, behavioural assays were performed on untreated cockroaches (age matched, after the last moult). Next, they were introduced to the wasp for the sting. Wasp sting duration was quantified as a measure of a normal stinging behaviour (Gal et al., 2014). For testing short-term effects of the venom, cockroaches were taken 10 min after the sting for dissection, and for the long-term effect of the venom, cockroaches were taken 24 h after the sting for behavioural assays. For this time point, only cockroaches that showed classic symptoms of hypokinesia (lack of escape response and decreased spontaneous walking) were taken for brain, SEG, or CX dissections and proteomic analysis.

3. Results

3.1. Mass spectrometry identification of venom targets isolated by venom affinity column

For both SEG and brain samples, peaks detected at 280 nm were observed after approximately 3 ml of elution buffer. Separation of these fractions by gel electrophoresis revealed multiple bands ranging from 28 to 250 kDa (Fig. 1a). Because of this broad range of protein sizes, all lanes showing visible proteins were sent for identification by mass spectrometry. MS data were screened to limit identification of proteins to those that were identified by at least 3 razor and unique peptides (Tyanova et al., 2016). This resulted in 967 proteins that were identified in at least one of the brain samples and 438 proteins that were identified in at least one of the SEG samples; 236 proteins were identified in all brain samples and 207 proteins were identified in all SEG samples. In total, 165 proteins were found in both brain and SEG. 71 proteins were unique to brain samples and 42 proteins were unique to SEG samples (a total of 278 proteins, Fig. 1b). 273 venom target proteins were annotated using BLAST against the SWISS-PROT database.



b.

	Brain	SEG
Number of repeats (each with 12 ganglia)	4	3
Proteins identified in at least one sample	967	438
Proteins identified in all samples	236	207
Unique to ganglion	71	42

Fig. 1. Isolation of CNS targets of wasp venom by venom affinity column and identification by mass spectrometry. a. One chromatogram of one cycle of affinity purification exhibits an absorption peak (fraction 21) following elution with high salt concentration. **b.** Summary of the mass spectrometry identification of venom targets.

Searches against the *D. melanogaster* SWISS-PROT database identified homolog proteins to 224 of the protein venom targets. Especially solid identification was found for several proteins in brain and SEG (proteins identified with the largest number of razor and unique peptides), including: Spectrin, Apolilipophorins and Microtubule associated Futsch. Proteins with highest intensity (largest signal intensity for identified peptides) in both SEG and brain included Tubulin, Spectrin and Sodium/potassium ATPase.

3.2. *The venom targets database is enriched in proteins associated with synaptic processes*

In order to characterize venom protein targets in brain and SEG, we performed functional annotation and gene enrichment analysis using DAVID for each ganglion separately (Huang da et al., 2009; Huang et al., 2009). This analysis revealed that many Gene Ontology (GO) terms are enriched in the database (ease score < 0.05). When including only biological processes GO terms (level 5, for high specificity), 240 terms were found enriched in brain and 209 in SEG. A total of 186 enriched terms are common between brain and SEG. Using the annotation clustering report on DAVID with manual corrections and adjustments, we constructed clusters, which display similar annotations together, and therefore, genes with similar function or involvement in similar processes and pathways. Two of the most enriched clusters are shown in Figs. 2 and 3. One of those clusters (Fig. 2) contains proteins that are involved in synaptic processes, such as neurotransmitter release and synaptic vesicle endocytosis and exocytosis. This group includes, for example, in both brain and SEG, the clathrin heavy chain, dynamin, synapsin, and synaptotagmin 1. While endophilin-A was found only in the brain, the AP-2 complex alpha was found only in SEG (Fig. 2).

3.3. *The venom targets database is enriched in proteins associated with cytoskeleton organization and synapse assembly*

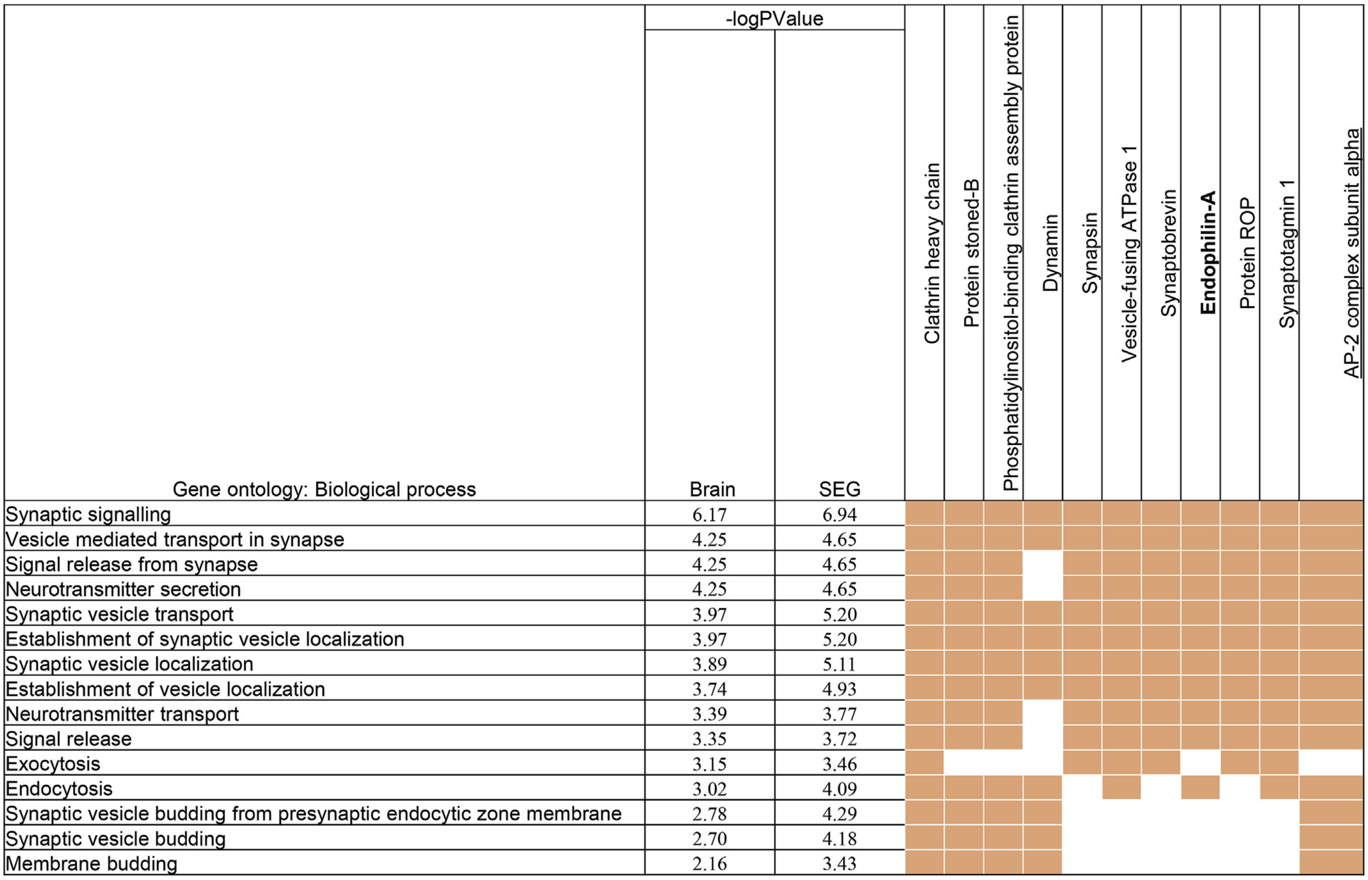
Another cluster of venom targets includes proteins related to cell or neuron development, cell morphogenesis, cytoskeleton organization, synapse assembly and regulation of those processes (Fig. 3). In both brain and SEG, this group includes spectrins and microtubule-associated protein futsch.

3.4. *Quantitative mass spectrometry identifies changes in protein expression in both time points*

In order to find differentially expressed proteins in the cockroach cerebral ganglia, we used label-free quantitative MS. We found that 10 min and 24 h after the sting, approximately 2% of the total proteins are differentially expressed in stung compared to control cockroaches (Fig. S1). These results suggest that, although stung cockroaches 10 min after the sting do not display hypokinesia, their proteome is different from that of control, unstung cockroaches.

3.5. *Changes in protein expression in the CX associated with hypokinesia*

In total, 39 proteins were differently expressed in the CX of stung cockroaches 24 h after the sting (P value < 0.05; difference of at least two-fold change). Changes observed were consistent between individual samples (Fig. 4) and the difference in expression between stung and control for those proteins was remarkable (average fold change of ~1370). Only the proteins Glutaredoxin-3, Cytochrome P450 9e2-like, Phenoloxidase 2 and U1 small nuclear ribonucleoprotein 70 kDa, had differential expression of around 2-fold change, while for



Gene ontology: Biological process	-logPValue		Clathrin heavy chain	Protein stoned-B	Phosphatidylinositol-binding clathrin assembly protein	Dynamin	Synapsin	Vesicle-fusing ATPase 1	Synaptobrevin	**Endophilin-A**	Protein ROP	Synaptotagmin 1	AP-2 complex subunit alpha
	Brain	SEG											
Synaptic signalling	6.17	6.94	■	■	■	■	■	■	■	■	■	■	■
Vesicle mediated transport in synapse	4.25	4.65	■	■	■	■	■	■	■	■	■	■	■
Signal release from synapse	4.25	4.65	■	■	■		■	■	■	■	■	■	■
Neurotransmitter secretion	4.25	4.65	■	■	■		■	■	■	■	■	■	■
Synaptic vesicle transport	3.97	5.20	■	■	■	■	■	■	■	■	■	■	■
Establishment of synaptic vesicle localization	3.97	5.20	■	■	■	■	■	■	■	■	■	■	■
Synaptic vesicle localization	3.89	5.11	■	■	■	■	■	■	■	■	■	■	■
Establishment of vesicle localization	3.74	4.93	■	■	■	■	■	■	■	■	■	■	■
Neurotransmitter transport	3.39	3.77	■	■	■		■	■	■	■	■	■	■
Signal release	3.35	3.72	■	■	■		■	■	■	■	■	■	■
Exocytosis	3.15	3.46	■				■	■	■		■	■	
Endocytosis	3.02	4.09	■	■	■	■		■		■		■	■
Synaptic vesicle budding from presynaptic endocytic zone membrane	2.78	4.29	■	■	■	■							■
Synaptic vesicle budding	2.70	4.18	■	■	■	■							■
Membrane budding	2.16	3.43	■	■	■	■							■

Fig. 2. The venom targets database is enriched with proteins that are associated with synaptic processes. The GO terms for brain and SEG identified venom targets with their corresponding EASE score (presented as a normalized –log10Pvalue) and their associated proteins (proteins are annotated according to their *D. melanogaster* homologs description). Protein names marked by **bold font** were found only in brain and underlined were found only in SEG.

all the other identified, differentially expressed proteins, the difference was at least one order of magnitude. Annotation of those proteins indicates that some are involved in the Rho GTPase pathway (Fig. 5a). These proteins include the guanine nucleotide exchange factor (GEF) (Yang and Bashaw, 2006), SLIT-ROBO Rho GTPase-activating protein (GAP) (Lucas and Hardin, 2017), Cappuccino/Formin (Vizcarra et al., 2011) and tyrosine kinase Src64B (Billuart et al., 2001). Additional differentially expressed proteins are the PRKCA interacting protein (PICK1), which is implicated in synaptic plasticity and tyrosine phosphatase MEG2 which is implicated in regulation of synaptic vesicles (Fig. 5a) (Hanley, 2008; Saito et al., 2007; Zhang et al., 2016). Other proteins that are differentially expressed in the CX are those involved in gene expression regulation such as translation initiation factor eIF-2B (Williams et al., 2001).

3.6. Changes in protein expression in the entire brain associated with hypokinesia

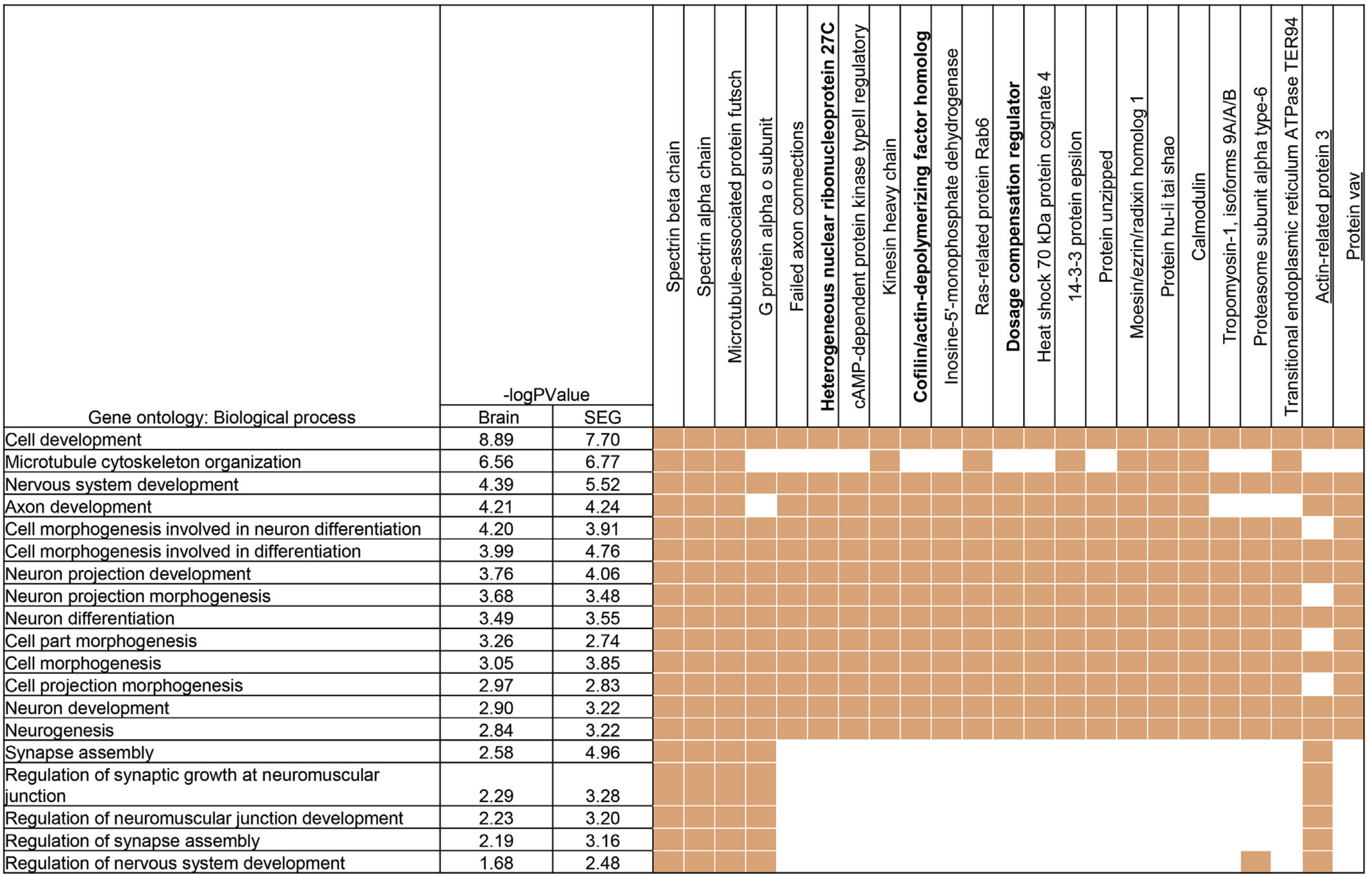
In the whole brain sample, a total of 23 proteins were differentially expressed in hypokinetic cockroaches (24 h after the sting; P value $<$ 0.05, at least two-fold change). Similar to the CX, changes observed were consistent between individual samples (Fig. S2). Expression differences between stung and control for these proteins were lower compared to those found in the CX (an average fold change of ~800), but still constituted a substantial difference. The greatest difference was found for UDP-glucuronosyltransferase, Heterochromatin protein 1 (Or Chromodomain Y 2), Troponin C, Ankyrin repeat KH domain-containing protein mask, and Fibroblast growth factor receptor. Among the differentially expressed proteins are the ryanodine receptor and the sodium and chloride dependent GABA transporter (GAT) (Fig. 5a). Fibroblast growth factor receptor, Ankyrin repeat KH domain-containing protein mask, and SHC adaptor protein, which are differentially expressed in the brain after sting, are involved in receptor tyrosine kinase signalling (Luschnig et al., 2000; Muha and Muller, 2013; Smith et al., 2002). Similar to the proteins found in the CX, some identified proteins are involved in regulation of gene expression, such as Heterochromatin protein 1. Notably among differentially expressed proteins are those related to the cell cytoskeleton, such as Troponin C, Myosin regulatory light chain 2, Myosin heavy chain and Tropomyosin-2 (Fig. 5a).

3.7. Changes in protein expression in the SEG associated with hypokinesia

Nine proteins were differentially expressed in the SEG of hypokinetic cockroaches (P value $<$ 0.05, difference of at least two-fold change). Changes observed were consistent between individual samples (Fig. S3) and expression differences between stung and control for these proteins were lower than those observed in the CX and brain (an average fold change of ~360). The protein identified as heterochromatin protein 1 found to be upregulated in brain is also upregulated in the SEG (Fig. 5a).

3.8. Changes in protein expression in brain prior to hypokinesia

Cockroaches do not exhibit hypokinesia 10 min after the sting, but some behavioural changes are observed, such as grooming (Weisel-Eichler et al., 1999). A similar number of proteins are differentially expressed 10 min after the sting compared with 24 h after the sting. To test whether changes observed 24 h after the sting are similar to those observed shortly after the sting, we searched for differentially expressed



	-logPValue		Spectrin beta chain	Spectrin alpha chain	Microtubule-associated protein futsch	G protein alpha o subunit	Failed axon connections	**Heterogeneous nuclear ribonucleoprotein 27C**	cAMP-dependent protein kinase typeII regulatory	Kinesin heavy chain	**Cofilin/actin-depolymerizing factor homolog**	Inosine-5'-monophosphate dehydrogenase	Ras-related protein Rab6	**Dosage compensation regulator**	Heat shock 70 kDa protein cognate 4	14-3-3 protein epsilon	Protein unzipped	Moesin/ezrin/radixin homolog 1	Protein hu-li tai shao	Calmodulin	Tropomyosin-1, isoforms 9A/A/B	Proteasome subunit alpha type-6	Transitional endoplasmic reticulum ATPase TER94	Actin-related protein 3	Protein vav
Gene ontology: Biological process	Brain	SEG																							
Cell development	8.89	7.70	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Microtubule cytoskeleton organization	6.56	6.77	■	■	■					■			■			■		■	■	■			■		
Nervous system development	4.39	5.52	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Axon development	4.21	4.24	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■				■	■
Cell morphogenesis involved in neuron differentiation	4.20	3.91	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
Cell morphogenesis involved in differentiation	3.99	4.76	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Neuron projection development	3.76	4.06	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Neuron projection morphogenesis	3.68	3.48	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
Neuron differentiation	3.49	3.55	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Cell part morphogenesis	3.26	2.74	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
Cell morphogenesis	3.05	3.85	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Cell projection morphogenesis	2.97	2.83	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
Neuron development	2.90	3.22	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Neurogenesis	2.84	3.22	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Synapse assembly	2.58	4.96	■	■	■	■																		■	
Regulation of synaptic growth at neuromuscular junction	2.29	3.28	■	■	■	■																		■	
Regulation of neuromuscular junction development	2.23	3.20	■	■	■	■																		■	
Regulation of synapse assembly	2.19	3.16	■	■	■	■																		■	
Regulation of nervous system development	1.68	2.48	■	■	■	■																■		■	

Fig. 3. The venom targets database is enriched with proteins that are associated with cytoskeleton organization and synapse assembly. The GO terms for brain and SEG identified venom targets with their corresponding EASE score (presented as the normalized $-\log_{10}$Pvalue) and their associated proteins (proteins are annotated according to their *D. melanogaster* homologs description). Protein names marked by **bold font** were found only in brain and underlined were found only in SEG.

proteins 10 min after the sting. In total, 27 proteins were found to be differentially expressed 10 min after sting in the brain (P value $<$ 0.05, difference of at least two-fold change). The difference in expression between stung and control for these proteins was ~1000 fold. Two proteins were differentially expressed in the brain at both 10 min and 24 h after the sting. One of these is Myosin heavy chain, which was found to be up-regulated at both time points. However, it is possible that they are different isoforms despite a similar annotation, as they were identified by different sequences in the transcriptome. The second protein in common is Apolipo D, which is down-regulated in the brain 10 min after the sting and upregulated in CX 24 h after the sting. Notable among differentially expressed proteins in the SEG 10 min after the sting are proteins involved in regulation of gene expression, such as 40S ribosomal protein S17, RNA-binding protein squid, probable 39S ribosomal mitochondrial and chromodomain-helicase-DNA-binding. The Takeout protein, whose expression is circadian clock regulated, is down-regulated in brain (Fig. 5b) (So et al., 2000).

3.9. Changes in protein expression in SEG prior to hypokinesia

A larger number of proteins (68) was differentially expressed in the SEG 10 min after the sting: (P value $<$ 0.05, difference of at least two-fold). The difference in expression between stung and control for those proteins was ~500 fold. The protein Esterase-6 was down-regulated in the SEG 10 min after the sting and upregulated in the brain 24 h after the sting; however, those proteins seem to be different isoforms. Among differentially expressed proteins are those related to cation channels and their regulation: voltage-gated potassium channel Shaw, sodium channel, clathrin linker 1, and Neurogenic protein big brain. In addition, acetylcholinesterase, which catalyses breakdown of acetylcholine, is upregulated in the SEG. As observed in the brain, the Takeout protein is down-regulated in the SEG 10 min after the sting (Fig. 5b).

3.10. The highest count of enriched GO terms is found in hypokinetic cockroaches

In order to better characterize differentially expressed protein groups that are associated with each time point, we used functional gene enrichment analysis. We found that a higher number of enriched GO terms was found in hypokinetic animals (ease score $<$ 0.05, level 5 biological processes for high specificity) (Fig. 6).

GO terms that were found in hypokinetic animals include actin cytoskeleton organization, neurogenesis, synapse assembly, neuron differentiation and development, and neuron projection morphogenesis (Fig. 6). Among the proteins involved in those processes are the Rho GTPase-activating protein and tyrosine-protein kinase Src64B, which are down regulated in the CX 24 h after the sting, and are involved in the Rho GTPase pathway. In addition, among those proteins are PICK1 and tyrosine-protein phosphatase MEG2. More proteins that are involved in those processes are Ankyrin repeat KH domain-containing protein mask and SHC adaptor protein, which are involved in receptor tyrosine kinase signalling and are differentially expressed in the entire brain 24 h after the sting. Proteins involved in gene regulation and differentially expressed in the whole brain and SEG 24 h after the sting, such as the Heterochromatin protein 1, were also among the proteins associated with those terms.

Most of the GO terms do not overleap between both groups. Among

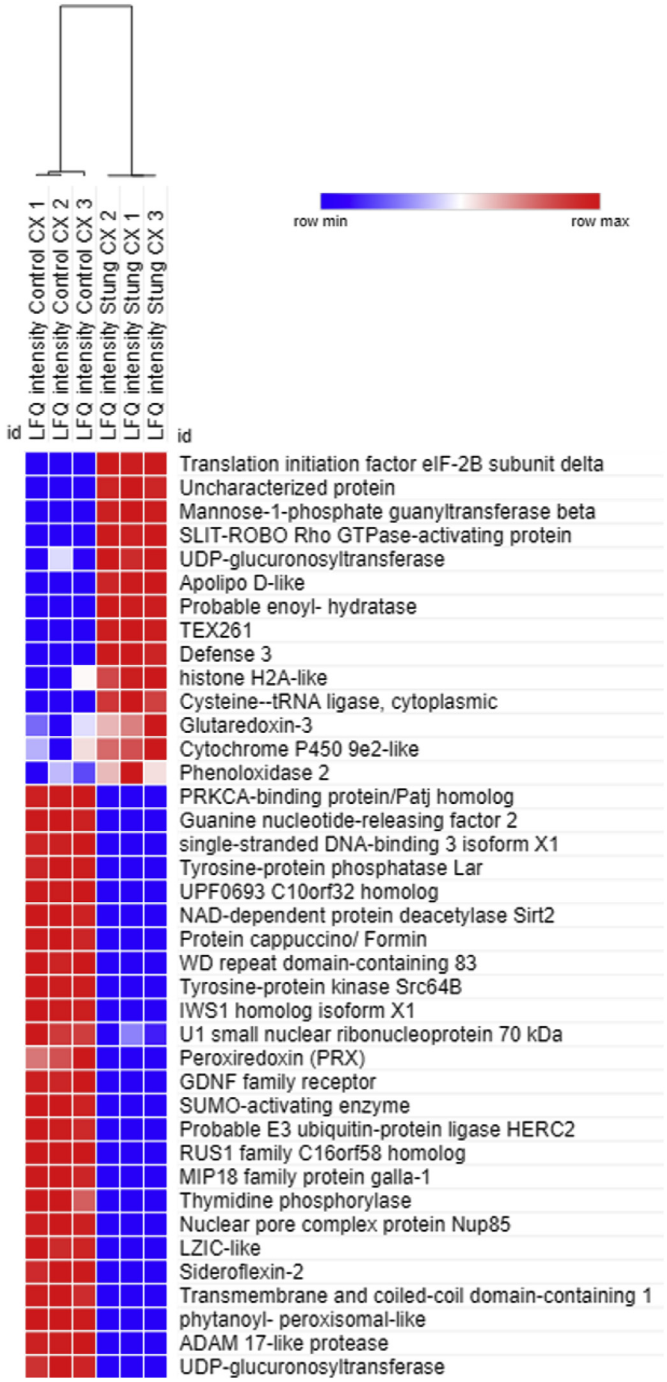


Fig. 4. Heat map of differentially expressed proteins in CX, 24 h after the sting. Protein descriptions are shown next to their normalized LFQ intensities.

the GO terms that are associated with the short-term effect of the venom are histone methylation, protein complex assembly, protein methylation and synaptic target inhibition (Fig. 6).

4. Discussion

4.1. Protein targets of wasp venom

Through use of affinity chromatography, we succeeded in identifying molecular targets of venom components in the CNS of cockroaches. Although we have not ascertained which venom proteins were bound to the column, the calculated binding efficiency was high and no proteins were bound to the control column. Therefore, it is reasonable to consider that proteins isolated from the cerebral ganglia using this column are candidate venom targets.

As expected from the large number and diversity of venom proteins (Arvidson et al., 2018), we find a similarly large number of molecular targets. Considering that there are at least 264 proteins in the venom, among them, proteases or proteins that are subject to post-translational modifications that could bind non-specifically to different protein targets, it is not surprising to find so many protein targets in cockroach cerebral ganglia. Due to the large number of identified target proteins, we focused on those that were consistently found in all brain or SEG samples, which are more likely to be venom targets.

Most proteins that were found in all brain samples were found also in all SEG samples, while a small set of proteins were found only in the brain or only in SEG. Although the number of identified proteins is quite large, many are isoforms or subunits of the same protein. Proteins with the strongest intensity and strongest identification by MS include spectrins and microtubule associated Futsch (homolog to the mammalian family of microtubule-associated proteins (MAPs) (Hummel et al., 2000; Lepicard et al., 2014)), cytoskeletal elements crucial for normal synaptic function (Goellner and Aberle, 2012). Spectrins (alpha and beta) are membrane-associated proteins implicated in several aspects of synaptic function, including neurotransmitter release from presynaptic terminals. Absence of spectrins disrupts subcellular localization of numerous synaptic proteins, suggesting that defects in presynaptic neurotransmitter release result from inappropriate assembly, transport, or localization of proteins required for synaptic function (Featherstone et al., 2001). Studies in *D. melanogaster* show that the microtubule associated Futsch has a role in microtubule stability, regulation of active zone and neurotransmitter release (Bettencourt da Cruz et al., 2005; Lepicard et al., 2014; Zhang et al., 2001).

Because of the large number of affinity-isolated proteins, we chose to characterize candidate venom targets as a group using gene ontology (GO) enrichment analysis against the well-annotated *D. melanogaster* genome. If the isolated proteins were simply randomly bound to the column by non-specific binding, no specific GO term would be enriched in the database. However, our analysis revealed that many GO terms are enriched in the venom targets database, indicating that a specific set of cerebral ganglia proteins were retained by the venom column. These proteins have related associated GO terms that include synaptic signalling, vesicle mediated transport at the synapse (or synaptic vesicle transport), establishment of synaptic vesicle localization (or synaptic vesicle localization), neurotransmitter secretion and transport, signal release, exocytosis, endocytosis, and synaptic vesicle and membrane budding, consistent with the venom targeting synaptic processes specifically. Proteins involved in those terms are mainly presynaptic proteins such as Clathrin, Stoned proteins, Dynamin, Synapsin, Vesicle-fusing ATPase 1, Synaptobrevin and Synaptotagmin 1, all of which participate in endocytosis and/or exocytosis of synaptic vesicles (Fig. 7) (De Camilli et al., 1995; Edelmann et al., 1995; Geppert et al., 1994; Hilfiker et al., 1999; Littleton et al., 2001; Mayor and Pagano, 2007; Stimson et al., 2001). Even if a specific venom component binds to only one of those proteins, it could dramatically affect synaptic transmission, by inhibiting protein function or by interfering with protein-protein interactions. If the wasp venom targets those synaptic proteins, it could interfere with exocytosis or/and endocytosis in all synapses of the CX or SEG, rather than affecting a specific type of synapse.

4.2. Venom components could enter the cell by 'hijacking' endocytosis machinery

Venom targeting of these synaptic proteins also could also serve as a mechanism promoting internalization of venom proteins, one of the most significant observations to emerge from this study. We propose that wasp venom components target these synaptic proteins with two possible outcomes: 1. interference with exocytosis and/or endocytosis;

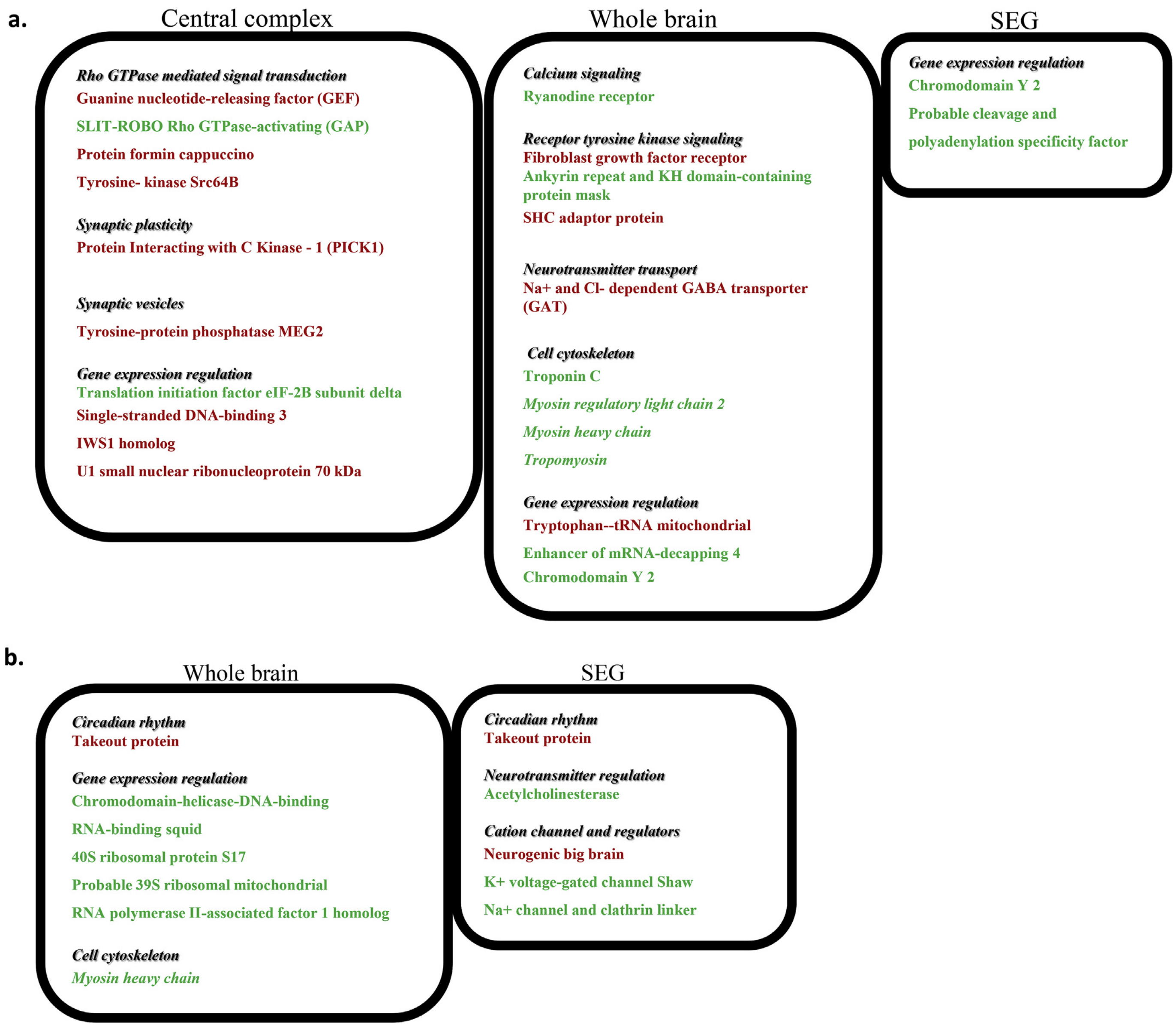


Fig. 5. Changes associated with the effect of the venom. Partial list of proteins with significant differential expression in cerebral ganglia of stung cockroaches when green indicated upregulated proteins and red indicates downregulated proteins. **a.** Changes registered at the 24 h time point that are associated with hypokinesia in the isolated CX (Central complex), whole brain and SEG, sorted by common predicted function. **b**. Changes registered at the 10 min time point that are associated with the short term effect of the venom in the whole brain and SEG, sorted by common predicted function. Green marks up-regulated protein and red marks down-regulated proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. 'hijacking' the endocytosis machinery. In both cases, targeting synaptic vesicle proteins could have a devastating effect on neuronal activity.

This leads to one important question in this behavioural manipulation strategy: do venom components enter and act inside target cells? The data collected in this study suggests they do. Many venom proteins are predicted to act inside the cell and most of the identified venom targets are cytoplasmic proteins. It seems reasonable to speculate that wasp venom components could 'hijack' endocytosis mechanisms to enter the cell as such is the case for Botulinum toxin (Dong et al., 2007; Verderio et al., 2006, 2007). It is possible that, similar to Botulinum toxin, proteins from the wasp venom bind synaptotagmin when it is exposed to the extracellular space during exocytosis. However, it remains uncertain which venom proteins target these endocytosis/exocytosis proteins. Novel uncharacterized venom peptides/proteins are promising candidates for this function, but so are several other candidates. The venom vesicular glutamate transporter interacts with Endophilin and Clathrin adaptor protein 2 (AP-2 complex) (Li et al., 2017; Santos et al., 2014). The FXNPXY motif in the low-density lipoprotein receptor-related protein 2 (LRP2) from the venom binds directly to Clathrin (Kibbey et al., 1998), which is known to mediate internalization of a variety of ligands/cargos (Strickland et al., 2002). Another venom protein, which contains a seven transmembrane receptor domain, bears similarity to vertebrate GPR107, a G-protein coupled receptor that is involved in Golgi-to-ER retrograde transport. It functions as a host factor required for infection by *Pseudomonas aeruginosa* exotoxin A and *Campylobacter jejuni* CDT toxins (Tafesse et al., 2014). Similar to LRP2, this protein was shown to interact with Clathrin (Zhou et al., 2014). In effect, Clathrin-mediated endocytosis is exploited by many bacterial and viral pathogens and their toxins (Cossart and Helenius, 2014). For example, tetanus, shiga, diphtheria, and anthrax toxins utilize clathrin-mediated endocytosis to enter cells (McMahon and Boucrot, 2011). It is therefore possible that receptors from the venom could 'hijack' clathrin mediated endocytosis and to mediate the

Gene ontology: Biological process	
10 minutes after the sting	24h after the sting
Columnar/cuboidal epithelial cell development	Actin cytoskeleton organization
Eepithelial cell development	Actomyosin structure organization
Epithelial cell differentiation	Actin filament organization
Eye pigment biosynthetic process	Myofibril assembly
Female gamete generation	Striated muscle cell development
Pigment metabolic process involved in developmental pigmentation	Muscle cell development
Eye pigment metabolic process	Animal organ development
Ovarian follicle cell development	Nervous system development
🟩 Cell-cell junction assembly	Regulation of actin cytoskeleton organization
Histone methylation	Neurogenesis
Gamete generation	Actin filament bundle assembly
Protein complex assembly	Synapse assembly
Oogenesis	🟩 Cell development
🟩 Cell development	Sarcomere organization
Protein methylation	Striated muscle cell differentiation
Regulation of gliogenesis	Positive regulation of cellular protein metabolic process
Synaptic target inhibition	Muscle cell differentiation
	Neuron differentiation
	Regulation of cytoskeleton organization
	Positive regulation of protein metabolic process
	Morphogenesis of embryonic epithelium
	Regulation of actin polymerization or depolymerization
	Regulation of actin filament length
	🟩 Cell-cell junction assembly
	Cellular protein modification process
	Protein modification process
	Neuron development
	Cell morphogenesis involved in neuron differentiation
	Cell morphogenesis involved in differentiation
	Cellular component assembly involved in morphogenesis
	Regulation of cellular protein metabolic process
	Covalent chromatin modification
	Embryo development ending in birth or egg hatching
	Open tracheal system development
	Neuron projection morphogenesis

🟩 Common to both groups

Fig. 6. Functional enrichment analysis of the differentially expressed proteins reveals enriched GO terms for each time point. Enriched GO terms for the differentially expressed proteins in the cerebral ganglia of stung cockroaches (EASE score < 0.05, level 5 biological processes for high specificity, at least 2 proteins), at the different time points after the wasp's sting. Color fill indicates terms that are in common between groups (as detailed at the legend); terms that are not marked are unique for each time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

entrance of venom proteins into the cell. In this parasitic host evolutionary arms race, the wasp has evolved a powerful arsenal in order to defeat the cockroach. Indeed, targeting the endocytosis/exocytosis pathway in a manner similar to the most lethal pathogenic toxins represents a powerful strategy for controlling behaviour of the cockroach for the benefit of its offspring. This strategy, rather than employing toxins, instead modulates synaptic integrity and efficacy for the purpose of inducing a “sleep-like” hypokinetic state in the host. The wasp venom cocktail thus constitutes a broad-based molecular “blizzard” that penetrates and assumes command of locomotory centers within the cockroach cephalic ganglia for the benefit of developing wasp progeny.

Our analysis also revealed additional GO terms, including microtubule cytoskeleton organization, axon development, cell or neuron differentiation and morphogenesis, neurogenesis, and synaptic assembly. Many of the proteins associated with those terms (as we mentioned above: Spectrins and microtubule associated Futsch) could provide a link between synaptic function and morphological changes (caused by changes in cytoskeleton organization) at the synapse.

Although we can only predict the function of isolated proteins based on sequence similarity, our results support a hypothesis that the wasp venom targets synaptic proteins and synaptic cytoskeleton-related proteins in cockroach cerebral ganglia, thereby leading to decreased synaptic efficacy (Fig. 7).

4.3. *Characterization of changes in protein expression in cerebral ganglia*

The second step for identifying proteins mediating venom actions was to characterize changes in protein expression in cerebral ganglia. To find differentially expressed proteins, we used label-free quantitative MS. Although we refer throughout this paper to the identified changes in the relative measured proteins level as differential protein expression, those changes can occur not only by changes of gene expression, but also by changes in the overall turnover rate of such proteins, especially considering the abundance of proteases in the venom.

This analysis revealed changes in protein expression in both tested time points: shortly after the sting (before hypokinesia effect of the

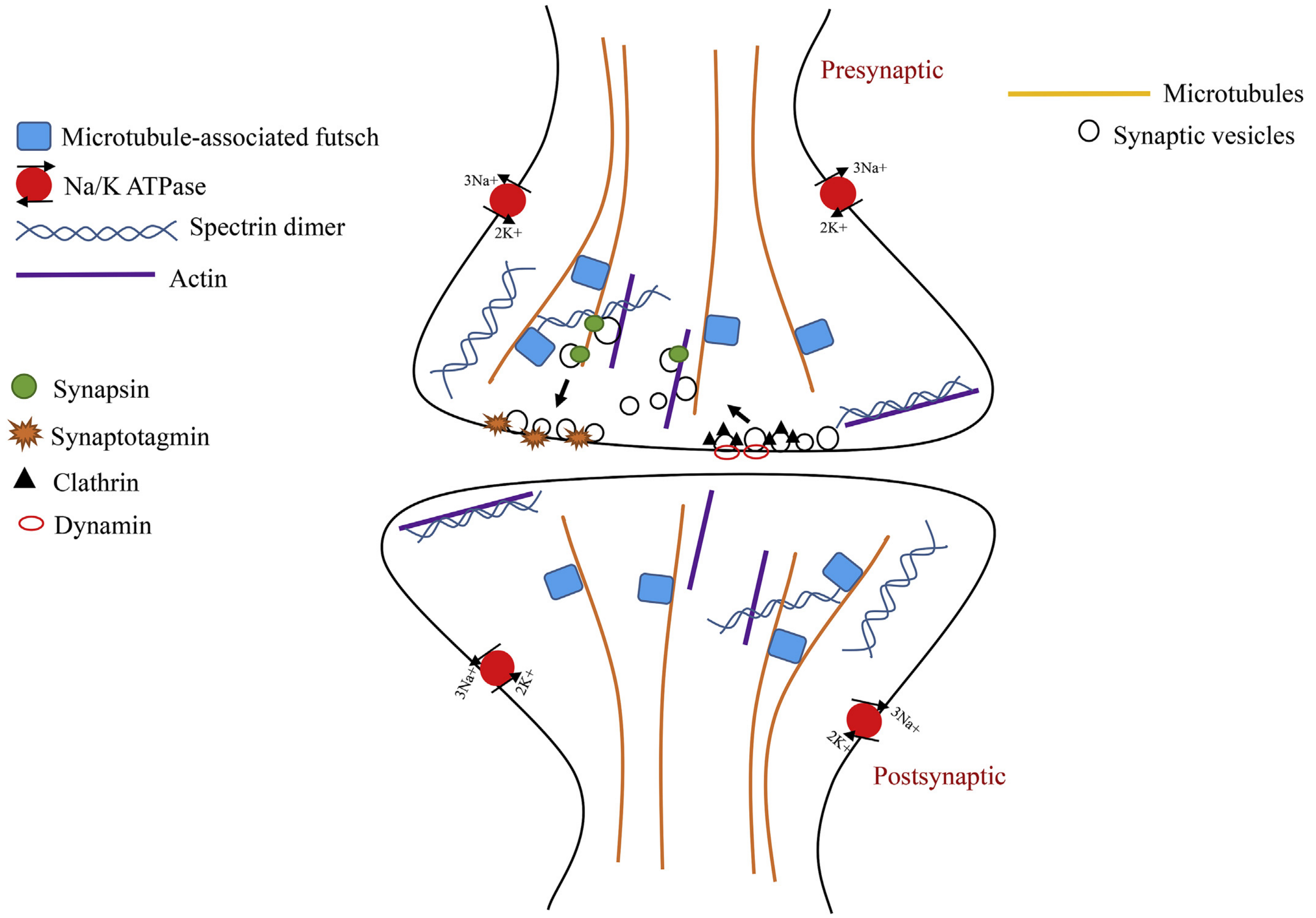


Fig. 7. Proposed molecular targets of Ampulex venom at the synapse. Many venom targets are located at the synapse and could interfere with processes such as endocytosis, exocytosis, and ionic homeostasis.

venom sets in), and 24 h after (under full effect of the venom). These results suggest that, although immediately after the sting, cockroaches do not present symptoms of hypokinesia, they display a protein expression profile different from that of control cockroaches. This means that the wasp venom causes changes in protein expression in the cerebral ganglia, and that some of these changes occur quickly (proteins associated with the short-term effect of the venom), while other changes are associated with the long term hypokinetic state (proteins associated with the long-term effect of the venom).

In most cases, the brain and SEG have different venom-induced differential protein expression profiles; most proteins that are differentially expressed in the brain are not the same proteins that are differentially expressed in the SEG (and vice versa). This is in contrast to venom targets, which are mostly common to the brain and SEG, implying that although the targets are the same, the downstream effect of the venom is different in each ganglion.

The relatively high total difference between tested groups and controls in all groups (with an average of at least 2 orders of magnitude) shows that identified differentially expressed proteins are not only slightly down- or up-regulated, but that there is a substantial shift in their expression in the tested groups.

Changes in protein expression were identified shortly following the sting (10 min after). The *takeout* protein, which is implicated in circadian control of feeding behaviour, is down-regulated in both brain and SEG (So et al., 2000). Most differentially expressed proteins in the brain are related to protein expression regulation, whereas changes in SEG are more related to neurotransmitter release, cation channels, and their regulators (Fig. 5b). This could indicate that shortly after the sting, proteins that could have direct effect on the neuronal activity in SEG are differentially expressed and that the response to the venom is divided into different phases, quick and short lasting changes followed by slow and long lasting changes.

Since the hypokinetic state is a long-lasting and drastic change in cockroach behaviour, we expected to find changes in protein expression in the cerebral ganglia underlying such a behavioural change. Differentially expressed proteins associated with long-term effects of the venom were different between the entire brain and the isolated CX (24 h after the sting). Most of the venom is injected in and around the CX, which is a relatively small central region of the brain (Haspel et al., 2003). Isolating this region for a more focused analysis revealed changes that were not apparent through analysis of the whole brain, since the significance of such changes in the CX were possibly reduced when analysed against the high background of other regions of the brain not targeted by the venom.

Several differentially expressed proteins in the CX are involved in the Rho GTPase signalling pathway. Rho GTPases are known to be directly involved in synaptic plasticity through direct interaction with actin and microtubule organizing proteins for regulation of axonal growth and dendritic spine formation (Ojelade et al., 2013; Sit and Manser, 2011; Tolias et al., 2011). Interestingly, similar proteins were found to be differentially expressed in the *dipteran* host of the ectoparasitoid wasp, *Nasonia vitripennis* (Danneels et al., 2013). This wasp injects venom and eggs into pupae, leading eventually to arrested development and death of the envenomated host. Although the mode of

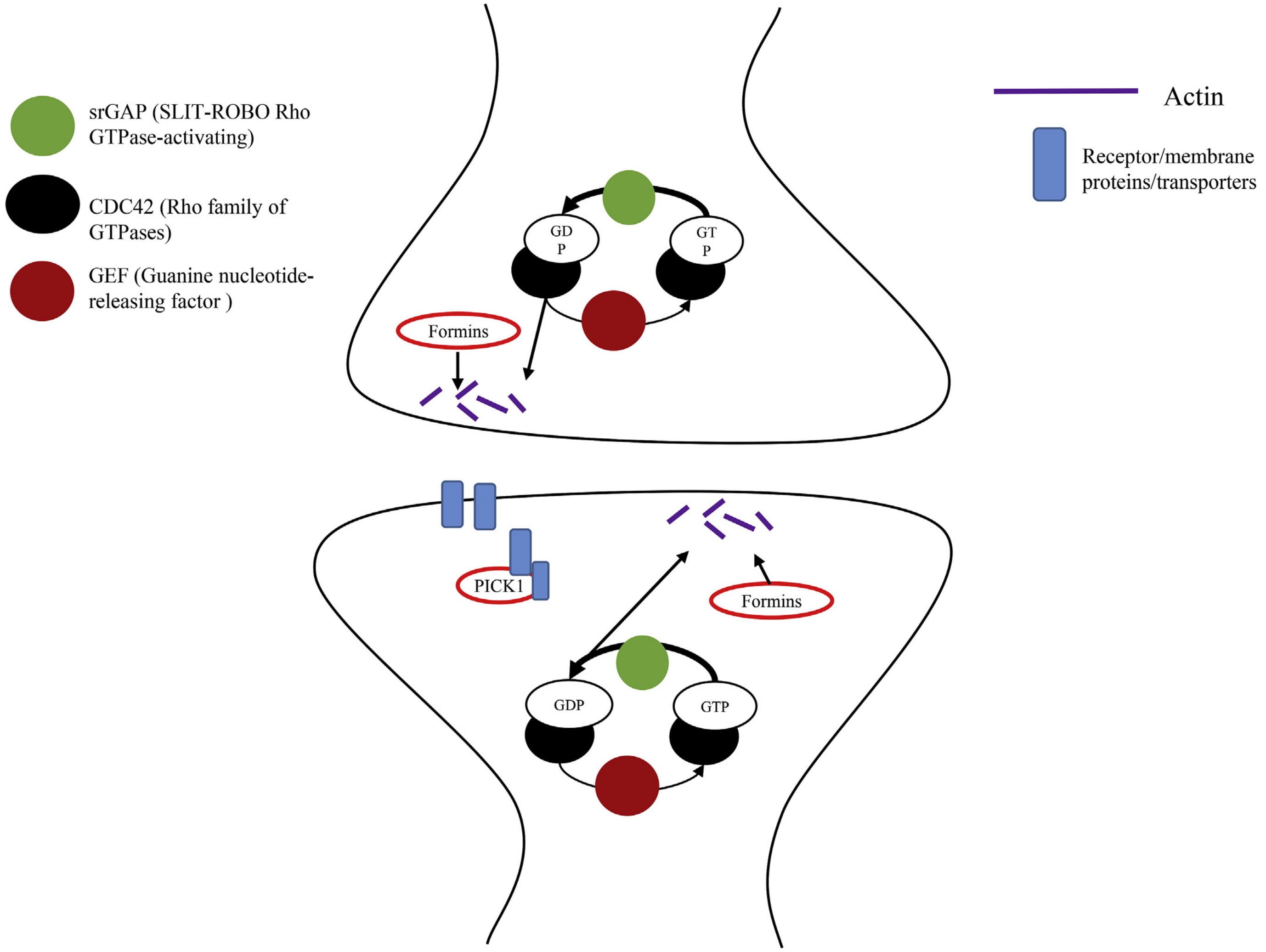


Fig. 8. Representation of differentially expressed proteins in the CX of stung cockroaches (24 h after the sting). Many of the differentially expressed proteins in CX depicted are involved in the Rho GTPase pathway and are implicated in synaptic plasticity. Upregulation of srGAP and downregulation of GEF could lead to less active Rho GTPase (probably CDC42). Together with the down-regulation of formins, this probably leads to less action polymerization at the synapse. PICK1, which is involved in regulation of the Rho GTPase pathway, is also implicated in regulation of receptors levels of in the membrane.

envenomation and its effect on the host is considerably different in *Nasonia* and *Ampulex*, there is still a possibility that similar venom components from each species target the Rho GTPase pathway (Arvidson et al., 2018).

These small GTPases are regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs are regulatory proteins that bind to activated G-proteins to promote GTPase-mediated inactivation, while GEFs activate small GTPases by stimulating release of GDP to allow binding of GTP, pushing the GTPase to an active state (Tolias et al., 2011). More specifically, differentially expressed proteins in the CX seem to be related to the Robo-Slit signalling pathway. Roundabout receptors (Robo) and their Slit ligands form one of the most important ligand-receptor pairings among axon guidance molecules (Blockus and Chedotal, 2016; Ypsilanti et al., 2010). Robo signalling depends upon a range of secondary molecules, which affect neurite growth through control of cytoskeletal rearrangements. We found that a SLIT-ROBO Rho GTPase-activating protein (srGAP), which is known as a downstream effector of the Slit- Robo signalling pathway, is upregulated in the CX (Lucas and Hardin, 2017; Wong et al., 2001). This protein has a higher similarity to the vertebrate srGAPs (Identity of 45–46%) than to *Drosophila* GAPs (33%). While the Slit-Robo pathway was first discovered in *Drosophila*, most of what is known about srGAPs comes from studies on vertebrates (Lucas and Hardin, 2017). The closest studied relatives in *Drosophila* are Rho-GAP93B and RhoGAP92B (Lundstrom et al., 2004; Nahm et al., 2010) which, like vertebrate srGAPs, seem to target Cdc42 Rho GTPase (Lundstrom et al., 2004; Nahm et al., 2010; Wong et al., 2001). Therefore, it is likely that increased expression of this specific GAP in the CX increases the intrinsic GTPase activity of Cdc42 to convert the GTP-bound form of Cdc42 into its GDP-bound form, thereby inactivating Cdc42 (Fig. 8). Inactivation of Cdc42 in turn leads to reduced activation of the Neuronal Wiskott-Aldrich Syndrome protein (N-WASP), thus decreasing the level of active Arp2/3 complex. Because active Arp2/3 promotes actin polymerization, reduction of active Cdc42 eventually decreases actin polymerization (Wong et al., 2001).

Also, significantly down-regulated in the CX of stung cockroaches is a GEF protein, which contains a conserved RasGEF domain (found in the CDC25 family of guanine nucleotide exchange factors for Ras-like small GTPases) and a REM domain (GEF for Ras-like GTPases; N-terminal domain). This protein shares 61% of the amino acid sequence with the C3G guanyl-nucleotide exchange factor of *Drosophila*. While some GEFs are specific to a single GTPase, others have multiple GTPase substrates (Schmidt and Hall, 2002). The functional domains of these GEF families are not structurally related and do not share sequence homology. These GEF domains appear to be evolutionarily unrelated, despite similar function and substrates. Thus, it is harder to predict GEF

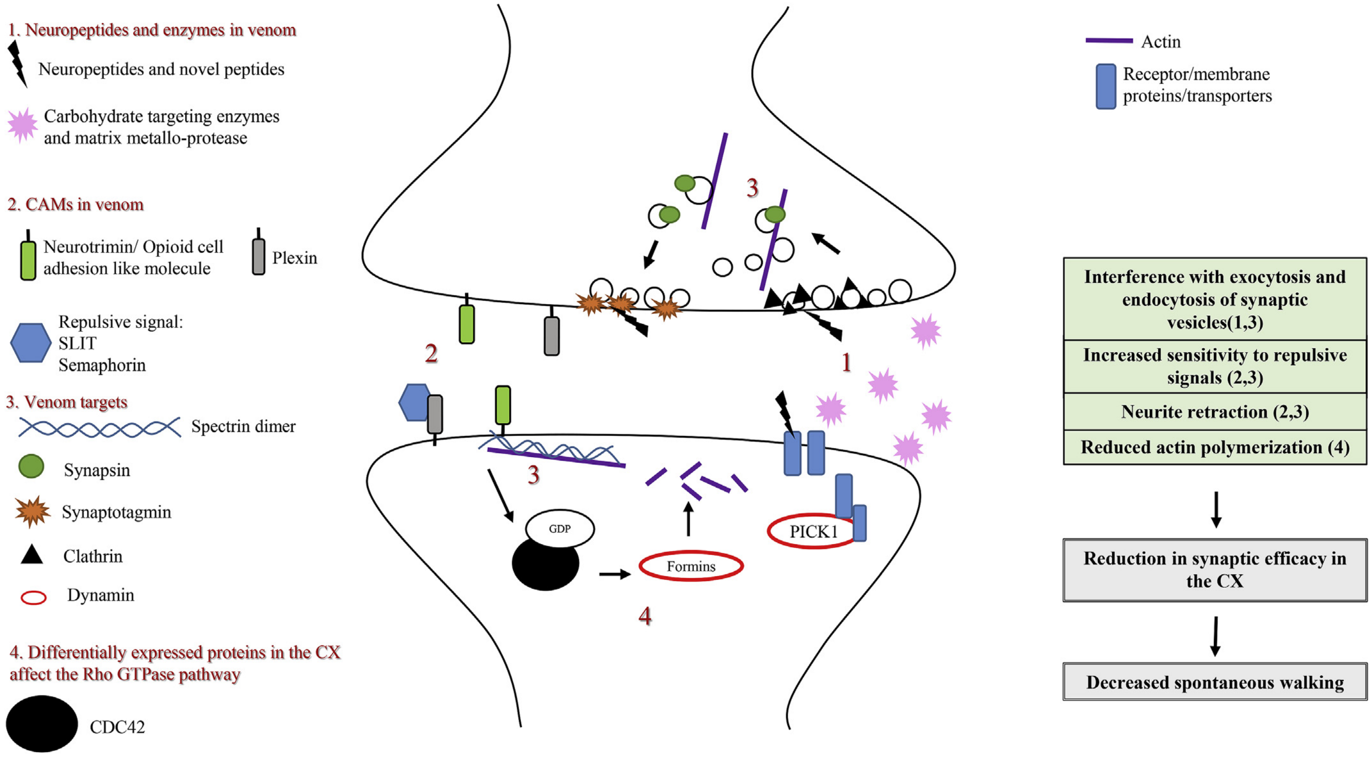


Fig. 9. An integrated model for the molecular mechanisms underlying the venom-induced behavioural manipulation. Possible mechanisms based on the proteomics data from venom, venom targets and changes in protein expression at the cerebral ganglia. 1. Neuropeptides and novel peptides/proteins can target specific proteins (like receptors, membrane protein and synaptic proteins) in the membrane and carbohydrate targeting enzymes and matrix metalloprotease can interfere with the extracellular matrix. 2. Intercalation of CAMs from the venom into the membrane could affect neurite responses to growth repulsive signals like SLIT or Semaphorin. This could potentially increase sensitivity to repulsive signals and/or cause neurite retraction. 3. Spectrins might be involved in regulation of Slit-Robo pathway components and therefore are possible mediators of this signalling cascade and candidate targets of the CAMs from the venom. Novel peptides/ proteins from the venom could targets synaptic proteins and interference with exocytosis and endocytosis of synaptic vesicles. 4. In the CX, less active Rho GTPase (CDC42) and downregulation of PICK1 and formins could lead to decreased actin polymerization, changes in synaptic plasticity, and possible neurite retraction. Taken together, molecular cross-talk between venom components and CNS targets depicted here could lead ultimately to decreased neuronal activity and synaptic efficacy in the CX, the outcome of which is hypokinesia.

substrates based on their sequence (Schmidt and Hall, 2002). However, it is possible that this GEF, which is downregulated in the CX, targets GTPases such as CDC42, similar to the way a familiar member of the Ras-GEF, Son of Sevenless (SOS) regulates slit-robo signalling (Yang and Bashaw, 2006). Downregulation of this GEF could lead to inactivation of Cdc42 (Fig. 8).

More down-regulated proteins in the CX are involved in the Rho GTPase pathway, such as tyrosine kinase Src64B and Cappuccino/ Formin. Tyrosine kinase Src64B has a strong homology to *Drosophila* tyrosine-protein kinase Src64B (73% identity) and similarity to tyrosine protein kinase Fyn in vertebrates (58–59% identity). It contains a catalytic domain of Src kinase-like protein tyrosine kinases and a Src homology 2 (SH2) domain found in the Src family of non-receptor tyrosine kinases. This Src kinase was shown to negatively regulate p109 RhoGAP in *Drosophila* and therefore affects the Rho GTPase pathway (Billuart et al., 2001). The protein Cappuccino/Formin is similar to *Drosophila* Cappuccino (46% identity) and vertebrate Formin (32–34%). The Cappuccino/Formin protein is known to be an effector of the Rho GTPase pathway and acts as an actin nucleation factor to promote assembly of actin filaments (Vizcarra et al., 2011) (Fig. 8).

In addition, PICK1, which is involved in regulation of Rho GTPase and synaptic plasticity, is differentially expressed in the CX (Aspenstrom, 2014; Hanley, 2008). The identified PICK1 protein has high homology to *Drosophila* and vertebrate PICK1 (68–69% identity). It contains Bin/Amphiphysin/Rvs (BAR) and PDZ conserved domains. Although PICK1 has not been thoroughly studied in *Drosophila,* a study suggests that PICK1 could regulate levels of glutamate receptors at the neuromuscular junction (NMJ). PICK1 reduction leads to smaller synapse size and reduced number of boutons per unit muscle area at the NMJ (Mallik et al., 2017). In mammals, PICK1 interacts directly with AMPA receptors and is involved in their regulated removal from the synaptic membrane. PICK1 has the ability to functionally interact with a number of cellular processes, including calcium signalling, actin polymerization and phospholipid membrane architecture (Aspenstrom, 2014; Hanley, 2008; Li et al., 2016; Xu and Xia, 2006).

When analysing the entire brain, we found differential expression of proteins that are related to receptor tyrosine kinase signalling, such as the fibroblast growth factor receptor (Muha and Muller, 2013; Stork et al., 2014), Ankyrin repeat KH domain-containing protein mask (Smith et al., 2002) and SHC adaptor protein (Luschnig et al., 2000). In addition, the ryanodine receptor, which is an important regulator of calcium signalling, is upregulated in the brain of stung cockroaches, 24 h after the sting (Vazquez-Martinez et al., 2003).

Interestingly a sodium/chloride-dependent GABA transporter is down-regulated in the entire brain, which can lead to increased GABA (or taurine) levels in the synaptic cleft and prolong the response to GABA (Scimemi, 2014). In fact, presence of GABA has been revealed in high concentration in the venom (Moore et al., 2006).

4.4. A model integrating molecular mechanisms and signalling pathways underlying the behavioural manipulation

Our extensive database of putative venom targets has allowed us to build a model that combines massive -omics information that could account for behavioural changes observed in stung cockroaches (Fig. 9). The venom is a rich mixture of different proteins, many of which could affect synaptic efficacy (Arvidson et al., 2018). For example, neuropeptides and novel peptides/proteins can target specific proteins in the membrane (such as the Na/K ATPase or the GABA transporter) and carbohydrate-targeting enzymes, such as hyaluronidase, and matrix metalloproteases could disrupt integrity of the extracellular matrix. Some venom proteins are expected to be immunoglobulin-like (Ig) cell adhesion molecules (CAMs), among which are homologs to OBCAM, Plexin (Semaphorin receptor) and Roundabout (SLIT receptor). These proteins can potentially intercalate into the membrane, and affect neurite responses to growth repulsive signals like SLIT or Semaphorin (Fig. 9). Their incorporation into the membrane at synapses of the CX or SEG could increase their sensitivity to repulsive signals and/or cause neurite retraction. Even a slight neurite retraction could affect synaptic efficacy and therefore reduce neuronal activity in the CX and SEG, which would cause the aforementioned decrease in spontaneous walking displayed by stung cockroaches. Interestingly, a CX slit-ROBO GAP (srGAP, Fig. 8), a downstream effector in the SLIT signalling pathway, is upregulated 24 h after the sting. Upregulation of srGAP could lead to less active Rho GTPase (probably CDC42), and therefore to decreased actin polymerization, changes in synaptic plasticity, and possible neurite retraction. Spectrins, here identified as venom targets, might be involved in regulation of Slit-Robo pathway components and therefore are possible mediators of this signalling cascade (Garbe et al., 2007). Spectrins have been shown to mediate the effect of CAMs and might be targets of the CAMs from the venom. Interaction of CAMs with signal transduction pathways could have a long-lasting effect, at least in the CX where the Rho GTPase pathway seems to be affected. We suggest this pathway could lead to changes in synaptic plasticity, synaptic protein assembly, neurite retraction and decreased actin polymerization (Fig. 9). This hypothesis is supported by the downregulation of other proteins in the CX, namely PICK1 and Cappuccino/Formin, which could lead ultimately to decreased synaptic efficacy. Reduced synaptic efficacy in the CX fits perfectly with the stung cockroach phenotype; that is, decreased neuronal activity in the CX leads to decreased spontaneous walking (Kaiser and Libersat, 2015).

Completing this molecular database for the wasp-cockroach interaction opens new avenues for deciphering molecular mechanisms underlying this unique behavioural manipulation. The challenge now will be determining a causal relationship between individual venom components, their protein targets, and their impacts on host behaviour. Advances in the field of MS and bioinformatics have allowed us to conduct this study and rapid developments in this field hold promise for future studies. Since preparing this manuscript, the genome of the American cockroach was finally completed, providing great opportunities for future research (Li et al., 2018). Understanding such mechanisms is at the heart of applied aspects of parasitology such as epidemiology and medicine and is important for evolutionary and ecological reasons. In addition, this study contributes to the field of Neuroethology by uncovering basic neural mechanisms underlying decision making in the insect brain. Specifically, since the wasp seems to alter decision making in the cockroach or the motivation of the cockroach to escape, uncovering the mechanisms responsible will shed light on how cockroaches make decisions, from the neuronal site of this process to the molecules and pathways that are involved.

Acknowledgements

We thank the Smoler Proteomics Center at the Technion for their service and guidance regarding the mass spectrometry analysis. This work is currently supported by the United States-Israel Binational Sciences Foundation (2015161) to F.L. and M.A. The experiments performed comply with Principles of Animal Care, NIH publication no. 86-23, revised in 1985, and with the current laws of the State of Israel. The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.11.009.

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